ENZYMIC MODIFICATION OF CELLULOSE-XYLOGLUCAN NETWORKS

Implications for fruit juice processing

CENTRALE LANDBOUWCATALOGUS
0000 0714 4799

Promotor:	dr. ir. A.G.J. Voragen Hoogleraar in de levensmiddelenchemie
Co-promotor:	dr. G. Beldman Universitair docent in de levensmiddelenchemie

NN08201,2085

Jean-Paul Vincken

ENZYMIC MODIFICATION OF CELLULOSE-XYLOGLUCAN NETWORKS

Implications for fruit juice processing

Enzymatische modificatie van cellulose-xyloglucaan netwerken

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 24 mei 1996
des namiddags om half twee in de Aula
van de Landbouwuniversiteit te Wageningen.

ISN 925701

Those parts of this thesis which have been published elsewhere have been reproduced with the permission of both publishers and authors.

The research described in this thesis was financed by Gist-brocades (Delft, The Netherlands).

Cover photo: cell wall ghosts of apple cells (∅ ≈250 μm), stained with calcofluor, were obtained after partial degradation of apple tissue; Boudewijn van Veen (Media Service, Wageningen).

BIBLIOTHEEK
LANDBOUWUNIVERSITEIT
WAGENINGEN

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Vincken, Jean-Paul

Enzymic modification of cellulose-xyloglucan networks :
implications for fruit juice processing / Jean-Paul
Vincken. - [S.l.: s.n.]
Thesis Wageningen Agricultural University. - With ref. - With
summary in Dutch.
ISBN 90-5485-510-X
Subject headings: apples / xyloglucan / cellulose.

NN08201, 2085.

STELLINGEN

1. Ishii mag op basis van afwezigheid van feruloylesterase activiteit in Driselase niet aannemen dat dit enzympreparaat geen andere esterases bevat. De conclusie dat rhamnogalacturonaan uit bamboescheuten uit drie regios met verschillende acetyleringsgraad bestaat, is daarom niet gerechtvaardigd.

   Ishii, T. (1995) *Mokuzai Gakkaishi* **41**, 561-572.

2. Te vaak worden enzymen op basis van hun sequenties geclassificeerd, zonder de substraat specificiteit te toetsen.

3. Het is onjuist om de aanwezigheid van xylose in pectine klakkeloos te interpreteren als behorende tot een xylaan-pectine complex.

   Coimbra, M.A., Rigby, N.M., Selvendran, R.R., and Waldron, K.W. (1995) *Carbohydr. Polym.* **27**, 277-284.

4. Het ligt niet voor de hand dat een exo-galacturonidase nevenactiviteit in de door Lerouge *et al.* gebruikte preparaten inwerkt op zijketens van rhamnogalacturonaan I.

   Lerouge, P., O'Neill, M.A., Darvill, A.G., and Albersheim, P. (1993) *Carbohydr. Res.* **243**, 359-371.

5. Het tegen beter weten in toeschrijven van ß-glucanase activiteit aan CBHII door Henriksson *et al.* roept twijfels op omtrent hun wetenschappelijke integriteit.

   Henriksson, K., Teleman, A., Suortti, T., Reinikainen, T., Jaskari, J., Teleman, O., and Poutanen, K. (1995) *Carbohydr. Polym.* **26**, 109-119.
   Reinikainen, T., Henriksson, K., Siika-aho, M., Teleman, O., and Poutanen, K. (1995) *Enzyme Microb. Technol.* **17**, 888-892.

6. De door Shao *et al.* gebruikte methodieken, alsmede de interpretatie van de resultaten *t.a.v.* de substraatspecificiteit van α-glucuronidase zijn ontoereikend.

   Shao, W., Obi, S.K.C., Puls, J., and Wiegel, J. (1995) *Appl. Environ. Microbiol.* **61**, 1077-1081.

7. Nieuwe ontwikkelingen als "combinatorial chemistry", "direct evolution" en "good laboratory practice" vragen om wetenschappers met goede administratieve vaardigheden.

8. Het feit dat vele conflicten hun oorsprong in godsdienst vinden, komt de *geloof*waardigheid van godsdiensten niet ten goede.

9. In de slag om de kijkdichtheid heeft het "NOS 8 uur journaal" haar zakelijk karakter verloren.

10. De amerikaanse "National Park Service Act" is gebaseerd op twee niet verenigbare doelstellingen: natuurbehoud en volksvermaak.

The National Park Service Act (1916) *U.S. Statutes at Large* **39**, 535.

11. Gezien de veranderende taak van de krijgsmacht zou een "peace-keeping" stage in oorlogsgebied onderdeel van de militaire opleiding moeten zijn.

12. Uit commercieel oogpunt verdient het aanbeveling om de reclameblokken op de diverse televisiekanalen te synchroniseren, zodat ook de aandacht van de "zappende" kijker gevangen wordt.

Stellingen behorende bij het proefschrift
'ENZYMIC MODIFICATION OF CELLULOSE-XYLOGLUCAN NETWORKS
Implications for fruit juice processing'
Jean-Paul Vincken
Wageningen, 24 mei 1996

VOORWOORD

Het in dit proefschrift beschreven onderzoek is geworteld in de collegiale sfeer en kameraadschap die ik bij de sectie Levensmiddelenchemie en -microbiologie genoten heb. Slechts een aantal personen hebben zichtbaar een stempel op dit onderzoek gedrukt maar het is de verdienste van velen dat ik tot op de dag van vandaag nog veel plezier in mijn werk heb. Met z'n allen hebben jullie de afgelopen periode tot een onvergetelijke gemaakt! Een aantal mensen wil ik graag met naam noemen.

Fons Voragen, bij de aanvang van mijn project heb je me verteld dat ik op een rijdende trein sprong. Ik heb wel eens het gevoel gehad dat ik bij de eerste halte afgesprongen ben en vervolgens met een boemeltje naar een onbekende bestemming vertrokken ben. Jij hebt je in deze een "inducible promot*or*", en tevens een bekwaam wisselwachter getoond. Ik waardeer het vertrouwen dat je me gegeven hebt en de wijze waarop je me steeds hebt weten te motiveren.

Gerrit Beldman, jij hebt mij een fantastische erfenis (bestaande uit zes endo- en twee exoglucanases) uit jouw promotietijd nagelaten. Jouw oprechte betrokkenheid en kritische kanttekeningen hebben dit proefschrift gelouterd. Met plezier denk ik terug aan onze gezamenlijke congresbezoeken.

Ab van Ooijen, jij hebt je ontpopt tot mijn trouwste fan bij voordrachten. Jij bent het *vnl.* geweest die me aangespoord heeft om over de toepasbaarheid van mijn bevindingen na te denken. Marjo Searle-van Leeuwen, Felix Kormelink, Fred Lahouse, Landry le Chevanton, Yves Coutel, Marga Herweijer, Dick Schipper, Onno Misset, en Joop van der Laan, jullie bijdrage aan het Gist-brocades project werd op prijs gesteld.

Henk Schols, het is goed toeven in ons "boomhok". Met zijn tweeën bestrijken we beide netwerken van de appelcelwand. Al onze boeiende discussies ten spijt hebben we de "link" tussen beide netwerken nog steeds niet gevonden. Zonder jou zou hoofdstuk 7 er beslist anders uitgezien hebben!

Wilfried Niessen (Center for Drug Research, Leiden), wil ik bedanken voor zijn massaspectrometrische ondersteuning in de hoofdstukken 3 en 5. Tinka Spronk, Geert-Jan Rademaker, Jane Thomas-Oates, prof. J.P. Kamerling (Rijksuniversiteit Utrecht), ^{1}H-NMR en FAB-MS bleken onmisbaar bij de volledige karakterisering van xyloglucaan oligosachariden uit appel. Arie de Keizer (Fysische en Kolloidchemie, LUW), jij hebt mij ingewijd in de geheimen der treinen, lussen en staarten bij de binding van xyloglucaan aan cellulose.

Een tweetal studenten zien hun bijdrage in dit boekje verwerkt. Sascha Wijsman, jouw grondigheid heeft gestalte gegeven aan de opheldering van de structuur van aardappelxyloglucaan. David van der Lei, jouw enthousiasme heeft van de jacht naar XET een enerverende aangelegenheid gemaakt!

Miranda Huisman, bedankt voor het leveren van (on)rijpe appels uit eigen boomgaard en je (bere)gezelligheid op lab 504. Ben van den Broek, samen zijn we erachter gekomen dat experimenten met weefsel veel lastiger zijn dan die met geïsoleerde celwanden. Ons

speurwerk is echter niet voor niets geweest! Olga Zabotina (Institute of Biology, Kazan, Russia), I appreciate your patience and determination in quantifying XET activity in apples. Harry Raaijmakers, zonder jouw adviezen waren we waarschijnlijk nooit aan het derivatiseren van oligosachariden begonnen.

Marian, jij bent degene die de "ongekuiste" versie van mijn ideeën voor de kiezen krijgt. Je helpt me steeds het kaf van het koren te scheiden, hetgeen veel belangrijker voor me is dan je denkt.

Pa, Anton, Cor, Hennie, Chantal, Isabel, Joop, Rob, ..., ..., ik hoop dat mijn tijdgebrek van voorbijgaande aard is.

ABSTRACT

Xyloglucans play an important role in connecting cellulose microfibrils in the primary cell wall of plants, and the resulting cellulose-xyloglucan network is thought to determine the strength of these walls. Xyloglucans were isolated from apple fruit and potato tuber cell wall material by alkaline extraction and their primary structures were determined. Major differences between these two polysaccharides were their degree of backbone branching and the presence of fucosyl and arabinosyl residues.

The substrate specificity of three endoglucanases from *Trichoderma viride* (endoI, endoIV and endoV) was investigated. The target substrate of endoI is cellulose, that of endoIV xyloglucan, whereas endoV is the most versatile endoglucanase having the ability of degrading both substrates. EndoIV and endoV differ in their mode of action towards potato xyloglucan. Further, strong indications were obtained that xyloglucanase activity is related to a long array of substrate-binding sites.

The degradation of the cellulose-xyloglucan network in isolated cell wall material from apple fruit involves several glucanase activities. Xyloglucanase activity is important to make cell wall embedded cellulose more accessible to true cellulolytic enzymes such as endoI and cellobiohydrolase. Extensive degradation is required because xyloglucan fragments having a backbone of 20 glucosyl residues (five building units) still bind to cellulose surfaces. These results might explain why fungi excrete so many different kinds of endoglucanases.

When the cell walls of living apple fruit tissue were treated with pectin lyase and a mixture of glucanases from *Trichoderma viride* (liquefaction), the ease with which the apple tissue disintegrated, seemed to depend on the maturity of the fruit. The disintegration of apple fruit tissue during liquefaction correlates to the level of (ripening-related) xyloglucan endotransglycosylase activity in apple fruit. An hypothesis for the synergism of fungal and plant glucanases is put forward. Under certain circumstances (controlled liquefaction) the cellulose-xyloglucan network can be modified in such a way, that a stable cellulose-based cloud is formed in the resulting apple juice. The significance of these observations for juice manufacturing is discussed.

List of Abbreviations

APfxg	apple fucoxyloglucan
APxg	apple xyloglucan after removal of fucose
BSA	bovine serum albumine
CBD	cellulose-binding domain
CBH	cellobiohydrolase
CDTA	1,2-cyclohexylene-dinitrilotetra acetic acid
CMC	carboxymethyl cellulose
DA	degree of acetylation
DEAE	diethyl amino ethyl
DM	degree of methylation
DMSO	dimethylsulphoxide
DP	degree of polymerization
DTT	dithiothreitol
EG	endoglucanase described in literature (various sources)
endoI, *etc.*	endoglucanase I from *Trichoderma viride*, *etc.*
exoI, *etc.*	exoglucanase I from *Trichoderma viride*, *etc.*
GC	gas chromatography
^{1}H-NMR	proton nuclear magnetic resonance
HOAc	acetic acid
HPAEC	high-performance anion-exchange chromatography
HPLC	high-performance liquid chromatography
HPSEC	high-performance size-exclusion chromatography
MHR	modified hairy regions
mol wt	molecular weight
NaOAc	sodium acetate
PAD	pulsed amperometric detection
PED	pulsed electrochemical detection
PG	polygalacturonase
PL	pectin lyase
PME	pectin methylesterase
POsxg	potato arabinoxyloglucan
PS→PS	polysaccharide to polysaccharide
PS→OS	polysaccharide to oligosaccharide
PVPP	polyvinylpolypyrrolidone
TFA	trifluoro acetic acid
WUS	water-unextractable solids
XET	xyloglucan endotransglycosylase

Ara, Ara*f*	arabinose, arabinofuranose
Fuc, Fuc*p*	fucose, fucopyranose
Gal,Gal*p*	galactose, galactopyranose
GalA	galacturonic acid
Glc, Glc*p*	glucose, glucopyranose
Man	mannose
Rha	rhamnose
Xyl, Xyl*p*	xylose, xylopyranose
G_2, G_3, *etc.*	cellobiose, cellotriose, *etc.*
XXXG, *etc.*	xyloglucan oligosaccharides according to the commonly applied nomenclature which is explained in the introduction of this thesis
[●●]G	xyloglucan oligosaccharide with, between brackets, two elements (G, X, L, F, S) of which the exact order is unknown
$[xg]_n$	xyloglucan fragments that are composed of *n* building units
$[xg]_\infty$	polymeric xyloglucan
pa-$[xg]_1$	population of xyloglucan oligosaccharides containing molecules that are labelled with 2-aminopyridine by reductive amination

CONTENTS

CHAPTER 1

General Introduction

FRUIT JUICE PROCESSING, RETROSPECT AND PERSPECT

Since the 1930's there has been a gradual increase in the use of enzymes in fruit juice processing. This was partly due to low capital investments involved in introducing this new technology, but also socio-economic changes have played a role. An example of the latter is that fruits were no longer processed during 24 hours a day during green season, but rather over a longer time span with shorter working-days. Therefore, storage of the fruits was inevitable and during this period structural changes could take place which hampered fruit juice manufacturing [1,2].

An increasing knowledge of plant cell walls in combination with identification and characterization of technological relevant enzymes, as well as more sophisticated methods for producing enzymes and protein purification, triggered new developments in fruit juice processing, which are outlined below in chronological order. Conventional *pressing* often yielded cloudy juices due to the stabilization of pulp particles by pectin. Also, aroma stripping followed by refridgeration sometimes resulted in gelatinization and subsequent retrogradation of starch. *Clarification* of these juices could easily be done by application of pectinases (such as PL[1] or PG plus PME, active towards highly methoxylated pectin) and α-amylases, respectively [2]. After this, *pulpenzyming* was introduced, a process using similar pectinases as required for clarification [1]. Here, the enzymes were added to the pulp of, for instance, black currants or overripe apples. This fruit material contained such large amounts of soluble pectin that gelation occurred, which resulted in an impressable mass. Pectinases readily degraded this gel and only little pressure input drained the juice from the pomace. The *liquefaction* process goes beyond the previous treatment in that a complete degradation of the plant cell wall is realized and presses could be replaced by decanters [1-3]. Liquefaction is achieved by supplementing the pectinases with cellulases. Advantages of this operation were a high yield compared to pulpenzyming and low investments and costs. However, a major drawback was haze formation in, for instance, apple juice. When this haze was identified as being an α-(1→5)-arabinan [4], attention was drawn to the group of the arabinanases [5,6]. The haze appeared to be formed upon linearization of a branched arabinan by an arabinofuranosidase and could be effectively removed by an endo-arabinanase [7]. As a consequence of this, some existing enzyme preparations used for liquefaction were *enriched* in *limiting* endo-arabinanase activity. The example of the arabinan haze illustrates how ill-defined commercial enzyme preparations can be. In general, they contain many side-activities, some of which may be

[1] *Abbreviations:* see page viii.

desirable and others which are undesirable. Many of these activities have been purified and their mechanism of action has been fairly well established. At present, research is directed towards the large-scale production of relatively pure enzymes with genetically manipulated microorganisms. A goal for the near future is to combine these single enzyme components into *tailor-made mixtures*. With respect to liquefaction, these mixtures should be formulated in such a way, that the plant cell walls can be degraded with a minimum number of enzymes. Advantages of these new preparations are obvious. They can be easily adapted to any kind of application in fruit juice processing (different fruits, maturity, *etc.*), and additionally they contain much smaller amounts of undesirable side activities.

In order to formulate tailor-made preparations for liquefaction of apple fruit, Renard [8] has studied the degradation of isolated apple fruit cell walls by individual enzymes, as well as by their combinations. An important conclusion of that work was that one endoglucanase, in particular, acted synergistically with PL. It seemed that the action of the endoglucanase was directed predominantly towards xyloglucan. Although this polysaccharide is an important constituent of many plant cell walls [9], its degradation has received little attention compared to pectic polysaccharides and cellulose. The observations of Renard have prompted this more detailed study of the action of glucanases towards xyloglucan. In the next paragraphs, a short update on plant cell walls is given. Further, the structure of cellulose and xyloglucan, as well as their degradation is discussed.

A THREE-DIMENSIONAL CELL WALL CONCEPT

Recently, the architecture of the primary cell wall of plants has been reviewed by several authors [10,11], and new models for the structure of these walls have been put forward. These new models differ from the previous one proposed by Keegstra *et al.* [12] in that the coherence of the wall is not predominantly determined by covalent crosslinking of various wall components. McCann *et al.* [13] have analyzed shadowed replicas of onion cell walls by electron microscopy after graded extraction with several solutions. From these observations a scale model of the primary cell wall of onion parenchyma was constructed (Fig. 1) [10]. It is expected that the general features of this model are also applicable to parenchyma tissues other than onion. The primary cell wall (width $\approx$75 nm) is flanked by a plasma membrane and a middle lamella (width $\approx$50 nm). The latter is predominantly composed of low esterified pectins. The cell wall can accomodate three to four lamellae of cellulose microfibrils (diameter 5-12 nm; indeterminate length). The microfibrils in one layer run parallel, and no weaving between layers is observed. The pore size of unextracted walls is *c.* 10 nm which corresponded well to *in vivo* determinations performed by others [14,15]. After removal of pectin by mild extraction procedures, the pore size increased to *c.* 20-50 nm. Similar observations were done by Baron-Epel *et al.* [15] after treatment of soybean cell walls with pectinases. Extraction of

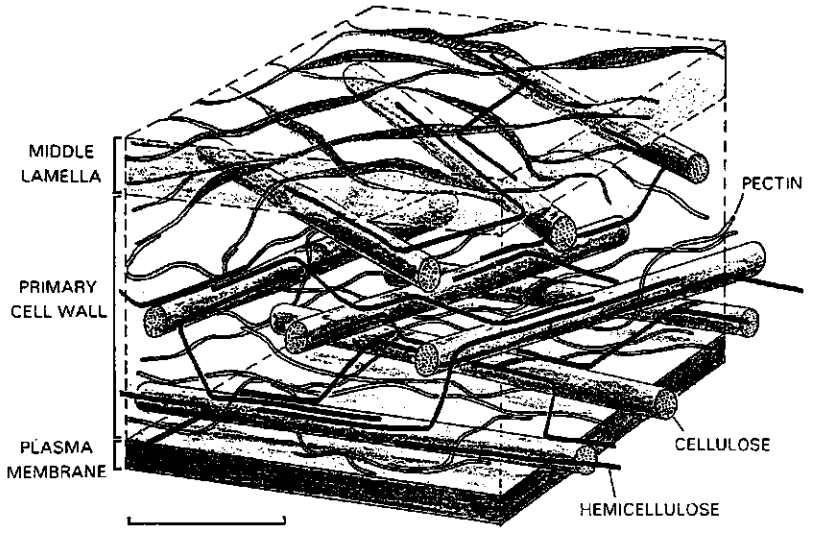

FIGURE 1. A model for the cell wall of onion parenchyma tissue [10]. The most important hemicellulose is xyloglucan. Scale bar represents 50 nm.

pectin did not affect the integrity of the wall. The spacing of *c.* 30 nm between lamellae was maintained. The cell wall framework collapses upon extraction of xyloglucans by strong alkali solutions. This is attended with swelling and lateral association of microfibrils [13]. The length of individual xyloglucan molecules (30-400 nm) generally exceeds the distance between lamellae many times, which suggests that these polysaccharides can interlink several microfibrils. Typically, xyloglucans seem to be built of blocks of 30 nm repeats; one such repeat corresponds to *c.* 60 Glc residues (or 15 xyloglucan oligosaccharides, see further) [16,17]. These observations fueled the hypothesis in which the cell wall is composed of two independent matrices. The strength of the wall is determined by the cellulose-xyloglucan network, while the porosity is controlled by pectins.

More evidence for two independent matrices was obtained by studying the cell wall structure of tomato cell which had been adapted to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile [18,19]. In these adapted cells, newly synthesized xyloglucan molecules have no cellulose surface to bind to and are not incorperated into the wall. Consequently, the adapted cells lack a cellulose-xyloglucan network, the load-bearing function of which is taken over by Ca^{2+} bridged pectate. The tensile strength of these adapted cells is reduced compared to that of normal cells whereas the porosity is similar. Typically, the thickness of the wall is not affected by the absence of cellulose and xyloglucan [20].

MODIFICATION OF CELLULOSE-XYLOGLUCAN NETWORKS

Enzymic degradation of cellulose

Cellulose is the most abundant natural polysaccharide. It is found in the cell walls of algae (for instance *Valonia*) and that of higher plants, but also as an excretion product from certain bacteria (for instance *Acetobacter xylinum*). Although cellulose is composed of only ß-(1→4)-D-Glc*p* residues, its actual structure is much more complicated than this single building unit suggests. Generally, glucan chains derived from the primary cell walls of higher plants have a DP of 2000-6000 [21]. Several glucan chains (10 to 250) aggregate laterally by hydrogen bonding to form microfibrils, the width of which is species-dependent. Native celluloses have in common that they are composed of cellulose I, *i.e.* the chains have a parallel arrangement (all reducing chain ends point in one direction) [22,23]. Next to different (crystalline) allomorphs (I_α and $I_ß$) [24], cellulose structure is further diversified by the presence of amorphous regions within microfibrils. The former have a highly ordered structure whereas the molecules in the latter have a completely random orientation. The degree of crystallinity depends on the origin of the cellulose: 40-50% in wood and bacterial cellulose, *c.* 60% in cotton and up to 70% for some algae [25].

The enzymic conversion of cellulose by cellulase has been extensively studied. Cellulase preparations contain several glucanases, the majority of which are built of two domains: a catalytic core and a CBD [26]. Both parts of the enzyme are essential for the degradation of water-insoluble substrates like cellulose [27]. Endoglucanases cleave the amorphous regions of cellulose. By doing this, new chain ends are generated which are readily attacked by CBHs [28]. The latter proceed along the glucan chain, while splitting off G_2. In principle, cellulose can be completely degraded by this concerted action of glucanases. The ratio between endoglucanases and exoglucanases is very important in this process [28]. Until now, relatively little is known about the degradation of cellulose which is buried in the primary cell walls of plants.

Enzymes active towards xyloglucan

Although the primary structure of xyloglucan is greatly species-dependent, they all share a cellulosic ß-(1→4)-Glc*p* backbone, with α-Xyl*p* residues attached to the 6-position of Glc, as a common structural feature [9]. Branching of the backbone often follows a

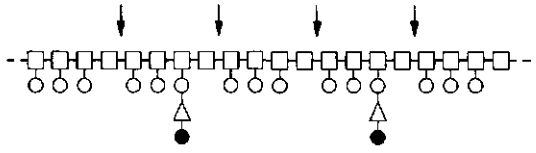

FIGURE 2. Schematic representation of pea xyloglucan [9]. □, ß-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; △, ß-D-Gal*p*-(1→2)-; ●, α-L-Fuc*p*-(1→2)-. Endoglucanase can cleave this xyloglucan molecule at the linkages indicated by arrows.

TABLE I. One-letter codes for differently-substituted ß-D-Glc*p* residues of xyloglucan oligosaccharides.

Structure represented	Code letter	Mnemonic
-ß-D-Glc*p*-	**G**	Glucose
-ß-D-Glc*p*- α-D-Xyl*p*-(1→6)-┘	**X**	Xylose
-ß-D-Glc*p*- ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-┘	**L**	gaLactose
-ß-D-Glc*p*- α-L-Fuc*p*-(1→2)-ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-┘	**F**	Fucose
-ß-D-Glc*p*- α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-┘	**S**	Solanaceae
α-L-Ara*f*-(1→2)-┐ -ß-D-Glc*p*- α-D-Xyl*p*-(1→6)-┘	**A**	Arabinose
ß-D-Xyl*p*-(1→2)-┐ -ß-D-Glc*p*- α-D-Xyl*p*-(1→6)-┘	**B**	Beta-xylose
α-L-Ara*f*-(1→3)-ß-D-Xyl*p*-(1→2)-┐ -ß-D-Glc*p*- α-D-Xyl*p*-(1→6)-┘	**C**	follows A and B

very regular pattern as is shown in Fig. 2. Next to α-D-Xyl*p*-(1→6)-sidechains, also ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-, α-L-Fuc*p*-(1→2)-ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-, and α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-sidechains occur. With the elucidation of more and more oligomeric structures, the configuration of these sidechains appeared to be very consistent for all xyloglucans studied. In the meantime, a complex system of trivial names for individual oligosaccharides was evolving as well. Because this was getting very confusing, an unambiguous nomenclature was developed [29]. As with peptide nomenclature, code letters were defined for slices of backbone, *i.e.* the sidechain(s) and the backbone ß-D-Glc residue itself. The code letters are explained in Table I. This nomenclature is used throughout this thesis.

Xyloglucans can be degraded by similar cellulase preparations as used for the degradation of cellulose. The glucan backbone is cleaved next to an unbranched Glc residue as is indicated in Fig. 2. Relatively little is known about the ability of the individual endoglucanases to degrade xyloglucan. The work of Renard [8] indicates that

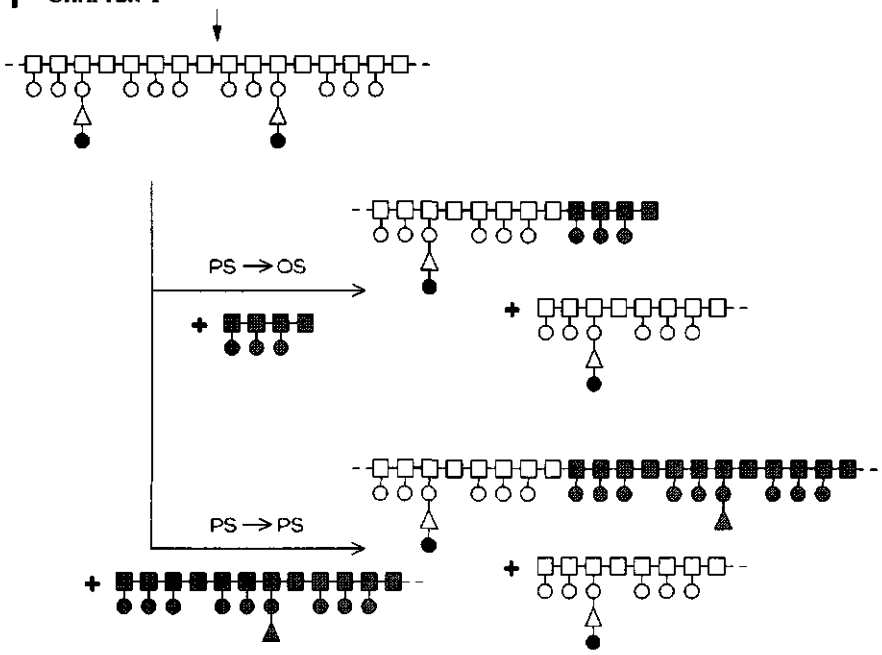

FIGURE 3. Schematic representation of transglycosylation reactions catalyzed by XET. Structure with open symbols represents the donor substrate; shaded molecules represent acceptor substrate. Symbols are explained in Fig. 2.

not all endoglucanases are equally efficient in doing this.

Whereas fungal endoglucanases are often active towards CMC and xyloglucan, plants seem to be equipped with highly specific xyloglucanases [30-33]. Until very recently, it was thought that some of these enzymes were activatable by xyloglucan oligosaccharides [31,32]. However, it appeared that these enzymes are actually transglycosylases [34-36]. The mechanism of action of xyloglucan endotransglycosylase (XET) is illustrated by Fig. 3. XET catalyzes the cleavage of a xyloglucan molecule (donor substrate), and attaches the nonreducing moiety of the donor molecule to a xyloglucan acceptor [37,38]. Only xyloglucan serves as a donor substrate, CMC and cellulose are not recognized [37]. XET activity decreases dramatically when the donor substrate becomes shorter [38]. Only very little activity was found on donor substrates of 10 kDa (consisting of *c.* 8 xyloglucan oligosaccharides). Polymeric xyloglucans (PS) as well as xyloglucan oligosaccharides (OS) serve as acceptor substrates for XET, although it is not known if these acceptors are equally effective. During PS→PS endotransglycosylation, the molecular weight distribution of xyloglucan molecules broadens, but the average molecular weight remains approximately the same [37,39]. PS→OS endotransglycosylation results in a strong decrease in molecular weight. This reaction has practically the same effect as endohydrolysis catalyzed by endoglucanases. The PS→OS endotransglycosylation also explains that some endoglucanases were thought to be oligosaccharide-activatable [31,32].

Several oligosaccharides have been tested as acceptor substrates. No transglycosylation was observed with G_2, G_4 to G_6, laminarihexaose, FG, XGG and GXG [37,38,40]. Some xyloglucan oligosaccharides appeared to be more efficient than others (efficiency in decreasing order XLLG, XXXG and XXFG) [37]. In a separate study it was shown that XXXG was a better acceptor than XXG [40]. It was concluded that XET requires two adjacent Xyl residues as in XXG, which is the smallest acceptor molecule.

AIM AND OUTLINE OF THIS THESIS

Two observations underlie the work described in this thesis. First, Renard [8] has shown that endoglucanases can differ in their specificity towards xyloglucans. With respect to liquefaction, this is an important observation because it implies that some endoglucanases are more suitable for tailor-made enzyme preparations than others. Beldman and coworkers [41] have purified six endoglucanases from a *Trichoderma viride* preparation. Only endoI and endoIV have been tested on isolated cell wall material from apple. There may be (a) better candidate(s) for tailor-made preparations among the four untested endoglucanases. Second, PG antisense tomatoes have failed to confirm that PG is primarily responsible for fruit softening [42]. Although a possible role for PME in the softening process was not taken into account, these experiments provide some evidence that pectin does not determine the strength of the cell wall. This is in accordance with new cell wall concepts, which predict an important role for xyloglucan in determining the coherence of the cell wall [10,11]. These new concepts suggest that xyloglucan crosslinks are the "Achilles heel" of the cell wall. It is expected that enzymes which are targeted to these crosslinks are of great importance for liquefaction.

The aim of this thesis is (i) to select the most suitable endoglucanase for liquefaction of apple fruit, (ii) to determine why some endoglucanases have xyloglucanase activity and others have little, (iii) to extrapolate our results, obtained by studying the degradation of isolated cell wall material, to native apple tissue. First, cell wall material was isolated from apples, and this material was treated with several combinations of glucanases (Chapter 2). It appears that xyloglucanase activity plays a role in fine-tuning the degradation of cellulose that is buried in the plant cell wall; cellulose degradation is enhanced considerably upon removal of its xyloglucan coating. The xyloglucan degradation products, which are released during these treatments are characterized, in Chapter 3 and 4. These results provide a "fingerprint" of apple xyloglucan, which is used throughout the rest of the thesis. Chapter 5 describes the isolation and (partial) characterization of a xyloglucan from potato. This polysaccharide has a less branched backbone and is used to study the mode of action of two endoglucanases with xyloglucanase activity. The question why some endoglucanases have xyloglucanase activity and others have little is addressed in Chapter 6. To assess this question, the number of substrate-binding sites of three different endoglucanases is determined, and

these results are related to their activity on xyloglucan. The adsorption of xyloglucan to cellulose was studied in Chapter 7. These experiments suggest that xyloglucan should be degraded extensively before cellulose surfaces in plant cell walls become accessible for cellulolytic enzymes. In order to investigate whether our results on isolated cell wall material could be extrapolated to the liquefaction process, the degradation of native apple tissue was studied in Chapter 8. In addition, it is shown that XET is present in apples and that this enzyme is active during liquefaction. Synergism between fungal xyloglucanases and XET is hypothesized, based on the observation that raw apple tissue disintegrates faster than blanched material. Compelling evidence for this theory is obtained in Chapter 9 where we show that XET in apple is ripening-related. Apples with a high XET level require less cellulase for liquefaction than those with low XET levels. A cellulose-based cloud can be formed under certain conditions in the liquefaction juice of apples having a high XET level. The implications of our work are discussed in Chapter 10.

REFERENCES

[1] Pilnik, W. (1982) Enzymes in the beverage industry. In *Use of Enzymes in Food Technology* (Dupuy, P., ed., Technique et Documentation Lavoisier, Paris), 425-449.

[2] Pilnik, W. (1988) From traditional ideas to modern fruit juice winning processes. In *International Congress of Fruit Juices* (Orlando, Florida), 159-179.

[3] Pilnik, W., Voragen, A.G.J., and de Vos, L. (1975) Enzymatische Verflüssigung von Obst und Gemüse. *Flüss. Obst* **42**, 448-451.

[4] Churms, S.C., Merrifield, E.H., Stephen, A.M., Walwyn, D.R., van der Merwe, K.J., Spies, H.S.C., Costa, N., and Polson, A. (1983) An L-arabinan from apple juice concentrates. *Carbohydr. Res.* **113**, 339-344.

[5] Voragen, A.G.J., Geerst, F., and Pilnik, W. (1982) Hemicellulases in enzymic fruit processing. In *Use of Enzymes in Food Technology* (Dupuy, P., ed., Technique et Documentation Lavoisier, Paris), 497-502.

[6] Rombouts, F.M., Voragen, A.G.J., Searle-van Leeuwen, M.F., Geraeds, C.C.J.M., Schols, H.A., and Pilnik, W. (1988) The arabinanases of *Aspergillus niger* - Purification and characterisation of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinanase. *Carbohydr. Polym.* **9**, 25-47.

[7] Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F., Schols, H.A., and Pilnik, W. (1987) The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. *Food Hydrocoll.* **1**, 423-437.

[8] Renard, C.M.G.C. (1989) Etude des polysaccharides parietaux de la pomme. Extraction et caracterisation par des methodes chimiques et enzymatiques. PhD Thesis, University of Nantes, France.

[9] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[10] McCann, M.C., and Roberts, K. (1991) Architecture of the primary cell wall. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed., Academic Press, New York), 109-129.

[11] Carpita, N.C., and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**, 1-30.

[12] Keegstra, K., Talmadge, K.W., Bauer, W.D., and Albersheim, P. (1973) Structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.* **51**, 188-197.

[13] McCann, M.C., Wells, B., and Roberts, K. (1990) Direct visualization of cross-links in the primary plant cell wall. *J. Cell Sci.* **96**, 323-334.

[14] Carpita, N.C., Sabularse, D., Montezines, D., and Delmer, D.P. (1979) Determination of the pore size of cell walls of living plants. *Sci.* **205**, 1144-1147.

[15] Baron-Epel, O., Gharyal, P.K., and Schindler, M. (1988) Pectins as mediators of wall porosity in soybean cells. *Planta* **175**, 389-395.

[16] Taylor, I.E.P., and Atkins, E.D.T. (1985) X-ray diffraction studies on the xyloglucan from tamarind (*Tamarindus indica*) seed. *FEBS Lett.* **181**, 300-302.
[17] McCann, M.C., Wells, B., and Roberts, K. (1992) Complexity in the spatial localization and length distribution of plant cell-wall matrix polysaccharides. *J. Microscopy* **166**, 123-136.
[18] Shedletzky, E., Shmuel, M., Delmer, D.P., and Lamport, D.T.A. (1990) Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. *Plant Physiol.* **94**, 980-987.
[19] Shedletzky, E., Shmuel, M., Trainin, T., Kalman, S., and Delmer, D.P. (1992) Cell wall structure in cells adapted to growth on the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile. A comparison between two dicotyledonous plants and a graminaceous monocot. *Plant Physiol.* **100**, 120-130.
[20] Wells, B., McCann, M.C., Shedletzky, E., Delmer, D., and Roberts, K. (1994) Structural features of cell walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. *J. Microscopy* **173**, 155-164.
[21] Delmer, D.P. (1987) Cellulose biosynthesis. *Annu. Rev. Plant Physiol.* **38**, 259-290.
[22] Kroon-Batenburg, L.M.J., and Kroon, J. (1995) The crystal and molecular structures of cellulose. *Carbohydr. in Eur.* **12**, 15-19.
[23] Delmer, D.P., and Amor, Y. (1995) Cellulose biosynthesis. *Plant Cell* **7**, 987-1000.
[24] Heiner, A.P., Sugiyama, J., and Teleman, O. (1995) Crystalline cellulose Iα and Iß studied by molecular dynamics simulation. *Carbohydr. Res.* **273**, 207-223.
[25] Lin, J.S., Tang, M.-Y., and Fellers, J.F. (1987) Fractal analysis of cotton cellulose as characterized by small-angle X-ray scattering. In *The Structures of Cellulose* (Atalla, R.H., ed., Washington), *ACS Symposium Series* **340**, 233-254.
[26] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., and Warren, R.A.J. (1991) Domains of microbial ß-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**, 303-315.
[27] Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. (1988) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* **170**, 575-581.
[28] Beldman, G., Voragen, A.G.J., Rombouts, F.M., and Pilnik, W. (1988) Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from *Trichoderma viride*. *Biotechnol. Bioeng.* **31**, 173-178.
[29] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.
[30] Edwards, M., Dea, I.C.M., Bulpin, P.V., and Reid, J.S.G. (1986) Purification and properties of a novel xyloglucan-specific endo-(1→4)-ß-D-glucanase from germinated nasturtium seeds (*Tropaeolum majus* L.). *J. Biol. Chem.* **261**, 9489-9494.
[31] Farkas, V., and Maclachlan, G. (1988) Stimulation of pea 1,4-ß-glucanase activity by oligosaccharides derived from xyloglucan. *Carbohydr. Res.* **184**, 213-219.
[32] Maclachlan, G., and Brady, C. (1992) Multiple forms of 1,4-ß-glucanase in ripening tomato fruits include a xyloglucanase activatable by xyloglucan oligosaccharides. *Aust. J. Plant Physiol.* **19**, 137-146.
[33] Fry, S.C. (1995) Polysaccharide-modifying enzymes in the plant cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 497-520.
[34] Farkas, V., Sulova, Z., Stratilova, E., Hanna, R., and Maclachlan, G. (1992) Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. *Arch. Biochem. Biophys.* **298**, 365-370.
[35] Fanutti, C., Gidley, M.J., and Reid, J.S.G. (1993) Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1→4)-ß-D-glucanase) from the cotelydons of germinated nasturtium seeds. *Plant J.* **3**, 691-700.
[36] Maclachlan, G., and Brady, C. (1994) Endo-1,4-ß-glucanase, xyloglucanase and xyloglucan endo-transglycosylase activities versus potential substrates in ripening tomatoes. *Plant Physiol.* **105**, 965-974.
[37] Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828.
[38] Nishitani, K., and Tominaga, R. (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J. Biol. Chem.* **267**, 21058-21064.
[39] Nishitani, K. (1992) A novel method for detection of endo-xyloglucan transferase. *Plant Cell Physiol.* **33**, 1159-1164.
[40] Lorences, E.P., and Fry, S.C. (1993) Xyloglucan oligosaccharides with at least two α-D-xylose residues act as acceptor substrates for xyloglucan endotransglycosylase and promote the depolymerisation of xyloglucan. *Physiol. Plant.* **88**, 105-112.

[41] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.

[42] Smith, C.J.S., Watson, C.F., Ray, J., Bird, C.R., Morris, P.C., Schuch, W., and Grierson, D. (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* **334**, 724-726.

CHAPTER 2

The Effect of Xyloglucans on the Degradation of Cell Wall Embedded Cellulose by the Combined Action of Cellobiohydrolase and Endoglucanases from *Trichoderma viride*[I]

Two endoglucanases of *Trichoderma viride*, endoI and endoIV, were assayed for their activity towards alkali-extracted apple xyloglucans. EndoIV was shown to have a 60-fold higher activity towards xyloglucan than endoI, whereas both enzymes showed a similar activity towards carboxymethyl cellulose. The enzymic degradation of cellulose embedded in the complex cell wall matrix of apple fruit tissue has been studied using cellobiohydrolase (CBH) and these two different endoglucanases. A high-performance liquid chromatographic method was used to monitor the release of cellobiose and oligomeric xyloglucan fragments. Synergistic action between CBH and endoglucanases on cell wall embedded cellulose was, with respect to their optimal ratio, slightly different from that reported for crystalline cellulose. The combination of endoIV and CBH solubilized twice as much cellobiose compared to a combination of endoI and CBH. Apparently, the concomitant removal of the xyloglucan coating from cellulose microfibrils by endoIV is essential for an efficient degradation of cellulose in a complex matrix. Cellulose degradation slightly enhanced the solubilization of xyloglucans. These results indicate optimal degradation of cell wall embedded cellulose by a three-enzyme system consisting of an endoglucanase with high affinity towards cellulose (endoI), a xyloglucanase (endoIV), and CBH.

Substrates such as the wood cellulose preparation Avicel have often been used to demonstrate the synergistic action of CBH[II] and endoglucanases in the enzymic degradation of cellulose to G_2. Although several mechanisms to explain this synergism have been proposed [1-4], there is still debate concerning the exact mechanism of cellulose hydrolysis. The adsorption behavior of the various endoglucanases on crystalline cellulose seems to play an important role and explains, at least qualitatively, the difference in optimal endoglucanase:CBH ratio in solution [2,3,5].

For the liquefaction of apple fruit tissue, efficient degradation of the bulk component of the primary cell wall, cellulose, is essential. Contrary to Avicel the accessibility of cell wall embedded cellulose is reduced by the presence of other cell wall constituents such as pectin and xyloglucan. Several authors [6,7] have demonstrated that the removal of pectin by purified pectolytic enzymes facilitates cellulose degradation by cellulases. Xyloglucans have received relatively little attention in this respect, although they hydrogen bond tightly to cellulose [8-10] and are also bound to other cell wall components [8,11]. Furthermore, xyloglucans seem to play a key role in cell growth [12-15] and ripening [16]. Therefore, their removal might not only facilitate cellulose degradation but might also be crucial for a

---

[I]Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) *Plant Physiol.* **104**, 99-107.

[II]*Abbreviations:* see page viii.

total collapse of the cohesion between cell wall polymers.

With few exceptions [17-19], endoglucanases from plant and fungal origin are capable of hydrolyzing xyloglucan as well as CMC. The cellulase complex of *Trichoderma viride* has been shown to contain six distinct endoglucanases [20], which can be subdivided into a specific (endoI, endoII, and endoIII) and a nonspecific (endoIV, endoV, and endoVI) class based on their ability to degrade xylans as well. A member of each class (endoI and endoIV) has been tested on apple cell wall material, and the latter was found to have a more pronounced action [7,21]. In the present study the ability of both enzymes to degrade apple xyloglucan was assessed, and we discuss the consequences thereof for the enzymic degradation of cell wall embedded cellulose.

EXPERIMENTAL

Materials

Raw materials.—Apples (*Malus malus* L., Rosaceae, *cv.* Golden Delicious) were harvested in mid-October, 1989, and stored in a controlled atmosphere (2°C, 2% O_2 and 5% CO_2) for 4 months. CMC (Akucell AF type 0305) was obtained from Akzo (Arnhem, The Netherlands).

Enzymes.—Two endoglucanases (endoI and endoIV; EC 3.2.1.4) and one CBH (EC 3.2.1.91), previously referred to as exoIII [20], were purified from a commercial preparation from *Trichoderma viride* (Maxazyme Cl; Gist-Brocades, Delft, The Netherlands) as described by Beldman *et al.* [20].

Purification of APfxg

Isolation of WUS.—After the cores were removed, the apples (5 kg) were microwaved (AKB 276/PH, 4.2 kW; Philips, Eindhoven, The Netherlands) for 2 min at 2 kW (effective power output) in 1-kg portions to inactivate endogenous enzymes (PG and PME). The resulting material was peeled, ground, and extracted extensively with warm distilled water (50 °C) until the wash water contained minimal amounts of sugars. Solids were collected by centrifugation (20 min, 50,000 *g*), and the residual material was freeze-dried and ground in a pulverisette 14 (sieve 1.0 mm; Fritsch, Idar-Oberstein, Germany) and designated as WUS.

Extraction of WUS.—WUS (2 g) was extracted with 0.05 M NaOH (200 mL) containing 5 mM CDTA for 16 h at 4 °C under continuous stirring. After the material was centrifuged (20 min, 50,000 *g*) the residue was resuspended in 1 M KOH (320 mL) containing 1% (w/w) $NaBH_4$, extracted for 16 h at 20 °C and centrifuged (20 min, 50,000 *g*). The latter procedure was repeated with this residue and 4 M KOH containing 1% (w/w) $NaBH_4$. Between sequential extraction steps, the residue was washed twice with distilled water. The final residue was acidified to pH 5 with HCl and freeze dried. The corresponding supernatants were collected, acidified to pH 5 with HCl and dialyzed extensively against distilled water [22].

Purification of the 4 M KOH extract.—The 4 M KOH extract was depectinized on a DEAE Sepharose CL-6B column (40 x 440 mm, Pharmacia, Uppsala, Sweden), equilibrated with 50 mM NaOAc buffer (pH 5.0). After the sample (200 mL) was applied, the column was washed with 400 mL of buffer. The fraction retained on the column was released by elution with 500 mL of 1 M NaOAc buffer (pH 5.0). Fractions (16.5 mL) were assayed for both uronic acids and neutral sugar, pooled accordingly, dialyzed and freeze-dried. The neutral fraction consisted of xyloglucan.

Incubations with purified enzymes

All incubations were carried out in 100 mM succinate buffer (pH 4) containing 0.01% (w/v)

NaN_3 at 40 °C. For each incubation the amount of protein (μg or ng) as well as the corresponding catalytic activity (mU; pH 4; 40 °C) towards CMC are indicated.

Xyloglucan degradation.—The purified xyloglucan (250 μg) was dissolved in 100 μL of buffer and incubated for 1 or 20 h with endoI (400 ng, 10 mU) or endoIV (30 ng, 1 mU), respectively. Enzyme dosage was such that no substrate limitation occurred. The samples were then diluted twice, and the increase in reducing sugars was determined according to the procedure of Somogyi [23] using Glc for calibration. Similar experiments were done using CMC as a substrate.

Enzymic degradation of WUS.—Incubations were carried out in triplicate with 20 mg of WUS, suspended in a total volume of 1.5 mL of buffer using head-over-tail mixing. Incubations contained 60 μg (29 mU) of CBH protein, and the dosage of endoI and endoIV {3 (78 mU) and 13 μg (383 mU), respectively} was based on their activities towards CMC at pH 4 and 40 °C, and was theoretically sufficient to hydrolyze the cellulose part to Glc within 12 h. After 24 h the reaction was either stopped or continued for another 24 h with or without enzyme addition. After incubation the samples were centrifuged (1 min, 20,000 *g*) and all supernatants were assayed for uronic acids and total neutral sugar. Sugar composition as well as mol wt distributions were determined only for the supernatants of the 48-h incubations with second enzyme addition.

Synergism between endoI and/or IV and CBH.—For time-course studies, 20 mg of WUS were suspended in a total volume of 1.5 mL of buffer containing 6.4 μg (3 mU) of CBH and 1 μg of endoI (26 mU) or endoIV (30 mU) {endoglucanase:CBH ratio of 0.16 ($\mu g\ \mu g^{-1}$)} and incubated for defined time intervals up to 24 h. A second series differed from the above in incubation time (3 h) and a varying endoglucanase (endoI or endoIV) amount {endoglucanase:CBH ratios ranging from 0.02-30 ($\mu g\ \mu g^{-1}$)}. In a last series endoI and endoIV were mixed at fixed endoglucanase (6.1 μg, 160-180 mU) and CBH (6.4 μg, 3 mU) quantities and also incubated for 3 h. The course of cellulose degradation was checked for linearity at a endoglucanase:CBH ratio of 5 ($\mu g\ \mu g^{-1}$). After the incubation mixtures were heated for 10 min at 100 °C to inactivate the enzymes and centrifuged (2 min, 20,000 *g*), the degradation products in the supernatant were analyzed by HPLC.

Analytical methods

Uronic acid content.—Uronic acids were estimated colorimetrically with an automated *m*-hydroxydiphenyl test [24] using concentrated H_2SO_4 containing 0.0125 M $Na_2B_4O_7$. Water-insoluble material was pretreated with 72% (w/w) H_2SO_4 (1 h at 30 °C) and, after dilution with water, degraded by 1 M H_2SO_4 (3 h at 100 °C) prior to analysis. GalA was used as a standard.

Total neutral sugar content.—The total neutral sugar content was determined colorimetrically with an automated orcinol/H_2SO_4 assay [25]. Glc was used as a standard.

Neutral sugar composition.—Water-insoluble material was subjected to a 72% (w/w) H_2SO_4 prehydrolysis (1 h at 30 °C) followed, after dilution with water, by a 1 M H_2SO_4 hydrolysis (3 h at 100 °C). Water-soluble material was hydrolyzed with 2 M TFA (1 h at 121 °C) [26]. Next, the released neutral sugars were converted into their alditol acetates [27] and separated on a 3-m x 2-mm i.d. glass column (packed with Chrom WAW 80-100 mesh, coated with 3% OV275, Chrompack, Middelburg, The Netherlands) in a Carlo Erba Fractovap 2300 gas chromatograph (Milan, Italy) operated at 200 °C and equipped with a flame ionization detector set at 270 °C. Inositol was used as the internal standard.

Glycosyl linkage composition.—The purified xyloglucan was methylated according to a modification of the Hakomori method [28] and subsequently dialyzed against water and dried by evaporation (airstream, room temperature). This procedure was repeated once. Next, the methylated xyloglucan was hydrolyzed using 2 M TFA (1 h at 121 °C), which was removed by evaporation (airstream, room temperature). Sugars were reduced by adding 0.2 mL of a freshly prepared 1.5 M ammonia solution containing 75 mg $NaBD_4$ mL^{-1}, and converted into alditol acetates [27]. The partially methylated alditol acetates (1 μL) were analyzed by on-column injections on a fused silica capillary column (30-m x 0.32-mm i.d.; wall coated with DB 1701;

0.25-μm film thickness; J & W Scientific, Folsom, CA, USA) in a Carlo-Erba Fractovap 4160 gas chromatograph equipped with a flame ionization detector set at 280 °C. The temperature program was 80→180 °C at 20 °C min^{-1}, 180→230 °C at 2 °C min^{-1}, and 230 °C for 3 min. Identification of the compounds was confirmed by GC-MS using a CP Sil 19 CB capillary column (26-m x 0.22-mm i.d., 0.18-μm film thickness; Chrompack Nederland B.V., Middelburg, The Netherlands) in an HP 5890 gas chromatograph coupled to a Hewlett-Packard mass-selective detector 5970-B and using a PAW-HP 300 Chem Station (Hewlett-Packard). The temperature program was 160→185 °C at 0.5 °C min^{-1}, 185→230 °C at 10 °C min^{-1}, and finally 230 °C for 5.5 min. Derivatives were quantified according to their effective carbon response [29].

Protein content.—The protein content of WUS (N x 6.25) was determined by a semiautomated micro-Kjeldahl method. Soluble proteins (enzyme preparations, and the 4 M KOH extract) were quantified according to the method of Sedmak and Grossberg [30]. In this case, BSA was used as a standard.

DA and DM.—The degree of acetyl and methyl esterification of the isolated WUS was estimated by HPLC using the procedure described by Voragen *et al.* [31].

Analysis of xyloglucan and cellulose degradation products.—HPLC was conducted at 85 °C with an SP 8800 HPLC pump system (Spectra Physics, San José, CA, USA) fitted with a Shodex SE-61 refractometer (Showa Denko K.K., Tokyo, Japan) and an Aminex HPX-22H column (7.8 x 300 mm; Bio-Rad, Richmond, CA, USA) combined with an AG 50W X4 guard column (7.8 x 50 mm, Bio-Rad). The solvent was 5 mM H_2SO_4 pumped at a flow rate of 0.2 mL min^{-1}. The injection volume was 20 μL. Maltodextrins were used for calibration. The mol wt distribution of released xyloglucan fragments was studied using HPSEC, which was performed on a SP 8800 HPLC pump system (Spectra Physics) equipped with three BioGel TSK columns in series (40XL, 30XL, 20XL; 300 x 7.5 mm; Bio-Rad) in combination with a TSK-XL guard column (40 x 6 mm) at 30 °C. Aliquots of 20 μL were injected and subsequently eluted with 0.4 M NaOAc (pH 3.0) at a flow rate of 0.8 mL min^{-1}. The effluent was monitored using a Shodex SE-61 refractometer. This system was calibrated using dextran standards (10, 40, 70, 500 kDa; Pharmacia).

RESULTS

Extraction of apple material

After extensive water extractions *c.* 1% of the microwaved, ground apple material was recovered as WUS. The WUS consisted predominantly of sugars {*c.* 90% (w/w)}; the sugar composition is shown in Table I. During heat treatment of the apple fruit material much of the protein probably denatured and failed to dissolve in subsequent water washes. This might explain the relatively high protein content of 8% (w/w). The DM and DA of the WUS were calculated as a molar percentage of the GalA moiety, although acetyl groups have also been reported to be present on the Gal residues of sycamore xyloglucans [32]. Approximately 60% of the GalA residues contained a methyl group, and 26% contained acetyl groups, based on monosubstitution of the GalA residues.

The WUS was subjected to a series of extractions. Most of the material was recovered in four different fractions: 0.05 M NaOH {5% (w/w)}, 1 M KOH {3% (w/w)}, 4 M KOH {8% (w/w)}, and the residue {80% (w/w)}. The 0.05 M NaOH extract consisted mainly of pectic material; the bulk of the xyloglucans was extracted by 4 M KOH (Table I and II). The residue contained mainly cellulose, although pectic material and xyloglucans

TABLE I. Sugar composition (mol%) of WUS and WUS fractions obtained by sequential extractions.

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
WUS	1	1	13	10	2	9	42	22
0.05 M NaOH	6	–	47	4	1	18	1	23
1 M KOH	1	3	15	21	4	13	31	12
4 M KOH	1	5	5	29	3	12	40	5
Residue	1	1	13	8	3	8	60	6

were still present in relatively large amounts. Both of the extraction yields and sugar compositions are in agreement with the findings of others [33,34].

Purification and degradation of apple xyloglucan

The pectic material of the 4 M KOH extract could effectively be removed from neutral polysaccharides by anion-exchange chromatography. The latter was further characterized by methylation analysis (Table II). The 1,4,6-linked Glc, 1,2-linked Xyl, terminal Xyl, 1,2-linked Gal, and terminal Gal, which are typical for xyloglucans, formed an important part of this fraction. Although the protein content of the WUS was relatively high, no protein was recovered in the neutral fraction of the 4 M KOH extract. The terminal Fuc (*c.* 6%) was therefore attributed to xyloglucan rather than to a glycoprotein [35]. Fucosylated oligosaccharides have been demonstrated in similarly extracted apple xyloglucan [36], which supports the above conclusion. This fraction is, therefore, referred to as APfxg. This xyloglucan had a degree of backbone substitution (C-6) of 65%. The APfxg was slightly contaminated with 1,4-linked Xyl (1%) and a branched (*c.* 10%) Man-

TABLE II. Glycosyl linkage composition of APfxg.

Deduced linkage	Molar ratio	Total
L-Ara*f*-(1→*	1	1
L-Fuc*p*-(1→	6	6
D-Gal*p*-(1→	5	
→2)-D-Gal*p*-(1→	8	13
D-Xyl*p*-(1→	18	
→2)-D-Xyl*p*-(1→	10	
→4)-D-Xyl*p*-(1→	1	29
→6)-D-Glc*p*-(1→	1	
→4)-D-Glc*p*-(1→	15	
→4,6)-D-Glc*p*-(1→	29	45
→4)-D-Man*p*-(1→	5	
→4,6)-D-Man*p*-(1→	1	6

* Determined as 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, *etc.*.

containing polysaccharide (*c.* 6%). It should be noted that a small percentage of undermethylation, especially of Glc, occurred. This was not further quantified because of poor separation of Glc and inositol.

APfxg eluted as one population (mol wt *c.* 2,000 kDa) on HPSEC (data not shown). Upon treatment with the highly purified endoIV, this material was almost completely degraded to yield oligomers that eluted in the shaded peaks, as shown in Fig. 1B (Aminex HPX 22H). Although the activity of endoI and endoIV towards CMC was of the same order of magnitude (Table III), the latter had a 60-fold higher activity towards APfxg.

Action of enzymes on WUS, individually and in combinations

The enzymic degradation of cell wall embedded cellulose by CBH is a slow process. Similar amounts of neutral sugars were solubilized during the first and second 24 h of incubation. Addition of fresh enzyme enhanced the degradation rate, indicating enzyme limitation (data not shown). The solubilization of almost 50% of the cell wall Glc after 48 h by CBH, together with large amounts of Xyl and Gal (Table IV) could not be explained by cellulose degradation alone. No oligomeric degradation products other than G_2 were observed (data not shown), but HPSEC revealed some polymeric material

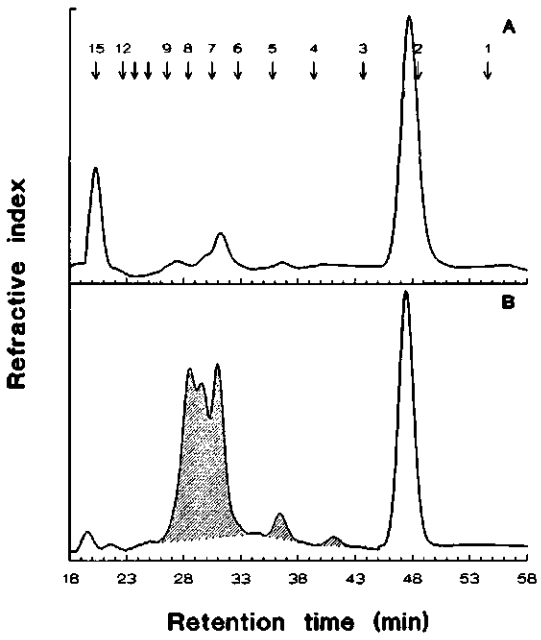

FIGURE 1. Typical chromatograms showing the degradation products of apple WUS solubilized by combinations of glucanases (24 h) and analyzed on an Aminex HPX 22H column. A, endoI plus CBH; B, endoIV plus CBH. The elution profile of a maltodextrin syrup is indicated by arrows, with the corresponding DP. Typical degradation products of endoIV are indicated by the shaded peaks (B). The sum of these areas was used for quantification of the xyloglucan oligosaccharides.

TABLE III. Some properties of the endoglucanases and CBH purified from *Trichoderma viride*.

Property	endoI	endoIV	CBH
Turnover number of CMC (min^{-1})	1310	690	nd*
Turnover number of APfxg (min^{-1})	9	562	≪1
Turnover number of Avicel (min^{-1})†	0.65	0.06	0.48
Adsorption (mg of protein mg^{-1} of cellulose)‡	0.126	0.003	0.063
Mol wt (kDa)†	50	23.5	62

* Not determined. † Values according to Beldman *et al.* [20]. ‡ Values according to Beldman *et al.* [5].

TABLE IV. Action of glucanases on apple WUS, individually and in combinations, at pH 4.0 and 40 °C.

Glucanase	Total % solub.*	Percentage of individual sugars solubilized†							
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
Blank	4	6	0	8	0	0	5	1	8
endoI	10	8	30	9	18	19	13	7	10
endoIV	16	15	56	14	38	6	22	13	15
CBH	32	10	55	9	45	69	23	46	16
endoI + CBH	41	13	30	11	54	100	29	62	19
endoIV + CBH	51	16	87	15	74	100	36	76	23

* Calculated as the sum of neutral sugars and uronic acid solubilized, divided by their quantities originally present in the WUS. † Calculated as the percentage of the individual sugars present in the WUS.

(mol wt > 2,000 kDa; data not shown). Possibly degradation of cellulose leads to structural changes, resulting in partial solubilization of the xyloglucan. The solubilization of Man seems somehow related to CBH, but the role of Man within the cell wall is still unclear.

The endoglucanases were mainly active towards xyloglucans. EndoIV was most effective; it solubilized *c.* 40% of the xyloglucans, whereas endoI solubilized only 20% as can be derived from the percentage of Xyl shown in Table IV. Supplementation with enzyme after 24 h did not affect this quantity significantly. Therefore, it can be concluded that not all xyloglucan is accessible to the enzymes. EndoIV also formed smaller end products than endoI, typically ranging from DP 5 to 9 (Fig. 1, A and B). Minor amounts of degradation products of endoI were found in the DP range of 6 to 7; however, most of the material excluding G_2 eluted in the void of the column (DP > 15). Neither endoI nor endoIV solubilized any G_2 from WUS (data not shown) demonstrating that both enzymes were free of CBH activity. In fact, no typical cellulose degradation products (Glc, G_2, and G_3), as reported by Beldman *et al.* [5] for Avicel crystalline cellulose, were found. However, the presence of xyloglucan, being a better substrate for the endoglucanases than cellulose (Table III) as well as a physical barrier for cellulose degradation, might explain

this discrepancy. Both factors reduce the probability of releasing cellodextrins by a endoglucanase-type of action. Unlike endoI, endoIV also solubilized some pectic material. Because both enzymes were devoid of pectolytic side activities, the release of pectic material indicated interactions between pectins and xyloglucans, as suggested by several authors [7,8].

Most effective in the degradation of WUS were combinations of endoglucanases and CBH, especially endoIV plus CBH (Table IV). Supplementation with fresh endoIV plus CBH and endoI plus CBH resulted in the solubilization of an additional 10 and 50% of the neutral sugars, respectively, during the period of 24 to 48 h, showing that the potential of endoIV plus CBH has been fully utilized (data not shown). Complete solubilization of the Glc-containing polysaccharides could not be achieved by this enzyme combination.

The effect of the endoglucanase:CBH ratio on G_2 solubilization

The rate of G_2 production from WUS by CBH remained constant during 24 h (Fig. 2A). Addition of equal amounts of endoI and endoIV to a similar quantity of CBH enhanced G_2 release more than 5-fold. After 12 h the rate of cellulose hydrolysis declined. Inhibition of CBH by G_2 [37] could be an explanation for this phenomenon.

The solubilization of oligomeric xyloglucans from WUS was monitored by integrating the shaded areas in Fig. 1B. Initially, the degradation of xyloglucans proceeded rather rapidly (Fig. 2B). After 5 h the rate of product formation started to level off, presumably due to a gradual decrease in accessibility of the remaining xyloglucan. A combination of

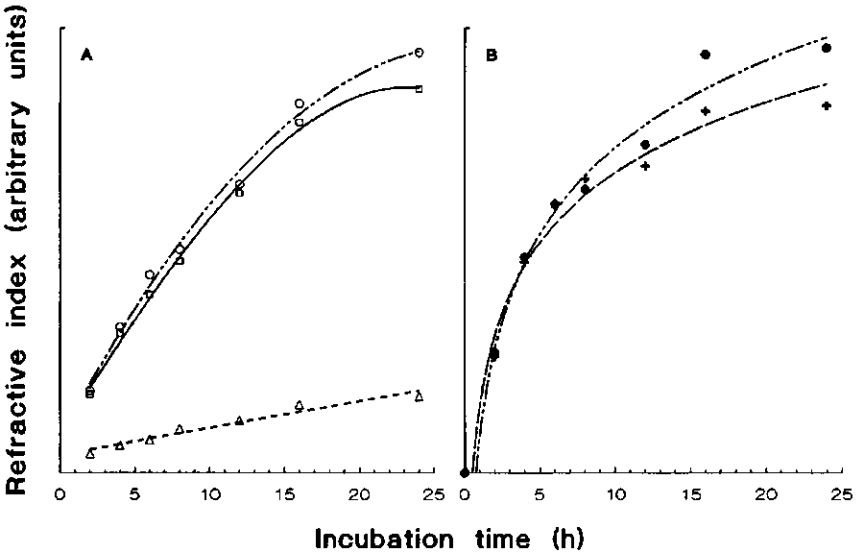

FIGURE 2. Time-courses of the release of G_2 (A) and oligomeric xyloglucan fragments (B) from apple WUS by various glucanase combinations. The endoglucanase:CBH mass ratio was 0.16 ($\mu g\ \mu g^{-1}$). △, CBH; □, endoI plus CBH; ○, endoIV plus CBH; ●, endoIV plus CBH; +, endoIV.

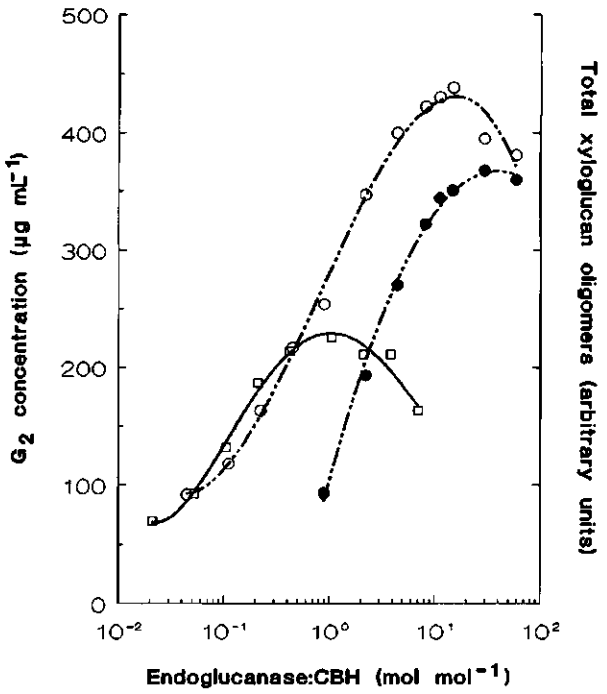

FIGURE 3. The influence of the endoglucanase:CBH ratio on the release of G_2 and oligomeric xyloglucan fragments from apple WUS. □, G_2 release by endoI plus CBH; ○, G_2 release by endoIV plus CBH; ●, release of xyloglucan oligosaccharides by endoIV plus CBH.

endoIV plus CBH solubilized more oligomers than endoIV alone. Although this is just a minor effect, it might indicate that concomitant degradation of cellulose slightly enhances xyloglucan degradation. This is consistent with the observation that the action of CBH alone solubilized only small amounts of polymeric xyloglucan. The action of endoI (plus CBH) on WUS was omitted from consideration because only prolonged incubations solubilized minor amounts of oligomeric material, mainly with a DP > 15.

In a second series of experiments the effect of the endoglucanase:CBH ratio on the rate of cellulose degradation was studied. At relatively low endoglucanase:CBH ratios the two endoglucanases showed similar behavior with respect to the degradation of cellulose, as shown in Fig. 2A. Higher ratios, however, revealed a marked difference between endoI and endoIV (Fig. 3). Addition of endoI to a fixed amount of CBH increased the rate of cellulose hydrolysis until a molar ratio of 1.1 {equal to a endoI:CBH mass ratio of 0.96 (μg μg^{-1})} was reached. After this point the hydrolysis rate decreased quickly. A combination of endoIV and CBH showed a similar pattern, with cellulose hydrolysis being optimal at a molar ratio of 14.9. Twice as much G_2 was produced under these conditions. Xyloglucan hydrolysis increased proportionally to the endoIV:CBH ratio (and thus the amount of endoIV) until a ratio of *c.* 17 was reached. After this point, formation of

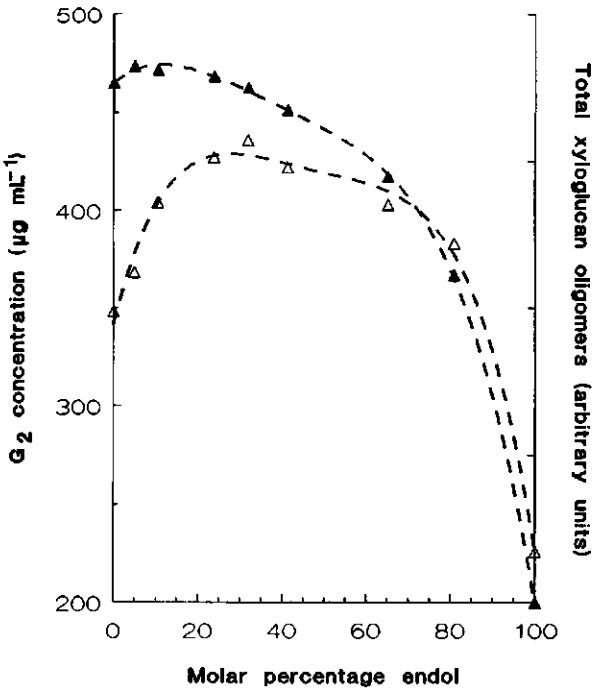

FIGURE 4. Formation of G_2 (△) and xyloglucan oligomers (▲) in a three-enzyme system at a fixed (endoI plus endoIV):CBH mass ratio of 0.96 ($\mu g\ \mu g^{-1}$) and varying amounts of endoI and endoIV.

oligomers from xyloglucan did not increase further. Typically, maximal xyloglucan hydrolysis coincided with optimal cellulose hydrolysis.

These data showed that optimal synergism with CBH was obtained with relatively small amounts of endoI, although cellulose hydrolysis proceeded less favorably than with endoIV plus CBH. The endoglucanases seemed to act differently on the cell wall material. Therefore, endoI and endoIV were added to a fixed amount of CBH at various molar endoI:endoIV ratios but at a constant endoglucanase:CBH mass ratio of 0.96, identical with the optimal endoI:CBH ratio (as shown in Fig. 3). Because of the difference in mol wt of the endoglucanases, the "endoglucanase-molarity" was not constant in this experiment. However, we showed previously that G_2 formation is attributable solely to CBH.

All combinations tested, using the two different endoglucanases in combination with CBH, increased the degree of cellulose hydrolysis (Fig. 4). At the optimal endoI:endoIV ratio for cellulose degradation of 0.33, the degree of hydrolysis was increased a factor 2 and 1.25 when compared to digestion by the two-enzyme systems endoI plus CBH and endoIV plus CBH, respectively. G_2 formation at this point equaled the amount reached at optimal endoIV:CBH ratio (Fig. 3); however, in this case a 7- to 8-fold smaller

endoglucanase dose was needed. Furthermore, it should be mentioned that the optimum is rather broad, indicating that a portion of only 20 mol% of either endoglucanase gave an almost maximal effect. The sharp decrease in the G_2 formation at 80% endoI is accompanied by a strong decrease in xyloglucan hydrolysis.

DISCUSSION

From the sugar composition (Table I) information concerning the constituent polysaccharides can be deduced as follows. Assuming all Xyl is present in xyloglucan [38], which has a Glc:Xyl:Gal:Fuc ratio of 7:5:2:1 (Table II), the xyloglucan fraction makes up about 24% (w/w) of the total amount of sugar, a value generally found for dicotyledons [6]. The sugar composition of the purified APfxg is consistent with the findings of others [6,21,33,39] for dicotelydons. All Ara and the remaining part of the Gal {16% (w/w)} are most likely associated with a rhamnogalacturonan in the pectic moiety of 41% (w/w). The remaining part of the Glc {33% (w/w)} is thought to be present as cellulose. This emphasizes the importance of the cellulose-xyloglucan complex, which accounts for *c.* 57% of the apple cell wall matrix. For some applications in fruit juice processing efficient enzymic degradation of this complex might be crucial. This principally involves at least two enzymes, *viz.* endoglucanase and CBH. Table IV shows that even the most effective combination of endoIV plus CBH is not able to solubilize all cellulose. One reason for this might be the inhibition of CBH by G_2. Lee and Fan [37] found that cellulose hydrolysis decreased more than 6-fold at a G_2 concentration of 30 g L^{-1}. The G_2 concentration in these experiments exceeded this value, indicating that a strong inhibitory effect is to be expected. Also, the presence of pectic material, limiting the accessibility of the cellulose-xyloglucan complex, will play a role regarding the strong synergism between pectolytic and cellulolytic enzymes observed by several authors [7,21,40].

Table III shows that, although endoglucanases have similar activities towards CMC, their activities towards xyloglucan differ. Hayashi *et al.* [41] tested two endoglucanases, extracted from pea epicotyls, for their ability to degrade CMC and pea xyloglucan. Unlike that of endoIV, the V_{max} values of these enzymes were lower for xyloglucan. Maclachlan and Brady [19] indicated that ripening tomato fruits contain multiple forms of 1,4-ß-glucanase, some of them being activated by xyloglucan oligosaccharides. Activation of endoI by oligosaccharides was not further investigated in the present study.

The degradation of cellulose has been the subject of numerous studies [1-3]. Generally, the synergistic action between endoglucanases and CBH is measured by determining the increase in reducing end groups upon degradation of Avicel. In a complex matrix like WUS, however, this is not a proper approach, because both G_2 and oligomeric xyloglucan fragments contribute to the amount of reducing end groups. Therefore, an HPLC method was used to determine both G_2 and xyloglucan oligomers quantitatively. Fig. 1B shows a

good separation of G_2 and xyloglucan oligosaccharides. The separation for the latter compounds is, however, less satisfactory. Therefore, only the total amount of oligomeric xyloglucan fragments was calculated. Many authors have reported the structure of oligomeric xyloglucan fragments of similar size solubilized from pea [9,13], sycamore [39], and apple [36] by (partially) purified endoglucanases. Considering the homology of xyloglucans in dicotelydons it seems most likely that the shaded peaks (Fig. 1B) contain some of these structures. The fine structure of these fragments is currently being investigated.

Degradation of cellulose involves the concerted action of endoglucanase and CBH whereby the former creates new chain ends for the latter to work on. At low endoglucanase:CBH ratios the amount of endoglucanase is limiting, whereas at high ratios endoglucanase starts competing with CBH for binding sites to cellulose. This implies that there is an optimal ratio, which is observed in Fig. 3, for both enzyme combinations. The optimal molar ratios for endoI and endoIV with respect to Avicel cellulose degradation determined by Beldman *et al.* [3] (1.9 and 13.2 respectively) could be explained partly by their adsorption behavior and were slightly different from the ones determined here on WUS (1.1 and 14.9, respectively). Compared with the results of Beldman *et al.* [3] the endoI:CBH ratio changed relatively most. This could be explained by assuming that the free surface area on the cellulose, to which these enzymes can adsorb, was limiting. The optimal endoIV:CBH ratio, on the contrary, shifted towards a higher value. This could be explained by assuming that some extra endoIV is needed for solubilizing the xyloglucans.

Apart from the different optimal endoglucanase:CBH ratios, the total amount of G_2 that was solubilized was particularly noteworthy. EndoIV had the ability to stimulate CBH to such an extent that the amount of G_2 released from WUS after 3 h of reaction time was twice as high compared to amounts released by incubations with endoI plus CBH. Cellulose degradation seems to relate to the solubilization of xyloglucan (Fig. 3), the former being optimal when the latter is maximal. It can be calculated that, at optimal endoI:CBH, the cellulose turnover exceeds the xyloglucan turnover. For optimal endoIV:CBH, the contrary is the case.

The fact that formulations with endoI plus CBH and even with only CBH still give considerable cellulose hydrolysis (Fig. 2A, Table IV) indicates that not all cellulose in apple WUS is coated with xyloglucan. This seems to be different from the results of Hayashi and coworkers [9,10], who found that *in vivo* pea stem cellulose was saturated with xyloglucan, whereas in a reconstitution experiment of pea stem cellulose and xyloglucan the deposition of xyloglucan was *c.* 10 times lower. They argued that the binding of xyloglucan to cellulose during synthesis prevented the fasciation of small fibrils into larger bundles. Apple WUS probably resembles the *in vivo* situation of pea stems, differing only in the presence of arabinogalactans and pectin, but these were shown not to influence the binding of xyloglucan to cellulose [10]. However, more data concerning the degree of occupation of the cellulose surface in apple WUS are needed to explain the action by the formulations mentioned above.

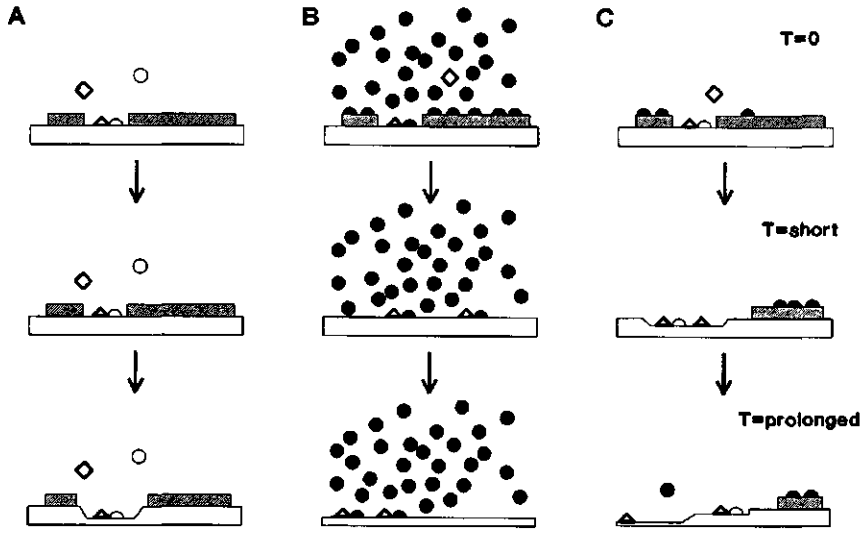

FIGURE 5. Schematic representation of the role of different endoglucanases in simultaneous cellulose and xyloglucan degradation. A, endoI plus CBH; B, endoIV plus CBH; C, endoI plus endoIV plus CBH. Situations A, B, and C present a true reflection of the molar enzymic composition at optimal endoglucanase:CBH ratios depicted in Fig. 3 and 4. Open rectangle, cellulose; shaded rectangle, xyloglucan; ○, endoI; ●, endoIV; ◇, CBH; half circles and triangle represent adsorbed enzymes.

The results indicate that the removal of a xyloglucan coating is essential for efficient cellulose hydrolysis. The degradation of the cellulose-xyloglucan complex is represented schematically in Fig. 5 at three different stages. The figure reflects the actual molar enzyme composition at the optimal ratios presented in Fig. 3 and 4.

Situation A.—The cellulose-degrading potential is not fully used because the surface area of uncoated cellulose to which the enzyme complex can bind is limiting. Because of the low turnover of xyloglucan by endoI, no fast improvement in this situation is to be expected. Despite the higher activity of endoI towards cellulose (Avicel, Table III) compared to endoIV, cellulose hydrolysis stays behind.

Situation B.—The turnover of xyloglucan exceeds the turnover of cellulose, and "bare" cellulose microfibrils are readily degraded. Full cellulose-degrading potential is used, although relatively large amounts of endoIV are needed because of its low adsorption onto the cellulose (Table III).

Situation C.—This situation combines the positive aspects of the previous two. By removing the xyloglucan coating, endoIV enhances the accessibility of the cellulose for endoI and CBH which display a larger activity towards this substrate than endoIV. In this case, cellulose turnover almost equals xyloglucan turnover. An advantage of this three-enzyme system is that much smaller quantities of enzyme are needed to reach a G_2 release similar to that in situation B.

Because precautions were taken to minimize any possible modification of the cell wall material by heat treatment, endogenous enzymes, and organic solvents, it is believed that the apple WUS chemically resembles intact apple tissue. However, physical parameters, such as enzyme diffusion, are not taken into account when studying the degradation of WUS, although they might be very important in applications such as fruit juice processing. With respect to this endoIV might have an additional advantage due to its low mol wt.

These results demonstrate that the endoglucanases of *Trichoderma viride* show different activities towards APfxg. Research is now focused on the fine structure of this polysaccharide and how it influences the specificity of the different endoglucanses.

REFERENCES

[1] Wood, T.M., and McCrae, S.I. (1979) Synergism between enzymes involved in the solubilization of native cellulose. In *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis* (Brown, R.D., and Jurasek, L., eds., Washington), *Advances in Chemistry Series* **181**, 181-209.

[2] Ryu, D.D.Y., Kim, C., and Mandels, M. (1984) Competitive adsorption of cellulase components and its significance in a synergistic mechanism. *Biotechnol. Bioeng.* **26**, 488-496.

[3] Beldman, G., Voragen, A.G.J., Rombouts, F.M., and Pilnik W. (1988) Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from *Trichoderma viride*. *Biotechnol. Bioeng.* **31**, 173-178.

[4] Walker, L.P., and Wilson, D.B. (1991) Enzymatic hydrolysis of cellulose: an overview. *Bioresource Technol.* **36**, 3-14.

[5] Beldman, G., Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F., and Pilnik, W. (1987) Adsorption and kinetic behaviours of purified endoglucanases and exoglucanases from *Trichoderma viride*. *Biotechnol. Bioeng.* **30**, 251-257.

[6] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[7] Renard, C.M.G.C., Searle-van Leeuwen, M.J.F., Voragen, A.G.J., Thibault, J.-F., and Pilnik, W. (1991) Studies on apple protopectin. II: Apple cell wall degradation by pure polysaccharidases and their combinations. *Carbohydr. Polym.* **14**, 295-314.

[8] Keegstra, K., Talmadge, K., Bauer, W.D., and Albersheim, P. (1973) The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.* **51**, 188-196.

[9] Hayashi, T., and Maclachlan, G. (1984) Pea xyloglucan and cellulose. I. Macromolecular organization. *Plant Physiol.* **75**, 596-604.

[10] Hayashi, T., Marsden M.P.F., and Delmer, D.P. (1987) Pea xyloglucan and cellulose. V. Xyloglucan-cellulose interactions *in vitro* and *in vivo*. *Plant Physiol.* **83**, 384-389.

[11] Talbott, L.D., and Ray, P.M. (1992) Molecular size and separability features of pea cell wall polysaccharides: implications for models of primary wall structure. *Plant Physiol.* **98**, 357-368.

[12] Fry, S.C., and Miller, J.G. (1989) Toward a working model of the growing plant cell wall: phenolic cross-linking reactions in the primary cell walls of dicotyledons. In *The Biosynthesis and Biodegradation of Plant Cell Wall Polymers* (Lewis, N.G., and Paice, M.G., eds., Washington), *Advances in Chemistry Series* **399**, 33-46.

[13] McDougall, G.J., and Fry, S.C. (1990) Xyloglucan oligosaccharides promote growth and activate cellulase: evidence for a role of cellulase in cell expansion. *Plant Physiol.* **93**, 1042-1048.

[14] Nishitani, K., and Tominaga, R. (1991) In vitro molecular weight decrease in xyloglucans by an apoplastic enzyme preparation from epicotyls of *Vigna angularis*. *Physiol. Plant* **82**, 490-497.

[15] Talbott, L.D., and Ray, P.M. (1992) Changes in molecular size of previously deposited and newly synthesized pea cell wall matrix polysaccharides: effects of auxin and turgor. *Plant Physiol.* **98**, 369-379.

[16] Redgwell, R.J., Melton, L.D., and Brasch, D.J. (1991) Cell-wall polysaccharides of kiwifruit *(Actinidia deliciosa)*: effect of ripening on the structural features of cell-wall materials. *Carbohydr. Res.* **209**, 191-202.

[17] Edwards, M., Dea, I.C.M., Bulpin, P.V., and Reid, J.S.G. (1985) Purification and properties of a novel xyloglucan-specific endo-1,4-ß-D-glucanase from germinated nasturtium seeds. *J. Biol. Chem.* **261**, 489-494.
[18] Durbin, M.L., and Lewis, L.N. (1988) Cellulases in *Phaseolus vulgaris*. *Meth. Enzymol.* **160**, 342-351.
[19] Maclachlan, G., and Brady, C. (1992) Multiple forms of 1,4-ß-glucanase in ripening tomato fruits include a xyloglucanase activatable by xyloglucan oligosaccharides. *Aust. J. Plant Physiol.* **19**, 137-146.
[20] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.
[21] Renard, C.M.G.C., Schols, H.A., Voragen, A.G.J., Thibault, J.-F., and Pilnik, W. (1991) Studies on apple protopectin. III: Characterization of the material extracted by pure polysaccharidases from apple cell walls. *Carbohydr. Polym.* **15**, 13-32.
[22] Selvendran, R.R., and O'Neill, M. (1987) Isolation and analysis of cell walls from plant material. In *Methods of Biochemical Analysis* (Click, D., ed., Wiley, New York), **32**, 25-154.
[23] Somogyi, N. (1952) Notes on sugar determination. *J. Biol. Chem.* **195**, 19-23.
[24] Thibault, J.-F. (1979) Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényl. *Lebensm. Wiss. Technol.* **12**, 247-251.
[25] Tollier, M.T., and Robin, J.P. (1979) Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Ann. Technol. Agric.* **28**, 1-15.
[26] Albersheim, P., Nevins, D.J., English, P.D., and Karr, A. (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. *Carbohydr. Res.* **5**, 340-345.
[27] Englyst, H.N., and Cummings, J.H. (1984) Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* **109**, 937-942.
[28] Sandford, P.A., and Conrad, H.E. (1966) The structure of the *Aerobacter aerogenes* A3(S1) polysaccharide. I. A reexamination using improved procedures for methylation analysis. *Biochem.* **5**, 1508-1517.
[29] Sweet, D.P., Shapiro, R.H., and Albersheim, P. (1975) Quantitative analysis of various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydr. Res.* **40**, 217-225.
[30] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.
[31] Voragen, A.G.J., Schols, H.A., and Pilnik, W. (1986) Determination of the degree of methylation and acetylation of pectins by hplc. *Food Hydrocoll.* **1**, 65.
[32] Kiefer, L.L., York, W.S., Darvill, A.G., and Albersheim, P. (1989) Xyloglucan isolated from suspension-cultured sycamore cell walls is *O*-acetylated. *Phytochem.* **28**, 2105-2107.
[33] Ruperez, P., Selvendran, R.R., and Stevens, B.J.H. (1985) Investigation of the heterogeneity of xyloglucans from the cell walls of apple. *Carbohydr. Res.* **142**, 107-113.
[34] Renard, C.M.G.C., Voragen, A.G.J., Thibault, J.-F., and Pilnik, W. (1991) Studies on apple protopectin. IV: Apple xyloglucans and influence of pectin extraction treatments on their solubility. *Carbohydr. Polym.* **15**, 387-403.
[35] Montreuil, J. (1975) Recent data on the structure of the carbohydrate moiety of glycoproteins. *Pure Appl. Chem.* **42**, 431-477.
[36] Renard, C.M.G.C., Lomax, J.A., and Boon, J.J. (1992) Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and alkali-extracted xyloglucans. *Carbohydr. Res.* **232**, 303-320.
[37] Lee, Y.H., and Fan, L.T. (1983) Kinetic studies of enzymatic hydrolysis of insoluble cellulose: (II). Analysis of extended hydrolysis times. *Biotechnol. Bioeng.* **25**, 939-966.
[38] Aspinall, G.O., and Fanous, H.K. (1984) Structural investigations on the non-starchy polysaccharides of apples. *Carbohydr. Polym.* **4**, 193-214.
[39] York, W.S., van Halbeek, H., Darvill, A.G., and Albersheim, P. (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and Fast-Atom-Bombardment Mass spectroscopy. *Carbohydr. Res.* **200**, 9-31.
[40] Heutink, R. (1986) Tomato juices and tomato juice concentrates: a study of factors contributing to their gross viscosity. PhD Thesis, Wageningen Agricultural University, The Netherlands.
[41] Hayashi, T., Wong, Y.-S., and Maclachlan, G. (1984) Pea xyloglucan and cellulose. II. Hydrolysis by pea endo-1,4-ß-glucanases. *Plant Physiol.* **75**, 605-610.

CHAPTER 3

Degradation of Apple Fruit Xyloglucan by Endoglucanase[I]

A purified, alkali-extractable apple fruit xyloglucan (APfxg) was treated by endoglucanase (endoIV) from *Trichoderma viride*. The degradation products were fractionated by size-exclusion chromatography on BioGel P-2; the pentamer to dodecamer fractions were further fractionated by semi-preparative high-performance anion-exchange chromatography. The purified oligosaccharides were characterized by monosaccharide analysis, mass spectrometry and degradation with Driselase. Based on these data, tentative structures were proposed for most products. APfxg is composed of a diversity of repeating units. Next to the major oligosaccharides XXXG, an octamer, XXFG and XLFG [Renard, C.M.G.C., Lomax, J.A., and Boon, J.J. (1992) *Carbohydr. Res.* 232, 303-320], XG, XXG, FG and two new fucose-containing oligosaccharide building units (hexasaccharide and dodecasaccharide) were found. Larger xyloglucan fragments were obtained by partial degradation of APfxg by endoIV, and treatment of apple cell wall material by endoI. These fragments were predominantly composed of fucose-containing oligosaccharides. Our results show that both endoIV and endoI are hindered by fucosylated sidechains.

Xyloglucans are extensively branched polysaccharides which occur in many primary cell walls of plants. Sidechains of α-D-Xyl*p*-(1→6)-[II], ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- or α-L-Fuc*p*-(1→2)-ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- are attached to *c.* 75% of the ß-(1→4)-D-Glc*p* residues of the backbone [1-3]. This consistency in chemical configuration led Fry *et al.* [4] to define a one-letter code for each different type of sidechain substitution which can unambiguously describe a xyloglucan structure. It should further be mentioned that the Gal residues can be *O*-acetylated [5].

A common repeating structure, found in xyloglucans of various origin, is that of a hexadecasaccharide (Fig. 1A). Treatment of xyloglucan with a crude cellulase preparation derived from *Trichoderma viride* does usually not accumulate XXFGXXXG; endoglucanase, an important constituent of such preparations, can cleave this fragment at the reducing side of an internal unsubstituted Glc residue to yield a nonasaccharide (XXFG) and a heptasaccharide (XXXG). Kiefer *et al.* [6] demonstrated that endoglucanase action was blocked when there was an additional substitution of the glucan backbone with α-L-Ara*f*-(1→2)- as is indicated in Fig. 1B. Removal of this residue by mild acid hydrolysis and subsequent endoglucanase treatment gave the expected XXFG and XXXG. Hisamatsu *et al.* [7] identified a whole group of these endoglucanase-resistant oligosaccharides. Next to an α-L-Ara*f*-(1→2)-sidechain, ß-D-Xyl*p*-(1→2)- and α-L-Ara*f*-(1→3)-ß-D-Xyl*p*-(1→2)-sidechains were also found; all extra sidechains were attached to

---

[I]Vincken, J.-P., Beldman, G., Niessen[*], W.M.A., and Voragen, A.G.J. *Carbohydr. polym.*, in press ([*] *Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden, The Netherlands*).

[II]*Abbreviations:* see page viii.

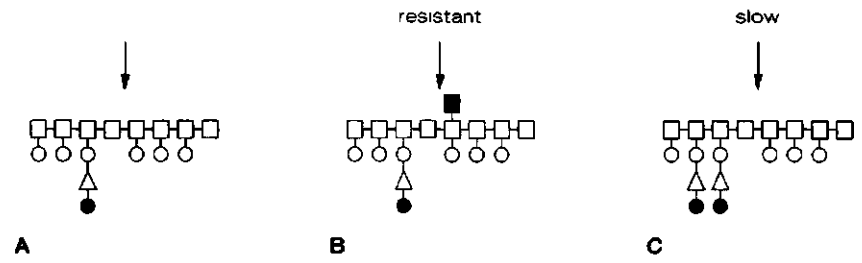

FIGURE 1. Schematic structures of xyloglucan composed of two building units. A, common, easily degradable, fragment (XXFGXXXG); B, endoglucanase-resistant fragment (XXFGAXXG); C, fragment (XFFGXXXG) which is partially resistant to cleavage by endoglucanase. □, ß-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; ▵, ß-D-Gal*p*-(1→2)-; ●, α-L-Fuc*p*-(1→2)-; ■, α-L-Ara*f*-(1→2)-. Sites of (possible) endoglucanase-attack are indicated by arrows.

the glucan backbone as is indicated in Fig. 1B. In addition, they showed that a structure containing two adjacent fucosylated sidechains (as in XFFG; Fig. 1C) could only be degraded with an overdose of endoglucanase. Apparently, Fuc residues play a role in the digestibility of xyloglucans which was further substantiated by York *et al.* [8].

The cellulase complex of *Trichoderma viride* contains several distinct endoglucanases [9], which differ in their activity towards APfxg; endoIV was shown to be a much better xyloglucanase than endoI [10]. Further, larger xyloglucan fragments (DP > 15) accumulated upon treatment of apple cell wall material (WUS) by endoI compared to those obtained by endoIV. Renard *et al.* [11] showed that APfxg is predominantly built from XXXG, XXFG and XLFG units; smaller oligosaccharides (DP *c.* 6) were also reported but these were not further characterized. The objective of the present study is to characterize the oligosaccharides, including the small ones, obtained by treatment of APfxg with endoIV. Larger fragments, obtained by treatment of WUS by endoI, were also purified and their oligosaccharide composition was determined by degradation with endoIV. The apparent endoI-resistancy of these xyloglucan fragments is discussed.

EXPERIMENTAL

Materials

Substrates.—Apple (*Malus malus* L., Rosaceae, var. Golden Delicious) cell wall material (WUS) was obtained after extensive extraction of apple pulp with water as described previously [10]. Xyloglucans were extracted from WUS by strong alkali (4 M KOH) and depectinized on a DEAE-Sepharose CL-6B column. This purified xyloglucan (further referred to as APfxg) contained small amounts of (1→4)-Man and (1→4)-Xyl suggesting a slight contamination with mannan and (arabino)xylan [10]. A mixture of cellodextrins (DP 1 to 6) was obtained from Merck (Darmstadt, Germany).

Enzymes.—Two endoglucanases (endoI and endoIV; EC 3.2.1.4) and CBH (EC 3.2.1.91), previously referred to as exoIII [9], were purified from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme CI, Gist-Brocades, Delft, The Netherlands) as described by

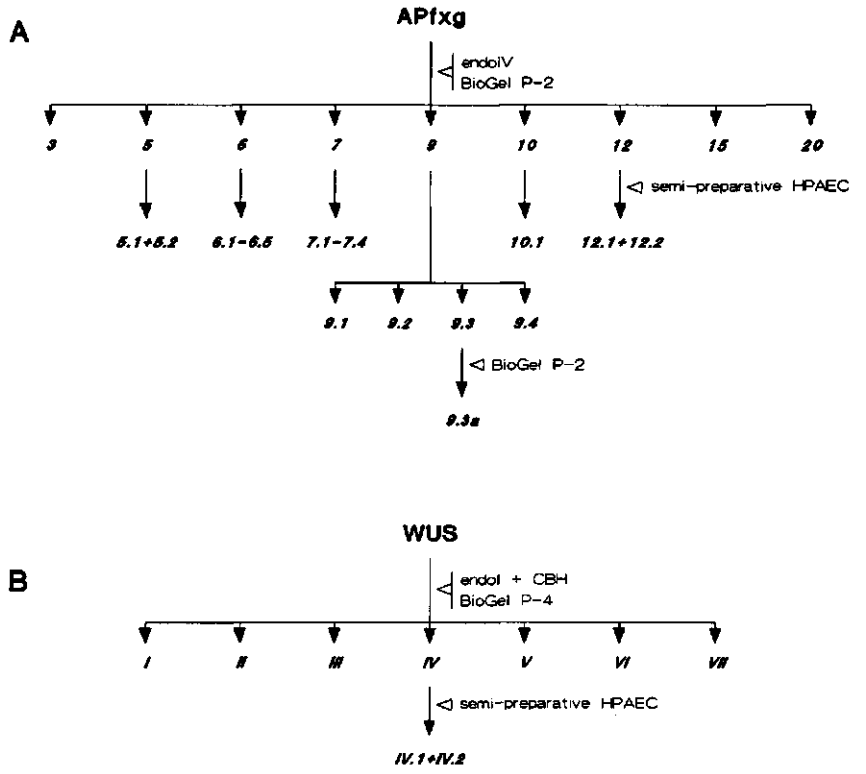

FIGURE 2. Purification scheme of xyloglucan fragments obtained after degradation of APfxg by endoIV (A), and released from apple WUS by endoI plus CBH (B).

Beldman *et al.* [9]. A Driselase preparation (Sigma, St Louis, MO, USA) was desalted on a Fast Desalting Column HR 10/10 (Pharmacia, Uppsala, Sweden) and used for "sequencing" of xyloglucan oligosaccharides without further purification.

Preparation of xyloglucan fragments

Nomenclature.—Two individual purification procedures of xyloglucan fragments were carried out. The first procedure used APfxg as starting material, which was degraded by endoIV. The digest was fractionated by BioGel P-2 chromatography and semi-preparative HPAEC (see further). Fractions obtained by this procedure are summarized in Fig. 2A. Larger xyloglucan fragments were obtained by the second fractionation procedure in which WUS was degraded by a combination of endoI plus CBH. The resulting fragments were fractionated by BioGel P-4 chromatography (see further). Roman numerals were used to refer to these fractions as is indicated in Fig. 2B.

Preparation of oligosaccharides from APfxg using endoIV.—APfxg (40 mg) was treated with endoIV (40 μg of protein) in 20 mL of a 50 mM NaOAc buffer pH 5 at 40 °C. After 24 h a similar amount of fresh enzyme was added. The enzyme was inactivated (10 min, 100 °C) after a total incubation time of 72 h. The digest was concentrated under reduced pressure, freeze-dried, dissolved in 2 mL of distilled water and applied onto a column (100 x 2.6 cm, i.d.) of BioGel P-2 (200-400 mesh, Bio-Rad, Richmond, CA, USA) at 60 °C and eluted with distilled water (18 mL h^{-1}). Fractions (2.0 mL) were assayed for total neutral sugar content. Appropriate fractions

were pooled; digits *3* to *20* were used to refer to these pools. The column was calibrated using a mixture of Glc, G_2, raffinose, stachyose and Dextran T150 (Pharmacia).

The BioGel P-2 fractions *5* to *12* were fractionated by semi-preparative HPAEC using a Dionex Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-1 column (250 x 9 mm, Sunnyvale, CA, USA). Samples (5 x 200 μL) were injected using a SP8780 autosampler (Spectra Physics, San José, CA, USA) equipped with a Tefzel rotor seal in a 7010 Rheodyne injector valve. The fractions were eluted (5 mL min^{-1}) at 20 °C with 100 mM NaOH containing different concentrations of NaOAc. The NaOAc gradients were adapted for the individual BioGel P-2 pools: *5*, 0→20 min, 40 mM NaOAc (isocratic); *6*, 0→30 min, 50 mM NaOAc (isocratic); *7* and *10*, 0→35 min, 60 mM NaOAc (isocratic); *9*, 0→35 min, linear gradient of 60→70 mM NaOAc; *12*, 0→30 min, 80 mM NaOAc (isocratic). After each run, the column was washed for 5 min by elution with 100 mM NaOH containing 1 M NaOAc, and subsequently equilibrated for 15 min with the starting eluent. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The eluate was monitored using a Dionex PED detector in the PAD mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1, 0.1 V and 0.5 s; E_2, 0.6 V and 0.1 s; E_3, -0.6 V and 0.1 s. The eluate was neutralized with 1 M HOAc and the appropriate fractions (*c.* 1 mL) were combined, desalted using columns (30 x 80 mm) of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) resins (Bio-Rad) in series (eluent: distilled water), and concentrated under reduced pressure. The oligosaccharides were analyzed for sugar composition and by mass spectrometry.

Preparation of fragments from WUS using endoI.—WUS (200 mg), suspended in 10 mL of a 50 mM succinate buffer pH 5, was treated with endoI (30 μg) and CBH (100 μg) for 48 h. After centrifugation (20 min, 50,000 *g*), the water-solubles were freeze-dried, dissolved in 2 mL of distilled water and applied onto a column (100 x 2.6 cm, i.d.) of BioGel P-4 (100-200 mesh, Bio-Rad) at 60 °C and eluted with distilled water (18 mL h^{-1}). Appropriate fractions were combined according to their neutral sugar and uronic acid content. BioGel P-4 pools are referred to by roman numerals *I* to *VII*. Fraction *IV* was further fractionated on HPAEC using 110 mM NaOAc (isocratic) for 20 min. Appropriate fractions were pooled, dialyzed against distilled water, concentrated under reduced pressure and dried under a stream of air. All fractions were analyzed for sugar composition. The BioGel P-4 column was calibrated as described for BioGel P-2.

Chemical and enzymic treatment of oligosaccharides

Reduction.—Oligosaccharides of *IV.2* were labelled at their reducing terminus by treating *c.* 100 μg of this fraction with 200 μL 1.5 M ammonia containing 75 mg mL^{-1} $NaBH_4$ for 1 h at 30 °C. The reduced oligosaccharides were dialyzed against distilled water and dried under a stream of air.

Mild acid hydrolysis.—Fuc was removed from *9.2*, *10.1* and *IV.2* by treating *c.* 50 μg of these oligosaccharides with 1 mL of 50 mM TFA for 1 h at 100 °C. The resulting defucosylated oligosaccharides were evaporated to dryness to remove TFA, and used for enzymic degradation studies without further purification. Removal of Fuc residues from polymeric xyloglucan was done using a different procedure. APfxg (40 mg) was treated with 8 mL of 25 mM TFA for 90 h at 60 °C. The defucosylated xyloglucan (referred to as APxg) was dialyzed extensively against distilled water and freeze-dried.

"Sequencing" of oligosaccharides with Driselase.—Oligosaccharides *7.4*, *9.2* and *10.1* (*c.* 50 μg) were treated with Driselase (*c.* 1 μg of protein) in 200 μL of a 50 mM NaOAc buffer pH 5 (40 °C). Release of Fuc and a Glc-Xyl disaccharide were monitored by analyzing aliquots of 10 μL by HPAEC.

Further degradation of larger fragments by endoglucanase.—Approximately 50 μg of *20*, *15*, *III*, *IV.1*, *IV.2* and reduced *IV.2* were incubated (24 h, 40 °C) with endoIV (1 μg of protein) in 100 μL of a 50 mM NaOAc buffer pH 5. Degradation products were analyzed by HPAEC. In a similar manner, *IV.2* and defucosylated *IV.2* were treated with endoI (1 or 20 μg of protein).

Analytical methods

Uronic acid content.—Uronic acids were estimated colorimetrically with an automated *m*-hydroxydiphenyl test [12] using concentrated H_2SO_4 containing 0.0125 M $Na_2B_4O_7$ for hydrolysis.

Total neutral sugar content.—The total neutral sugar content was determined colorimetrically with an automated orcinol/H_2SO_4 assay [13]. Glc was used as a standard.

Neutral sugar composition.—BioGel P-2 and P-4 fractions were hydrolyzed (1 h, 121 °C) using 2 M TFA. The released neutral sugars were converted into their alditol acetates and analyzed by GC as described previously [10]. Oligosaccharides, obtained after fractionation on HPAEC, were hydrolyzed as above and the sugar composition was determined by HPAEC as described by De Ruiter *et al.* [14].

Protein content.—The protein content of enzyme preparations was determined according to the procedure of Sedmak and Grossberg [15]. BSA was used as a standard.

Determination of mol wt of xyloglucan oligosaccharides.—The mol wt of the purified xyloglucan oligosaccharides was determined with a Finnigan MAT TSQ-70 mass spectrometer (San José, CA, USA), equipped with a 20 kV conversion-dynode and a Finnigan MAT electrospray interface as described previously [16].

Analysis of xyloglucan oligosaccharides.—Xyloglucan oligosaccharides and their degradation products were analyzed on a CarboPac PA-1 column (250 x 4 mm, Dionex) combined with PAD-analysis (as described above), eluted (1 mL min^{-1}) with the following NaOAc gradient in 100 mM NaOH: 0→5 min, linear gradient of 0→50 mM NaOAc; 5→30 min, linear gradient of 50→80 mM NaOAc; 30→35 min, linear gradient of 80→130 mM NaOAc; 35→50 min, linear gradient of 130→180 mM NaOAc. After each analysis, the column was washed for 5 min with 1 M NaOAc in 100 mM NaOH, and equilibrated in 100 mM NaOH for 15 min. Some samples were analyzed on CarboPac PA-100 which enabled a slightly better separation with the following NaOAc gradient in 100 mM NaOH: 0→5 min, linear gradient of 0→30 mM NaOAc; 5→45 min, linear gradient of 30→80 mM NaOAc; 45→55 min, linear gradient of 80→200 mM NaOAc. After each analysis the column was washed and conditioned as described for the CarboPac PA-1 column.

RESULTS

Characterization of xyloglucan oligosaccharides obtained by endoIV

APfxg was degraded with endoIV, and the resulting digest was subsequently fractionated by BioGel P-2 chromatography as is shown in Fig. 3. The fraction numbers ***3*** to ***20*** correspond to the DP of the oligosaccharides, which was extrapolated from the elution of standards on BioGel P-2. This DP was confirmed by mass spectrometry of the oligosaccharides (see further).

Fractions ***5*** to ***12*** were further fractionated by HPAEC (Fig. 4) with an optimized gradient for each pool. In this study we have focussed on the main oligosaccharides of APfxg (indicated by the shaded areas) but small amounts of other products were also present. Fraction numbers are indicated in Fig. 4. HPAEC analysis of ***5*** to ***12***, using the same gradient for all pools, showed that ***5.2***, ***6.1***, ***7.4***, ***9.2*** and ***10.1*** were present in more than one BioGel P-2 fraction. Comparison of retention times suggests that fractions ***6.2*** to ***6.5*** are similar to fractions ***7.1*** to ***7.3***. Fig. 4 indicated that ***9.3*** contained more than one component, and therefore this fraction was rechromatographed on a BioGel P-2 column. After removal of the main product of pool ***9*** (***9.2***) by HPAEC, a good separation of ***9.3a***

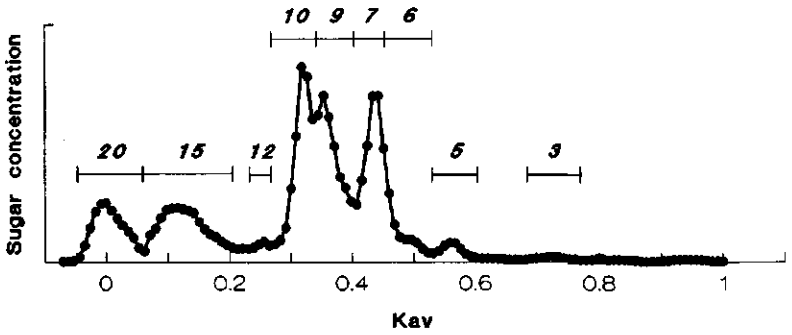

FIGURE 3. Elution pattern of an APfxg digest on BioGel P-2.

and a fraction similar to ***10.1*** was obtained by BioGel P-2 chromatography (data not shown). The purity of all oligosaccharides was confirmed by HPAEC and electrospray mass spectrometry (>90%); only fractions ***12.1*** and ***12.2*** contained *c.* 20% of contaminants (data not shown). Sugar composition and mol wt of all purified compounds are shown in Table I. Although Man was present in the starting material, this glycosyl residue was not recovered in any of the fractions. Monosaccharide composition of ***15*** and ***20*** showed that xyloglucan fragments are a major constituent of these fractions. The presence of these large xyloglucan fragments indicated that the degradation of xyloglucan by endoIV might be incomplete, which will be discussed later. The high Xyl and Ara content of ***20*** suggested that this pool contained an arabinoxylan contamination. Although HPAEC-analysis of ***15*** and ***20*** showed that these pools contained many compounds, no further fractionation was attempted.

Fuc residues of ***9.2*** and ***10.1*** could be removed by treatment with mild acid. This release of Fuc was analyzed by HPAEC; only minute amounts of Gal were released under these conditions (data not shown). Although the mol wt of the compounds was reduced, their retention times upon HPAEC increased. Fuc removal from oligosaccharides is therefore easily recognized by HPAEC. Similar observations were done by McDougall and Fry [17].

In order to obtain additional structural information, the most abundant oligosaccharide units in APfxg, ***7.4***, ***9.2*** and ***10.1***, were treated with a crude Driselase preparation. Apart from α-fucosidase and ß-galactosidase activity, this enzyme preparation has been shown to contain a "xyloglucosidase", an enzyme being able to release α-D-Xyl*p*-(1→6)-ß-D-Glc*p* (isoprimeverose) from the nonreducing terminus of xyloglucan oligosaccharides [3,17]. The substrate specificity of a similar enzyme, derived from a preparation of *Aspergillus oryzae*, was described in detail by Kato *et al.* [18]. This "xyloglucosidase" had a higher activity towards XXXG (*c.* 50 times) than towards G_4, and was unable to bypass Gal- and Fuc-containing sidechains. The degradation of fractions ***7.4***, ***9.2***, and ***10.1*** was monitored by taking samples at certain time intervals and analyzing these samples by HPAEC. A

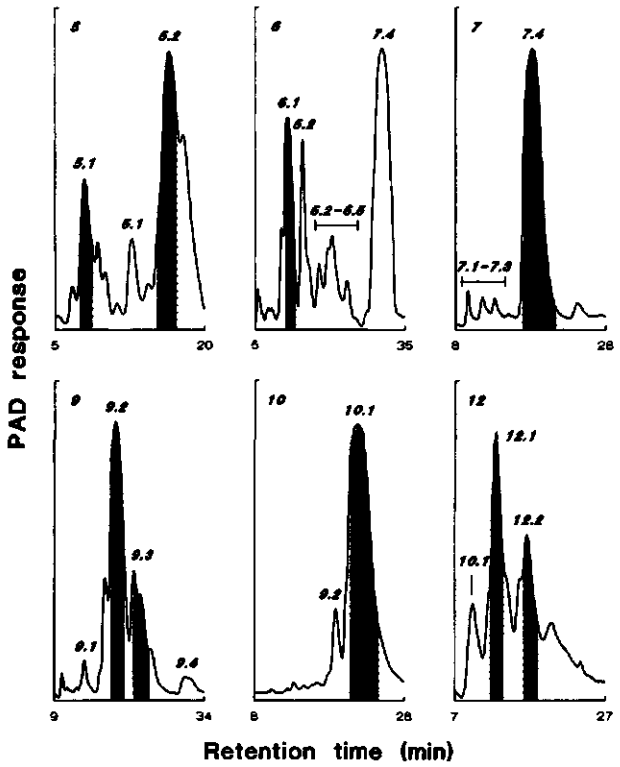

FIGURE 4. Elution profiles on semi-preparative HPAEC (CarboPac PA-1) of BioGel P-2 pools *5*, *6*, *7*, *9*, *10* and *12*. Products in shaded peaks were further characterized. Corresponding NaOAc gradients are described in the "Experimental" section.

representative elution profile after partial degradation of the individual oligosaccharides is shown in Fig. 5.

Considering sugar composition, mol wt (Table I) and the mode of action of endoglucanase, we conclude that *3*, *5.2* and *7.4* correspond to XG, XXG and XXXG, respectively. Treatment of XXXG (*7.4*) with Driselase yielded XXG and isoprimeverose at the early stages of degradation. The release of isoprimeverose confirmed that the X element was on the nonreducing side of the molecule. At later stages, XXG was further degraded to XG and isoprimeverose.

Treatment of *9.2* with Driselase gave an unknown intermediate product and isoprimeverose at the early stages of degradation. The intermediate product was further degraded to *5.1* and more isoprimeverose. These results demonstrate that there are two adjacent X elements at the nonreducing side of the oligosaccharide. This observation, the sugar composition, the mol wt and the known mode of action of endoglucanase indicated that *9.2* and *5.1* correspond to XXFG and FG, respectively. Some other products are formed as well. These are probably a result of α-fucosidase and ß-galactosidase activity in

TABLE I. Analysis of oligosaccharides obtained after degradation of alkali-extracted xyloglucan by endoIV and subsequent fractionation by BioGel P-2 chromatography and HPAEC.

BioGel P-2 fractions

	20	*15*
Glc (mol%)	25	44
Xyl (mol%)	45	32
Gal (mol%)	16	14
Fuc (mol%)	4	10
Ara (mol%)	10	0

HPAEC sub-fractions of *3* to *12*

	3	*5.1*	*5.2*	*6.1*	*7.4*	*9.2*	*9.3a*	*10.1*	*12.1*	*12.2*
Glc (mol%)	59	40	56	49	54	43	48	38	48	48
Xyl (mol%)	41	26	40	18	43	35	38	31	21	24
Gal (mol%)	0	16	2	16	1	11	12	19	16	14
Fuc (mol%)	0	18	2	17	2	11	2	12	15	14
mol wt*	nd.†	782	768	944	1062	1370	1224	1532	1870	nd.
$H_xP_yD_z$‡	nd.	$H_3P_1D_1$	H_3P_2	$H_4P_1D_1$	H_4P_3	$H_5P_3D_1$	H_5P_3	$H_6P_3D_1$	$H_8P_2D_2$	nd.
structure§	XG	FG	XXG	[FG]G	XXXG	XXFG	XLXG	XLFG	?	?

* Determined by electrospray mass spectrometry. † Not determined. ‡ Number of hexoses (H), pentoses (P) and deoxyhexoses (D), x, y and z, respectively, which correspond to the mol wt. § Tentative structure based on sugar composition, mol wt, defucosylation experiments and Driselase degradation patterns. Nomenclature according to Fry *et al.* [4].

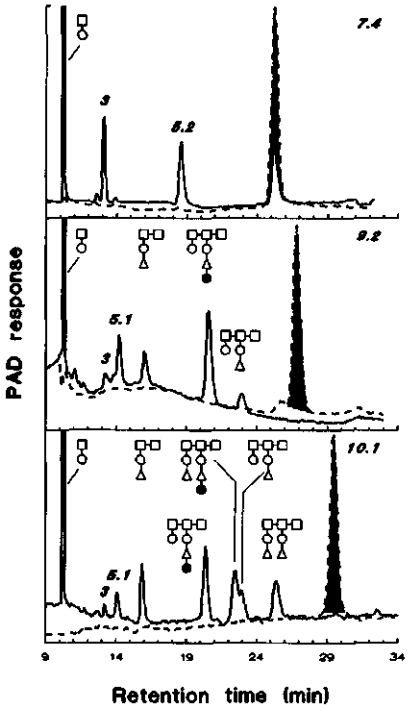

FIGURE 5. Elution patterns on HPAEC (CarboPac PA-1) of oligosaccharides ***7.4***, ***9.2*** and ***10.1*** after partial degradation with a crude Driselase preparation. Shaded peaks and dashed line indicate the original material; solid line shows a representative pattern during the course of degradation. Probable structures of degradation products are indicated. Symbols, as in Fig. 1.

Driselase. Tentative structures (LG, XFG and XLG) for these intermediate products are indicated in Fig. 5.

Isoprimeverose was released from ***10.1*** as an initial product, which showed that this disaccharide is at the nonreducing terminus. FG appeared much later during the course of degradation, which demonstrated that the penultimate Glc residue at the reducing side of the molecule carried the fucosylated sidechain. These observations and the results of Table I, show that ***10.1*** corresponds to XLFG. XLFG can be degraded according to one of the following routes: XLFG→ LFG→ XFG→ FG→ LG or XLFG→ XLLG→ LLG→ XLG→ LG or XLFG→ LFG→ LLG→ XLG→ LG. Tentative structures for all intermediates are proposed in Fig. 5.

Characteristics of the purified oligosaccharides are summarized in Table I. Based on these characteristics, and the degradation studies, tentative structures were proposed. Our experiments were not decisive on the structures of ***6.1***, ***9.3a*** and ***12.1***. In principle, there are three possible structures for octamer ***9.3a***: XXLG, XLXG and LXXG. Comparison of retention times of XXLG (defucosylated XXFG) and ***9.3a*** upon HPAEC showed that these oligosaccharides were different. Thus, ***9.3a*** does not correspond to XXLG. The existence of LXXG has never been reported before; thus, XLXG seems most probable for ***9.3a***. Fraction ***6.1*** and ***12.1*** did not fit in any of the Driselase degradation patterns. The mode of action of endoglucanase implies that both oligosaccharides have an unsubstituted Glc residue on the reducing terminus; however, the position of the trisaccharide sidechain(s) is

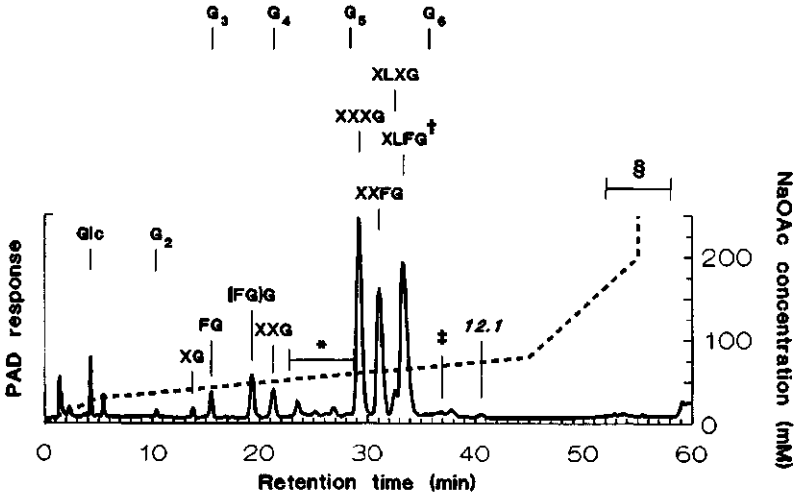

FIGURE 6. Elution profile of APfxg, which is completely degraded by endoIV, on HPAEC (CarboPac PA-100). The profile with solid line indicates PAD response; the profile with dashed line shows the NaOAc gradient. Products are indicated in the chromatogram by letter-codes or, in case of unknowns, by fraction number. *, degradation of XXFG (***9.2***) and XLFG (***10.1***) by Driselase suggests that XFG, LFG, XLG and LLG elute in this region. †, after defucosylation of XXFG, the resulting oligosaccharide coelutes with XLFG. ‡, elution of product obtained after defucosylation of XLFG. §, indicates the retention times where fragments of two repeating xyloglucan oligosaccharides (such as ***15***, ***20***, ***III*** and ***IV***) elute. Retention times of cellodextrins are indicated by Glc and G_2-G_6.

unknown. ***6.1*** will be further referred to as [FG]G.

The elution profile of APfxg, which is completely degraded to its oligosaccharide building units by endoIV, is shown in Fig. 6. Products are indicated according to Table I; the elution times of cellodextrins (Glc, G_2-G_6) are indicated for comparison. It should be noted that a CarboPac PA-100 column was used instead of a PA-1 column. Oligosaccharides have a tendency to elute at lower NaOAc concentrations on CarboPac PA-100 due to a smaller particle size and lower capacity of this packing material. However, an advantage of this material was that it allowed some separation of XLXG and XLFG. The presence of Glc and G_2 suggested that APfxg contained a small amount of contiguous ß-(1→4)-Glc residues. The asterisk indicates a group of unknown compounds, corresponding to ***6.2-6.5*** and ***7.1-7.3*** (see Fig. 4). Degradation of XXFG (***9.2***) and XLFG (***10.1***) by Driselase indicates that XFG, LFG, XLG and LLG elute at similar retention times (range 23 to 29 min).

Purification of xyloglucan fragments obtained by endoI plus CBH

In order to obtain larger xyloglucan fragments, apple WUS was degraded by a combination of endoI and CBH. Solubilized products were fractionated by BioGel P-4 chromatography into seven pools as is shown in Fig. 7. The monosaccharide composition

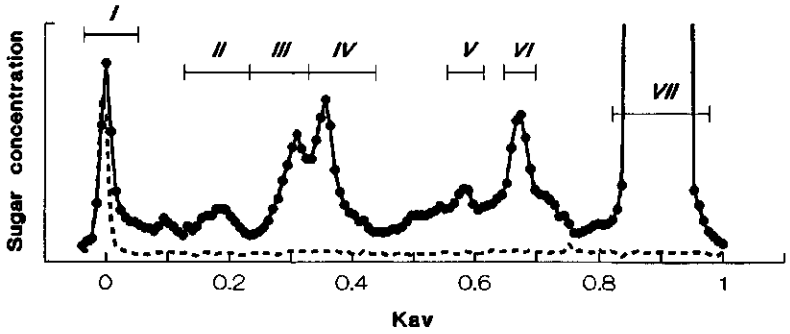

FIGURE 7. Elution profile on BioGel P-4 of the compounds solubilized from apple WUS after treatment with a combination of endoI and CBH. Neutral sugar response (—) and uronic acid response (- - -). Roman numerals are used to indicate these BioGel P-4 pools.

TABLE II. Neutral sugar composition (mol%) of BioGel P-4 fractions obtained after degradation of apple WUS by the combined action of endoI plus CBH.

	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Glc	19	43	44	47	49	54	96
Xyl	11	30	31	34	27	34	1
Gal	15	14	16	12	10	3	0
Fuc	1	8	7	5	4	1	0
Ara	49	3	1	1	1	1	0
Man	1	2	1	1	9	7	3
Rha	4	0	0	0	0	0	0

of these pools is given in Table II. Fraction *VII* contained predominantly G_2. The Glc to Xyl ratio suggested that xyloglucan fragments were present in *I* to *VI*; fraction *I* contained mainly pectic material, *V* and *VI* were contaminated with Man. HPAEC analysis showed that XXXG was the major component of *VI* (data not shown). Fractions *III* and *IV* eluted at retention times which are indicated by "§" in Fig. 6. Fraction *IV* was further fractionated by semi-preparative HPAEC (data not shown), which resulted in two pools, *IV.1* (*c.* 30% of the material) and *IV.2* (*c.* 70% of the material).

Further degradation of larger xyloglucan fragments

Both fractionation procedures described above have provided a number of fractions, containing fragments which consisted of more than one oligosaccharide building unit (*20*, *15*, *III*, *IV.1* and *IV.2*). To investigate if these fragments were resistant to endoglucanase, they were treated with a large dose of endoIV and analyzed by HPAEC. All fractions could be degraded to oligosaccharides, identical to those described in Table I; only in the case of fraction *20*, this degradation was incomplete. Time course studies demonstrated that no intermediary products were formed (data not shown), which showed that all larger

TABLE III. Summary of building units formed after degradation of some larger xyloglucan fragments by endoIV. Quantification of building units was based on peak areas using HPAEC (CarboPac PA-100 column). Symbols: −, not detected; ±, traces; +, present in small amounts; ++, most important building unit.

	*20**	*15**	*III*†	*IV.1*†	*IV.2*†	*IV.2*†‡
DP<7§	±	+	−	++	−	−
XXXG	±	+	±	±	++	−
XXFG	±	+	++	++	+	++
XLFG‖	++	++	++	++	+	++

* Pools from BioGel P-2 fractionation containing larger xyloglucan fragments. † Selected pools from BioGel P-4 fractionation. ‡ Fragment was reduced prior to treatment with endoIV. § Contains the smaller xyloglucan oligosaccharides: predominantly FG and [FG]G, but also XG and XXG. ‖ May contain small amounts of XLXG which can not be recovered as a separate peak when the XLFG to XLXG ratio is high.

fragments were built up of two oligosaccharide building units. Table III summarizes these oligosaccharide building units. The diversity of building units shows that each original fraction contains more than one compound. The main component of ***20*** is probably a dimer of XLFG. ***III*** and ***IV.2*** are composed of two oligosaccharides having four Glc residues in the backbone (such as XXXG, XXFG and XLFG). ***15*** and ***IV.1*** contain considerable amounts of smaller building units (such as XG, FG, XXG and [FG]G). The monosaccharide composition of the different fractions (Table I and II) is in agreement with this building unit composition. It should be noted that XXXG, the major oligosaccharide in APfxg, is not an important constituent of any of the above mentioned fractions (except ***IV.2***). Fucosylated oligosaccharides, however, were very abundant building units of these fractions. From these experiments it was concluded that Fuc-containing structures were poorly degradable, but that none of the fragments was endoglucanase-resistant.

Fraction ***IV.2*** was further studied because this fraction contained similar amounts of non-fucosylated (XXXG) and fucosylated (XXFG and XLFG) building units (Table III). It seemed unlikely that ***IV.2*** contains structures like XLFGXLFG, XLFGXXFG or XXFGXXFG, because these fragments would have eluted in fraction ***III*** (see also Table III). Therefore, it is expected that the fragments of ***IV.2*** are composed of XXXG and either XXFG or XLFG. To investigate which building unit was at the reducing end, ***IV.2*** was labelled by reduction, and subsequently degraded by endoIV. Oligosaccharides XXFG and XLFG were almost completely recovered, but no XXXG was found. This showed that XXXG is positioned predominantly at the reducing terminus, and that ***IV.2*** is composed of XXFGXXXG and XLFGXXXG. Apparently, cleavages like XXXG↓XXFG are preferred over those like XXFG↓XXXG in the degradation of APfxg.

Activity of endoglucanase towards defucosylated APfxg

Treatment of APfxg by mild acid, specifically removes Fuc residues from the

xyloglucan molecules (results not shown); this material is further referred to as APxg. The activity of endoI towards APxg was three times higher compared to that towards APfxg (data not shown). Defucosylation also enhanced endoIV activity, but to a much smaller extent (data not shown). It can be concluded that the action of endoI is more hindered by Fuc than that of endoIV.

The endoI-resistancy of XLFGXXXG (***IV.2***) was tested by treating this fraction with endoI (a similar amount of protein was used compared to endoIV). When the endoI dose was increased twenty times, this fraction was partially degraded. Removal of Fuc by mild acid had a positive effect on the ability of endoI to degrade this fragment. In this case, the material was completely degraded to XXXG, XXLG and XLLG by the lower dose of endoI. These results indicated that Fuc-containing sidechains of xyloglucans strongly hinder endoI.

DISCUSSION

In a previous paper [10], we have demonstrated that some endoglucanases (endoIV) display a much higher activity towards xyloglucans than others (endoI). This study investigates the products released by both enzymes in more detail. For this purpose, a purified, alkali-extracted APfxg was degraded by endoIV. The resulting digest could be fractionated satisfactorily by combining BioGel P-2 chromatography and HPAEC. Although the latter purification step is the most distinctive of the two, prefractionation by BioGel P-2 chromatography is necessary due to a, with respect to mol wt, rather unpredictable elution behavior of fucosylated compounds upon HPAEC. For instance, a Fuc-containing decasaccharide was coeluted (on CarboPac PA-1) with an octasaccharide lacking this residue.

Based on the BioGel P-2 elution pattern and Table I, it can be calculated that XXXG, XLXG, XXFG and XLFG form *c.* 80% of the building units of APfxg in a ratio of 4:2:3:4. Different responses of monosaccharides with the neutral sugar assay and the incomplete degradation of ***15*** and ***20*** were accounted for. Further, it was assumed that the PAD response factors of the compounds within one BioGel P-2 pool were equal. These results are in agreement with those reported by Renard *et al.* [11].

An important part of APfxg is composed of other, mainly smaller, oligosaccharides (*c.* 20% in total). Our study confirms that XXG is part of APfxg, but no evidence was found for the presence of a hexasaccharide composed of 4 Glc and 2 Xyl residues, as reported by Renard *et al.* [11]. This component might be among the unidentified oligosaccharides ***6.2*** to ***6.5*** or ***7.1*** to ***7.3***. Typically, our data suggest a diversity in fucosylated compounds: FG and two new structures, [FG]G and ***12.1***. Both unknown compounds contain a relatively high percentage of Glc. Since the degradation of xyloglucan was not complete, these might be intermediary products. A detailed characterization (^{1}H-NMR, MS-MS) of these structures will be reported in Chapter 4.

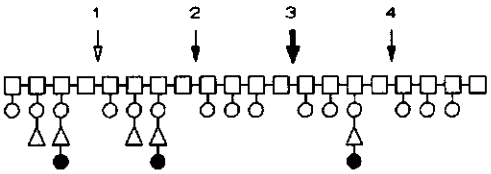

FIGURE 8. Schematic representation of the degradation by endoglucanases of an imaginary fragment consisting of five repeating xyloglucan oligosaccharide building units (XLFG-XLFG-XXXG-XXFG-XXXG). Cleavage of linkage "3" is preferred over "2" and "4", as is indicated by the thickness of the arrows. Linkage "1" is least preferred. Symbols, as in Fig. 1.

Our results do not suggest resistancy of certain xyloglucan fragments (DP > 15) to endoI. Accumulation of these fragments is merely due to the low specificity of endoI towards xyloglucan. Considering the accumulation of *e.g.* XLFGXLFG (***20***, poorly degradable by endoIV) and the facilitated cleavage of XLFGXXXG (***IV.2***) by endoI after removal of Fuc, our results suggest that fucosylated sidechains hamper the action of both endoglucanases. Similar observations have been made by Hisamatsu *et al.* [7] and York *et al.* [8] who showed that similar fragments from sycamore were partially resistant against a mixture of endoglucanases.

Levy *et al.* [19] indicated by modeling that the fucosylated sidechain of xyloglucan plays an important role in stabilizing certain molecular conformations. In solution, xyloglucan adopts a twisted conformation and the trisaccharide sidechain folds towards the reducing end. As a result of this the linkages indicated with "1", "2" and "4" (Fig. 8) might become poorly accessible to endoglucanases. Linkage "3" is much easier cleaved which explains that fragments like XXFGXXXG (***IV.2***) can accumulate. The presence of XLFGXLFG (***20***) or XLFGXXFG (***III***) shows that both endoglucanases are able to split linkage "2". Apparently, this cleavage site is preferred over "1", because XXFG or XLFG hardly accumulate upon treatment of WUS by endoI. Possibly Gal residues (as in XLFG) play a role in the relatively slow cleavage of linkage "1".

REFERENCES

[1] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[2] York, W.S., van Halbeek, H., Darvill, A.G., and Albersheim, P. (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and Fast-Atom-Bombardment Mass Spectrometry. *Carbohydr. Res.* **200**, 9-31.

[3] Hisamatsu, M., Impallomeni, G., York, W.S., Albersheim, P., and Darvill, A.G. (1991) A new undecasaccharide subunit of xyloglucans with two α-L-fucosyl residues. *Carbohydr. Res.* **211**, 117-129.

[4] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.

[5] Kiefer, L.L., York, W.S., Darvill, A.G., and Albersheim, P. (1989) Xyloglucan isolated from suspension-cultured sycamore cell walls is *O*-acetylated. *Phytochem.* **28**, 2105-2107.

[6] Kiefer, L.L., York, W.S., Albersheim, P., and Darvill, A.G. (1990) Structural characterization of an arabinose-containing heptadecasaccharide enzymically isolated from sycamore extracellular xyloglucan. *Carbohydr. Res.* **197**, 139-158.

[7] Hisamatsu, M., York, W.S., Darvill, A.G., and Albersheim, P. (1992) Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues. *Carbohydr. Res.* **227**, 45-71.

[8] York, W.S., Impallomeni, G., Hisamatsu, M., Albersheim, P., and Darvill, A.G. (1995) Eleven newly characterized xyloglucan oligoglycosyl alditols: the specific effects of sidechain structure and location on ^{1}H NMR chemical shifts. *Carbohydr. Res.* **267**, 79-104.

[9] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.

[10] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and glucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).

[11] Renard, C.M.G.C., Lomax, J.A., and Boon, J.J. (1992) Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and of alkali-extracted xyloglucans. *Carbohydr. Res.* **232**, 303-320.

[12] Thibault, J.-F. (1979) Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényl. *Lebensm. Wiss. Technol.* **12**, 247-251.

[13] Tollier, M.T., and Robin, J.P. (1979) Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Ann. Technol. Agric.* **28**, 1-15.

[14] De Ruiter, G.A., Schols, H.A., Voragen, A.G.J., and Rombouts, F.M. (1992) Carbohydrate analysis of water-soluble uronic acid-containig polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.* **207**, 176-185.

[15] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.

[16] Tinke, A.P., van der Hoeven, R.A.M., Niessen, W.M.A., van der Greef, J., Vincken, J.-P., and Schols, H.A. (1993) Electrospray mass spectrometry of neutral and acidic oligosaccharides: methylated cyclodextrins and identification of unknowns derived from fruit material. *J. Chromatogr.* **647**, 279-287.

[17] McDougall, G.J., and Fry, S.C. (1991) Purification and analysis of growth-regulating xyloglucan-derived oligosaccharides by high-pressure liquid chromatography. *Carbohydr. Res.* **219**, 123-132.

[18] Kato, Y., Matsushita, J., Kubodera, T., and Matsuda, K. (1985) A novel enzyme producing isoprimeverose from oligoxyloglucans of *Aspergillus oryzae*. *J. Biochem.* **97**, 801-810.

[19] Levy, S., York, W.S., Stuike-Prill, R., Meyer, B., and Staehelin, L.A. (1991) Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific folding. *Plant J.* **1**, 195-215.

CHAPTER 4

Dimers of a GFG Hexasaccharide Occur in Apple Fruit Xyloglucan[I]

Apple fruit xyloglucan is predominantly built up from XXXG, XXFG, and XLFG fragments. However, small amounts of oligosaccharides with a less heavily branched glucan backbone also occur. This paper describes the characterization of two such oligosaccharides, GFG and a dimer of GFG, using a combination of ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry. With the elucidation of these structures the structural-reporter groups for xyloglucans are extended. The peculiarity of finding GFGGFG as a structural element of apple fruit xyloglucan is discussed.

Xyloglucans play an important role in crosslinking cellulose microfibrils in the primary cell walls of plants [1]. These polysaccharides are composed of a ß-(1→4)-D-Glc*p*[II] backbone to which α-D-Xyl*p*-(1→6)-, ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- or α-L-Fuc*p*-(1→2)-ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-sidechains are attached [2,3]. Generally, *c.* 75% of a xyloglucan backbone is branched, although lower degrees of branching also occur, for instance, in rice seedlings (*c.* 30% [4]) and potatoes (*c.* 40% [5]).

The backbone of apple fruit xyloglucan is *c.* 65% branched [6] which suggests that, in addition to the three main oligosaccharide building units, XXXG, XXFG and XLFG [7,8] (for nomenclature, see [9]), oligosaccharides with less-substituted backbones might also occur. The first indication of this was provided by Renard *et al.* [7] with the detection of small amounts of Glc_4Xyl_2 (exact structure unknown) in a digest of apple xyloglucan. More evidence for relatively Glc-rich oligosaccharides as a structural element of apple fruit xyloglucan was obtained by Vincken *et al.* [8], who isolated two such fragments, $Glc_3Xyl_1Gal_1Fuc_1$ and $Glc_6Xyl_2Gal_2Fuc_2$, from a digest of apple fruit xyloglucan. This paper describes the detailed ^{1}H-NMR spectroscopic and mass spectrometric characterization of these and other fragments isolated from this digest. The ^{1}H-NMR spectroscopic data for these unreduced xyloglucan oligosaccharides provide an extension to the set of structural reporter-groups for xyloglucan oligosaccharides [3,10-12]. The biological significance of the Glc-rich oligosaccharides is discussed.

[I]Spronk*, B.A., Rademaker†, G.J., Haverkamp†, J., Thomas-Oates†, J.E., Vincken, J.-P., Voragen, A.G.J., Kamerling*, J.P., and Vliegenthart*, J.F.G. To be submitted (* *Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, 3508 TB Utrecht, The Netherlands*; † *Bijvoet Center, Department of Mass Spectrometry, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*).

[II]*Abbreviations:* CID, collision-induced dissociation; Deoxyhex, deoxyhexose; DQF-COSY, double-quantum filtered correlated spectroscopy; FAB, fast atom bombardment; Hex, hexose; HOHAHA, homonuclear Hartmann-Hahn; MS, mass spectrometry; NOESY, nuclear Overhauser enhancement spectroscopy; Pent, pentose; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; other abbreviations, see page viii.

EXPERIMENTAL

Materials

Xyloglucan oligosaccharides.—Xyloglucan oligosaccharides were obtained after enzymic degradation of an alkali-extracted, depectinized xyloglucan preparation derived from apple fruit material [6]. The resulting oligosaccharides were fractionated using BioGel P-2 chromatography and semi-preparative HPAEC [8].

Enzymes.—Endoglucanase (endoIV; EC 3.2.1.4) and exoglucanases (exoI; EC 3.2.1.91) were purified from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme CI, Gist-Brocades, Delft, The Netherlands) by Beldman *et al.* [13].

Chemical and enzymic treatment of oligosaccharides

Reduction of GFG.—In order to label GFG at its reducing terminus, *c.* 100 μg of this oligosaccharide were treated with 200 μL of 1.5 M ammonia containing 75 mg mL^{-1} of $NaBH_4$ for 1 h at 30 °C. After neutralization with HOAc, the reduced oligosaccharide was desalted using a mixture of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) resins (Bio-Rad, Richmond, CA, USA) in a ratio of *c.* 0.6 (v/v), and subsequently dried in a stream of air.

Degradation of oligosaccharides by endoIV and exoI.—Approximately 20 μg of the oligosaccharide-containing fractions were treated (12 h, 40 °C) with exoI (*c.* 100 ng of protein) in 100 μL of 50 mM NaOAc buffer (pH 5). Release of Glc was determined using HPAEC. Under similar conditions, *c.* 20 μg of GFGGFG was treated with *c.* 50 ng of endoIV. The resulting degradation products were analyzed by HPAEC.

Analytical methods

Protein content.—The protein content of enzyme preparations was determined according to the procedure of Sedmak and Grossberg [14], using BSA as a standard.

HPAEC analysis of xyloglucan oligosaccharides.—Degradation products of purified xyloglucan oligosaccharides were analyzed by HPAEC (CarboPac PA-100 column) as described previously [8].

NMR Spectroscopy.—Prior to ^{1}H-NMR analysis, xyloglucan oligosaccharides were exchanged twice in D_2O (99.9 atom% D, MSD Isotopes) with intermediate freeze-drying. Finally, samples were dissolved in 99.96 atom% D (MSD Isotopes). ^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AMX-500, or at 600 MHz on a Bruker AMX/2-600 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225). The 1D spectra were recorded with a spectral width of 5000 Hz at 500 MHz or 6000 Hz at 600 MHz, collecting 80-1000 free induction decays of 8K or 16K complex data points. Suppression of HOD was achieved by applying the WEFT pulse sequence as described [15]. The resolution of the 1D spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomial function when necessary.

2D NOESY was carried out with a 250 ms mixing time. 2D HOHAHA measurements [16] were performed using an MLEV-17 mixing sequence of 100-120 ms. For 2D DQF-COSY, 2D NOESY, and 2D HOHAHA spectra 512 measurements of 2K data points were recorded. The spectral width was 4032 Hz or 4500 Hz in each dimension.

2D ROESY was carried out using a spin-lock mixing pulse of 250 ms at a field strength corresponding to a 90° pulse-width between 100-110 ms. The carrier frequency was placed on the left side of the spectrum at δ 5.9 in order to minimize HOHAHA-type magnetization transfer. The spectral width was 5500 Hz in each dimension, and 512 experiments of 4K data points were recorded.

The 2D DQF-COSY, 2D NOESY, 2D HOHAHA, and 2D ROESY experiments were performed using the time-proportional phase-increment method to create t_1 amplitude modulation.

The HOD signal was suppressed by presaturation for 1.0 s. 2D NMR data were processed on Silicon Graphics Iris Indigo or 4D/35 stations, using Triton software (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University).

Mass spectrometry.—Positive ion FAB mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at 10 kV or 6 kV (for peracetylated fractions ***12.1*** and ***12.2***) accelerating voltage. The FAB gun was set at an emission current of 10 mA and generated a beam of 6 keV Xenon atoms. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running JEOL Complement software. CID mass spectra were recorded using the same instrument; after selecting the parent ion in the first mass spectrometer it was collided with helium in the third field free region collision cell, at a pressure sufficient to reduce the parent ion beam to one third of its original intensity. Daughter ion spectra were recorded by scanning the second mass spectrometer. Samples were dissolved in 10 μl of 5% aqueous HOAc (native samples), or DMSO (peracetylated samples), and 1.5 μl aliquots of sample solution were loaded into a matrix of mono-thioglycerol.

Per-*O*-acetylation was carried out on 1/20th of each fraction, which was dried under vacuum and then treated with 250 μL of a mixture {(2:1 (v/v)} of trifluoroacetic anhydride and HOAc (glacial). The reaction proceeded for 20 min at ambient temperature and the reagents were then removed under vacuum. The peracetylated samples were dissolved in chloroform and extracted with water (three times), and the solvent finally removed under vacuum.

Nomenclature

The xyloglucans are named according to the specific nomenclature for xyloglucans [9]. The Glc residues in the backbone of an oligosaccharide are designated by superscripts (a to e) starting from the Glc residue at the reducing terminus (*i.e.* Glc^{e}-Glc^{d}-Glc^{c}-Glc^{b}-Glc^{a}-Glc). The Xyl, Gal, and Fuc residues are denoted by the same superscript as the Glc residue to which they are attached. This is slightly different from the numbering system used by others [11] where in the larger structures the internal unbranched Glc is denoted Glc^{s}, bordered on both sides by three branched Glc residues. Since this situation does not apply here, a different notation is used.

RESULTS

Degradation of apple fruit xyloglucan by endoIV yielded a number of oligosaccharides which were fractionated using BioGel P-2 chromatography (Fig. 1) and semi-preparative HPAEC [8]. Based on monosaccharide analysis, mass spectrometric analysis and degradation with Driselase, tentative structures were proposed for most purified oligosaccharides. Next to the common oligosaccharides such as XXXG, XXFG, and XLFG, there were also indications for new structural elements (***12.1*** and ***12.2***) as building units of apple xyloglucan. In the present study, the proposed structures for the common oligosaccharides were confirmed. However, the accent of this study was on a detailed characterization of fractions ***12.1*** and ***12.2*** using spectrometric and spectroscopic techniques. Fraction ***6.1*** proved to be important for the elucidation of the structure of the compounds in these fractions. Therefore, the characterization of the compound in fraction ***6.1*** will be elaborated as well.

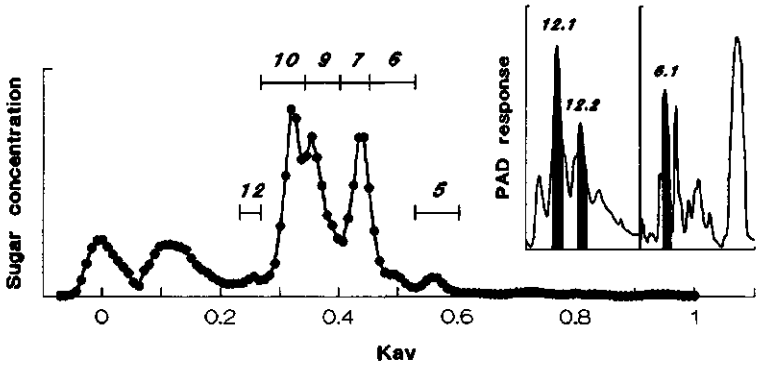

FIGURE 1. Elution pattern of an apple xyloglucan digest on BioGel P-2. Insets represent the elution profiles on semi-preparative HPAEC of pools *12* and *6*. The fractionation of the apple xyloglucan digest is described in more detail by Vincken *et al.* [8].

Characterization of common (unreduced) xyloglucan oligosaccharides

The current characterization of the xyloglucan fractions ***5.1***, ***5.2***, ***7.4***, ***9.3a***, ***9.2***, and ***10.1*** has been achieved on the basis of compositional [8], mass spectrometric and ^{1}H-NMR analysis. For the assignment of ^{1}H-NMR spectra, the structural-reporter groups for xyloglucans have been used [3,10-12,17]. It should be noted that in the present study the xyloglucan oligosaccharides are not reduced to oligoglycosyl alditols [3,10-12,17]. The branching patterns of XLXG and XXFG were identified using FAB-MS in conjunction with per-*O*-acetylation (data not shown). The ^{1}H-NMR H-1 chemical shift values are summarized in Table I. The chemical shift values for Glc^a and Glc^b, as shown in Table I, were assigned based on comparison with their reduced homologues [3,17]. It was assumed that the differences in chemical shift values between unreduced and reduced oligosaccharides were smaller for Glc^b than in Glc^a (close to the reducing end).

The fucosylated oligosaccharides, XXFG and XLFG, showed similar anomerization effects to those described by Levy *et al.* [18]. The H-1 resonance of Xyl^a, as well as the H-6 resonance of Fuc^a are split into two doublets. Typically, this is not observed in FG. Note that the anomerization effect only occurs (see further, Table II) when the backbone of FG is extended with one Glc residue at the nonreducing side (as in GFG).

Fraction *6.1*

Fraction ***6.1*** was examined using positive ion-mode FAB-MS. A single $[M+Na]^+$ pseudomolecular ion was observed at m/z 967, corresponding to a $Hex_4Deoxyhex_1Pent_1$ hexasaccharide. The presence of a single hexasaccharide was in agreement with the intensities of the anomeric signals, as observed in the ^{1}H-NMR spectrum (Fig. 2). The Fuc H-1 signal at 5.273 ppm indicates the presence of one F element in this oligosaccharide. The chemical shifts of the anomeric signals of this F element are almost identical to those in XXFG and XLFG (compare Tables I and II). In addition, two β

TABLE I. ^{1}H-NMR H-1 chemical shifts (ppm) for the penta- to decasaccharides derived from apple fruit xyloglucan.

Residue	FG (*5.1*)‡	XXG* (*5.2*)‡	XXXG† (*7.4*)‡	XLXG* (*9.3a*)‡	XXFG† (*9.2*)‡	XLFG† (*10.1*)‡
Glcß	4.667	4.662	4.661	4.661	4.665	4.665
Glcα	5.221	5.223	5.221	5.221	5.221	5.220
Glc^a	4.513	4.561§	4.559§	4.534§	4.540§	4.548§
Glc^b		4.553§	4.574§	4.565§	4.533§	4.519§
Glc^c			4.550	4.534	4.547	4.533
$Xyl^a{}_\beta$‖	5.134	4.959	4.957	4.956	5.141	5.138
$Xyl^a{}_\alpha$‖	5.138				5.144	5.141
Xyl^b		4.941	4.957	5.176	4.954	5.175
Xyl^c			4.940	4.941	4.941	4.942
Gal^a	4.621				4.616	4.618
Gal^b				4.558		4.555
Fuc^a	5.286				5.272	5.275

* ^{1}H-NMR chemical shift values of the reduced form of this oligosaccharide were reported by York *et al.* [17]. † ^{1}H-NMR chemical shift values of the reduced form of this oligosaccharide were reported by York *et al.* [3]. ‡ Numbers correspond to fractions in purification procedure described by Vincken *et al.* [8]. § Might be interchanged, detailed assignment was not possible due to small amounts of material. ‖ Two Xyl^a signals due to α, ß anomerization.

anomeric signals appear at 4.537 and 4.479 ppm, which were assigned using 2D NMR spectroscopy as belonging to Glc residues. The scalar coupled networks of each residue starting from the anomeric protons are observed in the HOHAHA spectrum (Fig. 3), and the chemical shifts are summarized in Table II. The positions of the Glc residues have been identified on the basis of cross-peaks observed in the ROESY spectrum (Fig. 4), in which Glc^a H-1 at 4.537 ppm is assigned to the branched internal, and Glc^b H-1 at 4.479 ppm to the unbranched nonreducing terminal Glc residue. Other evidence for the assignment of Glc^b can be found in the fact that the chemical shift values of the ring protons are in agreement with those of the nonreducing terminus of methyl-ß-cellobioside [19]. The ^{1}H-NMR data show that the component in fraction ***6.1*** corresponds to GFG.

In order to confirm this assignment of the position of the extended sidechain at Glc^a, fraction ***6.1*** was also examined using FAB-MS. In order to improve sensitivity and obtain unambiguous fragmentation, fraction ***6.1*** was per-*O*-acetylated, and the salt removed on organic/aqueous partition. The organic fraction was analyzed using positive ion-mode FAB-MS. An $[M+H]^+$ pseudomolecular ion can be identified at m/z 1701, corresponding to the fully acetylated hexasaccharide. Abundant oxonium ions are present at m/z 273 (Fuc^+), 331 (Glc^+), 561 ($Fuc\text{-}Gal^+$), 777 ($Fuc\text{-}Gal\text{-}Xyl^+$), 1353 ($GF^+$), and 1641 ($GFG^+$), defining the branching pattern of the hexasaccharide, in which the extended sidechain is attached to the internal Glc residue in the backbone. CID tandem MS analysis of the first oxonium ion at m/z 1641 yields the same abundant oxonium fragments (see Fig. 5A) and confirms the assignment.

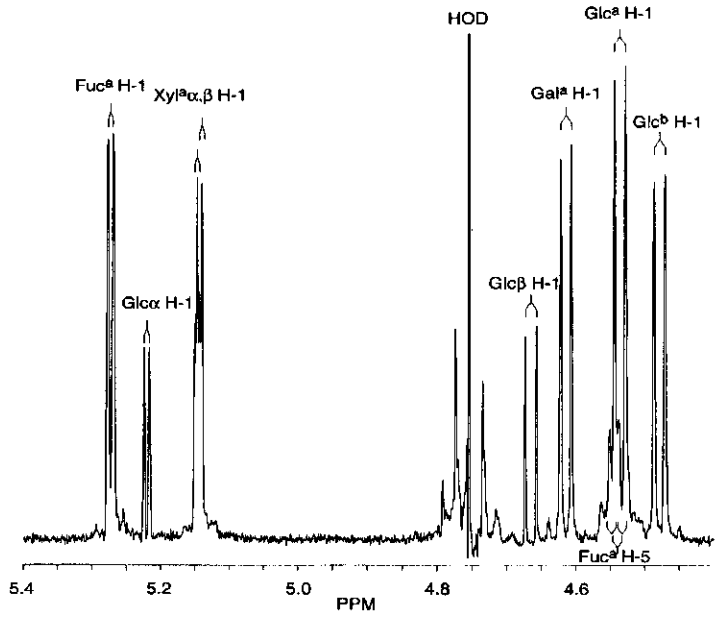

FIGURE 2. Resolution-enhanced 500 MHz ^{1}H-NMR spectrum of GFG.

TABLE II. ^{1}H-NMR full assignment of chemical shifts (ppm) of the xyloglucan fragment GFG.

Residue	H-1	H-2	H-3	H-4	H-5	H-5$_{eq}$	H-6	H-6'	CH_3
Glcß	4.666	3.276	3.615	3.615	3.615		3.810	3.946	
Glcα	5.221	3.567	3.615	3.857*	3.827*		3.940	3.940	
Glc^a	4.537	3.388	3.673	3.611	3.920		3.920	3.920	
Glc^b	4.479	3.331	3.505	3.422	3.488		3.736	3.912	
Xyl$^a_ß$†	5.144	3.658	3.884	3.615‡	3.597‡	3.690‡			
Xyl$^a_α$†	5.148								
Gal^a	4.615	3.741	3.850	3.905	3.670		3.750	3.800	
Fuc$^a_ß$†	5.273	3.791	3.886	3.806	4.545				1.262
Fuc$^a_α$†									1.257

* Assignments might be interchanged due to strong overlap. † Due to α, ß anomerization. ‡ Assignments might be interchanged due to strong overlap.

Fraction ***6.1*** was treated with exoI, and the products were analyzed by HPAEC; the reaction products coelute with Glc and FG. Upon reduction of fraction ***6.1*** and subsequent degradation by exoI, only Glc and no glucitol was detected in the reaction mixture. These data are consistent with the results that the Glc residue containing the fucosylated sidechain is flanked on both sides by an unbranched Glc residue.

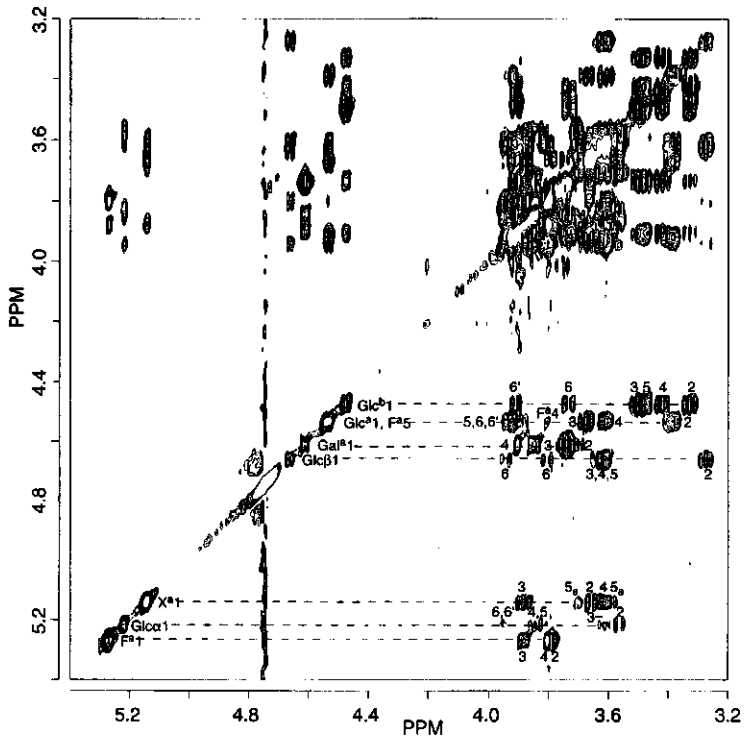

Figure 3. 500 MHz 2D HOHAHA spectrum of GFG. In the figure the diagonal peaks of the protons in the anomeric region are indicated. The numbers near cross-peaks in the figure refer to the protons of the scalar coupling network belonging to a diagonal peak.

Fraction *12.1*

Comparing the ^{1}H-NMR spectrum of fraction ***12.1*** with that of GFG, the two spectra are very similar except for the different peak intensity ratios (Fig. 6). The resonances of the anomeric signals are summarized in Table III. The 2D NMR spectra of fraction ***12.1*** are also very similar to those of GFG.

The positive ion-mode FAB-mass spectrum of the peracetylated derivative contains a low abundance signal corresponding to the $[M+H]^+$ pseudomolecular ion at m/z 3299, and to a composition of $Hex_8Deoxyhex_2Pent_2$ (Fig. 5B). The low mass region in the spectrum is very similar to that of GFG (Fig. 5A). Additionally, signals corresponding to oxonium ions are present at m/z 1929 ($GFGG^+$), m/z 2951 ($GFGGF^+$), and m/z 3239 ($GFGGFG^+$), defining the branching pattern (Fig. 5B). No signals are present to suggest a difference in structure between the two sidechains, or to indicate minor components with minor differences in the branching pattern. It can thus be concluded that the structure is GFGGFG. This is the first occasion where two neighbouring internal unbranched Glc residues are found to be present in a xyloglucan oligosaccharide.

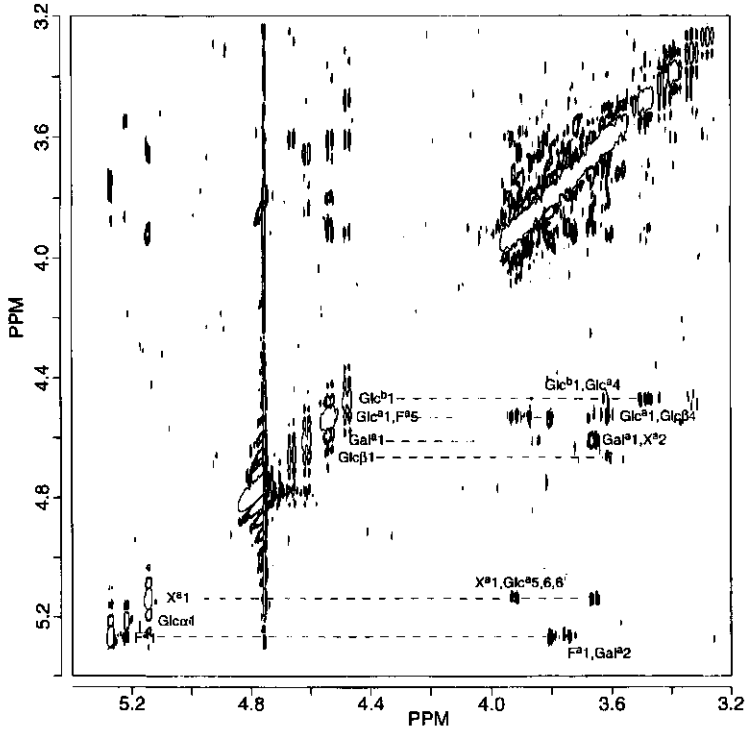

FIGURE 4. 500 MHz 2D ROESY spectrum of GFG. Solely the inter-residual NOE-connectivities along the H-1 tracks are denoted. F^a1,2 means a cross-peak between H-1 and H-2 of Fuc^a.

Fraction ***12.1*** is probably slightly contaminated with XLFG, considering the H-1 resonance at 5.175 ppm (see Table I) that corresponds to a Xyl^b residue to which a terminal Gal is attached. The slight contamination of fraction ***12.1*** with XLFG is further substantiated by the inset of Fig. 1. The retention time of the peak that flanks fraction ***12.1*** on the left side corresponds to that of XLFG. It should be mentioned that ions supporting the presence of such a contaminant are also observable at very low intensity in the FAB-mass spectrum of the peracetylated derivative.

In principle, GFGGFG should have been degraded by endoIV during the preparation of the apple fruit xyloglucan digest. In order to determine whether GFGGFG is endoIV-resistant, this oligosaccharide was incubated with endoIV. Analysis of the reaction products using HPAEC showed that GFGGFG was completely degraded to GFG. From this it was concluded that GFGGFG is not endoIV-resistant, but that cleavage of GFG↓GFG is more difficult for endoIV than that of, for instance, XXFG↓XXXG.

Fraction *12.2*

The FAB-mass spectrum of underivatized fraction ***12.2*** has $[M+Na]^+$ pseudomolecular

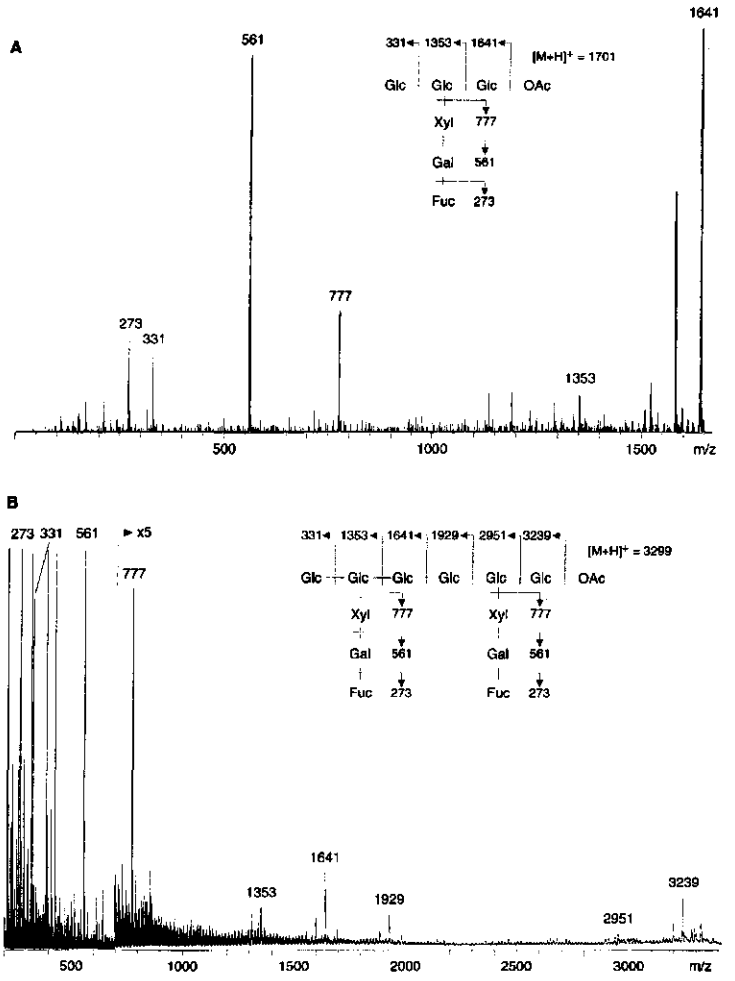

FIGURE 5. CID spectrum of oxonium ion at m/z 1641 of per-*O*-acetylated fraction ***6.1***, with fragmentation scheme (A). FAB mass spectrum of per-*O*-acetylated fraction ***12.1***, with fragmentation scheme (B).

ions of similar abundance at m/z 1893 and m/z 2025, corresponding to oligosaccharides having compositions $Hex_8Deoxyhex_2Pent_2$ and $Hex_8Deoxyhex_2Pent_3$, respectively. The ^{1}H-NMR spectrum is very similar to that of GFG and GFGGFG (Fig. 6).

Following per-*O*-acetylation, a FAB-mass spectrum was recorded. The spectrum contains several series of ions, indicating this fraction to be a mixture of several components. The major series of signals at m/z 273, 331, 561, 777, 1353, 1641 and 1929, corresponds to the GFGG- structural element described for GFGGFG. An additional fragment ion at m/z 1857 corresponds to an oxonium ion from a related species, bearing an additional Xyl residue; taken together with the fragment ions at m/z 273 (Fuc^+), 331

TABLE III. ^{1}H-NMR H-1 chemical shifts (ppm) for GFG, GFGGFG, and fraction *12.2*.

Residue	GFG	GFGGFG	fraction *12.2*
Glcß	4.666	4.666	4.666
Glcα	5.221	5.221	5.221
Glc^a	4.537	4.536	4.537
Glc^b	4.479	4.536	4.537
Glc^c		4.536	4.537
Glc^d		4.536	4.537
Glc^e		4.475	4.479
$Xyl^{a*}_{ß}$	5.144	5.144	5.144
$Xyl^{a*}_{α}$	5.148		
Xyl^b/Xyl^c			4.955/4.939†
Xyl^d		5.144	5.144
Gal^a	4.615	4.615	4.615
Gal^d		4.615	4.615
$Gal^{a,d‡}$			4.558
Fuc^a	5.273	5.273	5.273
Fuc^d		5.273	5.273

* Due to α, ß anomerization. † Not possible to assign, due to low amounts of material and strong overlap. ‡ Terminal Gal, no Fuc attached.

(Glc^+), 561 (Fuc-Gal^+), 777 (Fuc-Gal-Xyl^+), 1353 (GF^+) the fragment ion at m/z 1857 demonstrates the presence of a GFX- element. The possibility that the ion at m/z 1857 corresponds to an XFG- element can be excluded, since no signal corresponding to XF^+ is observed. Additionally, less abundant oxonium ions at m/z 547 (Gal-Xyl^+), 1123 (GL^+) and 1411 (GLG^+) are observed, corresponding to an additional GLG- structural element, in which the sidechain lacks its terminal Fuc residue. The oxonium ion at m/z 835 could correspond to either XG- or GX-, while a weak series of oxonium ions at m/z 331, 619, 907 and 1195 suggests the presence of a hexose polymer. The mass spectrometric data do not preclude the additional presence of a GFGX- structure. The FAB-MS data are supported by the NMR spectra where the anomeric protons at 4.954 and 4.939 ppm probably belong to terminal Xyl residues being attached to either Glc^b or Glc^c. A fragment ion containing terminal hexose is observed in the mass spectrum, and NMR evidence for this element can also be found in the signal at 4.558 ppm where a terminal Gal H-1 resonates, which is not present in the ^{1}H-NMR spectrum of GFGGFG. It can be concluded that fraction *12.2* is a mixture of several components, including GFGGFG (main component) and GFXGFG.

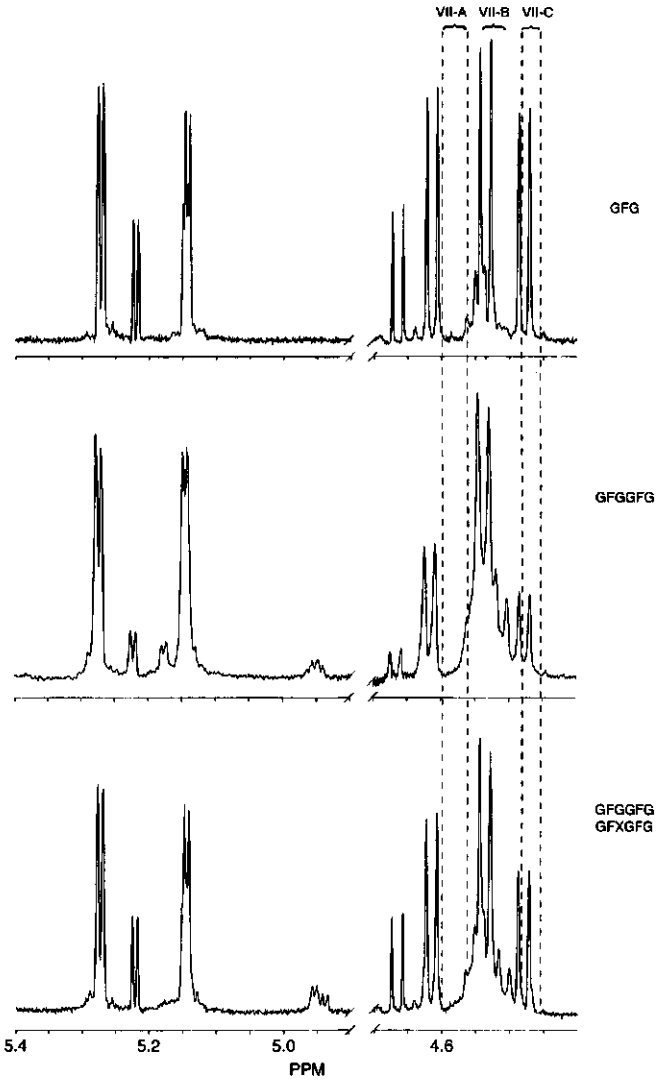

FIGURE 6. Region VII of the ^{1}H-NMR spectra of GFG, GFGGFG, and fraction ***12.2***. The subclasses of region VII are diagnostic for different structural features of the xyloglucans, as described in the text. The chemical shift range corresponds to the centre of the multiplet, and does not necessarily include all of the lines in the multiplet.

DISCUSSION

Although apple fruit xyloglucan is predominantly built up from XXXG, XXFG and XLFG, oligosaccharides with a less heavily branched glucan backbone can also occur [7,8]. In this investigation two such oligosaccharides were characterized using ^{1}H-NMR and mass spectrometric analyses. Their structures were shown unambiguously to correspond to GFG and GFGGFG.

On the basis of these new structures the chemical shift regions indicative of the structural elements, previously only reported for alditols, can be further extended. Our results show that the signals of nonreducing terminal unbranched Glc residues, flanked by a Glc residue containing a fucosylated sidechain appear in a very narrow region. Two examples of such residues are discussed in the present paper, *e.g.* Glc^b of GFG and Glc^e of GFGGFG with chemical shift values of 4.479 ppm and 4.475 ppm, respectively. Very recently, a chemical shift value of 4.483 ppm for Glc^b of GFGol has been reported [20]. Apparently, the distance from the reducing terminus (compare GFG, GFGGFG, and GFGol) does not affect the chemical shift values of these Glc residues much.

Hisamatsu *et al.* [11] distinguish three regions (VI, VII-A, and VII-B) which encompass all chemical shift values of different Glc residues present in the backbone of xyloglucan molecules. Region VI (4.600-4.660 ppm) includes H-1 signals of 4,6-linked ß-Glc attached to C-4 of Glcol (*e.g.* XXFG), and H-1 signals of 2,4,6-linked ß-Glc (*e.g.* XXFGAXXG). Region VII-A (4.560-4.600 ppm) includes H-1 signals of 4,6-linked ß-Glc^b when no α-Fuc-(1→2)-ß-Gal-(1→2)-moiety is present on Xyl^a (*e.g.* XXFGAXXG). Region VII-B (4.480-4.560 ppm) represents H-1 signals of 4-linked, 6-linked, and 4,6-linked ß-Glc not assigned in regions VI or VII-A (*e.g.* XXFGAXFG). Based on our results and one report in the literature [20], the structural-reporter groups for xyloglucans can be extended with a new region on the down-field side of region VII-B. This new region VII-C (4.475-4.485 ppm) includes the H-1 signals of nonreducing terminal unbranched Glc residues that are flanked by an F element (*e.g.* GFG). Our results show further that next to the H-1 signal of a single internal unbranched Glc residue (*e.g.* XXFGAXFG), the H-1 signals of two vicinal internal unbranched Glc residues (GFGGFG) are included in region VII-B.

Detection of a GFX structural element by mass spectrometry suggests that apple fruit xyloglucan contains small amounts of GFXG building units. Such an oligosaccharide might be formed as a result of α-xylosidase action on the nonreducing terminus of a xyloglucan molecule (XFXG→GFXG). These structures have not been previously described in the literature, although the presence of a Fuc-containing sidechain attached at a similar position in an oligosaccharide (*e.g.* XFFG [21]) has been demonstrated. Further, it should be mentioned that apple fruit xyloglucan contains reasonable amounts of XLXG. The results described in this study provide indications that fucosylation of such an oligosaccharide (for instance XFXG) is possible.

It is an intriguing question as to whether structures like GFG and GFGGFG are

synthesized as such or originate from post-depositional modifications in the cell wall. Unlike cellulose, xyloglucans are assembled entirely in the plant cell before being transported to, and excreted into the cell wall. Biosynthesis of xyloglucan involves an alternate transfer of Glc and Xyl to a nascent xyloglucan acceptor, by xyloglucan 4-ß-D-glucosyltransferase and a 6-α-D-xylosyltransferase, respectively [22-24]. This chain elongation, which is confined to the Golgi cisternae [25,26], depends on the presence of neither Gal nor Fuc [27]. Decoration of the xyloglucan (with Gal, Fuc) occurs predominantly in the *trans* Golgi network or dictyosomes [25,26]. The UDP-Xyl concentration plays an important role in the branching pattern of the glucan backbone. However, the exact mechanism for leaving unsubstituted Glc residues is still a matter of speculation. The structure of xyloglucan suggests that these units are introduced at regular intervals [28]. In apple fruit xyloglucan every fourth Glc residue in the backbone remains unbranched (XXXG-like structural elements).

Structural deviations from the basic apple fruit xyloglucan structure may be a result of the action of endogenous enzymes. A complete set of xyloglucan-modifying enzymes has been described in the literature, including ß-glucosidase [29], α-xylosidase [30,31], ß-galactosidase [32] and α-fucosidase [33]. It is expected that similar enzymes are also present in apple, and these could be responsible for generating XG, FG, XXG, GLG, and GFG building units at the nonreducing terminus of a xyloglucan molecule. For instance, GFG may be formed from XXFG by the consecutive action of α-xylosidase, ß-glucosidase, and α-xylosidase (XXFG→GXFG→XFG→GFG). The occurrence of GFGGFG is more difficult to explain because no α-xylosidases catalyzing a GFGXFG→GFGGGFG reaction have been reported to date.

XET [34] cleaves a xyloglucan molecule (donor), and transfers the nonreducing portion of the donor molecule to the nonreducing terminus of an acceptor molecule. Thus, in principle, XET action could be responsible for the eventual presence of XG, FG, XXG, and GFG in a mid-chain position, if these oligosaccharides are originally present as the nonreducing building unit of the acceptor molecule. However, not all oligosaccharides can be incorporated in this way. It appears that the acceptor molecule should meet certain requirements; two vicinal X elements (*e.g.* as in XXG) are required for XET action [35], so that FG, GXG, and XGG are not suitable acceptor substrates. Unfortunately, GFG has never been tested although it is expected that XET will not utilize this oligosaccharide as an acceptor, since it lacks vicinal X elements. A role for XET in forming GFGGFG seems, therefore, unlikely. With the current state of knowledge of biosynthesis and enzymic modification of xyloglucans no adequate explanation for the occurrence of GFGGFG in apple fruit xyloglucan can be given.

REFERENCES

[1] Carpita, N.C., and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**, 1-30.

[2] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[3] York, W.S., van Halbeek, H., Darvill, A.G., and Albersheim, P. (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and Fast-Atom-Bombardment Mass Spectrometry. *Carbohydr. Res.* **200**, 9-31.

[4] Kato, Y., Ito, S., Iki, K., and Matsuda, K. (1982) Xyloglucan and ß-D-glucan in cell walls of rice seedlings. *Plant Cell Physiol.* **23**, 351-364.

[5] Ring, S.G., and Selvendran, R.R. (1981) An arabinogalactoxyloglucan from the cell wall of *Solanum tuberosum*. *Phytochem.* **20**, 2511-2519.

[6] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and endoglucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).

[7] Renard, C.M.G.C., Lomax, J.A., and Boon, J.J. (1992) Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and alkali-extracted xyloglucans. *Carbohydr. Res.* **232**, 303-320.

[8] Vincken, J.-P., Beldman, G., Niessen, W.M.A., and Voragen, A.G.J. (1996) Degradation of apple fruit xyloglucan by endoglucanase. *Carbohydr. Polym.*, in press (this thesis, Chapter 3).

[9] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.

[10] Kiefer, L.L., York, W.S., Albersheim, P., and Darvill, A.G. (1990) Structural characterization of an arabinose-containing heptadecasaccharide enzymically isolated from sycamore extracellular xyloglucan. *Carbohydr. Res.* **197**, 139-158.

[11] Hisamatsu, M., York, W.S., Darvill, A.G., and Albersheim, P. (1992) Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues. *Carbohydr. Res.* **227**, 45-71.

[12] York, W.S., Impallomeni, G., Hisamatsu, M., Albersheim, P., and Darvill, A.G. (1995) Eleven newly characterized xyloglucan oligoglycosyl alditols: the specific effects of sidechain structure and location on ^{1}H NMR chemical shifts. *Carbohydr. Res.* **267**, 79-104.

[13] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.

[14] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.

[15] Hård, K., van Zadelhoff, G., Moonen, P., Kamerling, J.P., and Vliegenthart, J.F.G. (1992) The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and N-acetylgalactosamine-containing N-linked chains. *Eur. J. Biochem.* **209**, 895-915.

[16] Bax, A., and Davis, D.G. (1985) Practical aspects of two-dimensional transverse NOE spectroscopy. *J. Magn. Reson.* **63**, 207-213.

[17] York, W.S., Harvey, L.K., Guillén, R., Albersheim, P., and Darvill, A.G. (1993) Structural analysis of tamarind seed xyloglucan oligosaccharides using ß-galactosidase digestion and spectroscopic methods. *Carbohydr. Res.* **248**, 285-301.

[18] Levy, S., York, W.S., Stuike-Prill, R., Meyer, B., and Staehelin, L.A. (1991) Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific folding. *Plant J.* **1**, 195-215.

[19] Kroon-Batenburg, L.M.J., Kroon, J., Leeflang, B.R., and Vliegenthart, J.F.G. (1993) Conformational analysis of methyl ß-cellobioside by ROESY NMR spectroscopy and MD simulations in combination with the CROSREL method. *Carbohydr. Res.* **245**, (1993) 21-42.

[20] Guillén, R., York, W.S., Pauly, M., An, J., Impallomeni, G., Albersheim, P., and Darvill, A.G. (1995) Metabolism of xyloglucan generates xylose-deficient oligosaccharide subunits of this polysaccharide in etiolated peas. *Carbohydr. Res.* **277**, 291-311.

[21] Hisamatsu, M., Impallomeni, G., York, W.S., Albersheim, P., and Darvill, A.G. (1991) A new undecasaccharide subunit of xyloglucans with two α-L-fucosyl residues. *Carbohydr. Res.* **211**, 117-129.

[22] Hayashi, T., and Matsuda, K. (1981) Biosynthesis of xyloglucan in suspension-cultured soybean cells. Occurrence and some properties of xyloglucan 4-ß-D-glucosyltransferase and 6-α-D-xylosyltransferase. *J. Biol. Chem.* **256**, 11117-11122.

[23] Hayashi, T., Nakajima, T., and Matsuda, K. (1984) Biosynthesis of xyloglucan in suspension-cultured soybean cells. Processing of the oligosaccharide building blocks. *Agric. Biol. Chem.* **48**, 1023-1027.

[24] White, A.R., Xin, Y., and Pezeshk, V. (1993) Xyloglucan glucosyltransferase in Golgi membranes from *Pisum sativum* (pea). *Biochem. J.* **294**, 231-238.
[25] Brummell, D.A., Camirand, A., and Maclachlan, G.A. (1990) Differential distribution of xyloglucan glycosyl transferases in pea Golgi dictyosomes and secretory vesicles. *J. Cell Sci.* **96**, 705-710.
[26] Zhang, G.F., and Staehelin, L.A. (1992) Functional compartmentation of the Golgi apparatus of plant cells. Immunocytochemical analysis of high-pressure frozen- and freeze-substituted sycamore maple suspension culture cells. *Plant Physiol.* **99**, 1070-1083.
[27] Gordon, R., and Maclachlan, G. (1989) Incorporation of UDP-[^{14}C]glucose into xyloglucan by pea membranes. *Plant Physiol.* **91**, 373-378.
[28] Gibeaut, D.M., and Carpita, N.C. (1994) Biosynthesis of plant cell wall polysaccharides. *FASEB J.* **8**, 904-915.
[29] Edwards, M., Dea, I.C.M., Bulpin, P.V., and Reid, J.S.G. (1985) Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeolum majus* L. seeds following germination. *Planta* **163**, 133-140.
[30] O'Neill, R.A., Albersheim, P., and Darvill, A.G. (1989) Purification and characterization of a xyloglucan oligosaccharide-specific xylosidase from pea seedlings. *J. Biol. Chem.* **264**, 20430-20437.
[31] Fanutti, C., Gidley, M.J., and Reid, J.S.G. (1991) A xyloglucan-oligosaccharide-specific α-D-xylosidase or *exo*-oligoxyloglucan-α-xylohydrolase from germinated nasturtium (*Tropaeolum majus* L.) seeds. *Planta* **184**, 137-147.
[32] Edwards, M., Bowman, Y.J.L., Dea, I.C.M., and Reid, J.S.G. (1988) A ß-D-galactosidase from nasturtium (*Tropaeolum majus* L.) cotyledons. *J. Biol. Chem.* **263**, 4333-4337.
[33] Augur, C., Benhamou, N., Darvill, A., and Albersheim, P. (1993) Purification, characterization, and cell wall localization of an α-fucosidase that inactivates a xyloglucan oligosaccharin. *Plant J.* **3**, 415-426.
[34] Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828.
[35] Lorences, E.P., and Fry, S.C. (1993) Xyloglucan oligosaccharides with at least two α-D-xylose residues act as acceptor substrates for xyloglucan endotransglycosylase and promote the depolymerisation of xyloglucan. *Physiol. Plant.* **88**, 105-112.

CHAPTER 5

Potato Xyloglucan Is Built from XXGG-type Building Units[I]

Extraction of potato cell wall material with solutions of increasing strength of alkali yielded a xyloglucan-rich fraction which was further purified by anion-exchange chromatography and treatment with α-amylase and endogalactanase. Methylation analysis indicated that the purified xyloglucan contained a high percentage of unsubstituted glucosyl residues compared to, for instance, apple xyloglucan, and equal amounts of Xyl-(1→6)-, Gal-(1→2)-Xyl-(1→6)- and Ara-(1→2)-Xyl-(1→6)-sidechains. This xyloglucan was degraded with endoglucanase (endoV), purified from *Trichoderma viride*. The resulting digest was fractionated by BioGel P-2 chromatography, followed by preparative high-performance anion-exchange chromatography of the pentamer to nonamer fractions. The purified oligosaccharides were characterized by monosaccharide analysis, mass spectrometry and degradation with an exoglucanase. Degradation of potato xyloglucan by another endoglucanase (endoIV) of *Trichoderma viride* yielded a different set of products. EndoIV released predominantly oligosaccharides with two unbranched glucosyl residues at the reducing terminus, whereas endoV also released products containing unbranched glucosyl residues on both ends of the molecule. This is the first report in which a difference in mode of action of endoglucanases with xyloglucan-degrading activity is demonstrated.

Xyloglucans occur widely in the kingdom of plants [1], where they reinforce the primary cell wall by crosslinking cellulose microfibrils [2]. Their main structural characteristic is a (1→4)-ß-D-Glc*p*[II] backbone with α-D-Xyl*p*-(1→6)-sidechains. The presence of other glycosyl residues, like ß-D-Gal*p*, α-L-Fuc*p* or α-L-Ara*f*, as well as sidechain density, depend on the plant species. Xyloglucans can be degraded to oligosaccharides by endoglucanases, and usually a relatively crude cellulase preparation is used for this purpose. However, Vincken *et al.* [3] demonstrated that individual glucanases, derived from a *Trichoderma viride* cellulase preparation, can differ considerably in their ability to degrade apple fruit xyloglucan. It is unknown if these enzymes differ in their mode of action towards xyloglucan.

Apple fruit xyloglucan is built mainly from the structural elements XXXG, XLXG, XXFG and XLFG [4,5] (for nomenclature, see [6]), and hardly any sequences of unsubstituted Glc exist. Endoglucanases are "forced" to cleave right next to a branched Glc residue (for instance -XXXG↓XXXG-). When using such heavily branched polysaccharides such as apple xyloglucan, possible differences in mode of action of endoglucanases are likely to be masked. Therefore, we searched for a less branched

---

[I]Vincken, J.-P., Wijsman, A.J.M., Beldman, G., Niessen[*], W.M.A., and Voragen, A.G.J. Accepted for publication in *Carbohydr. Res.* ([*] *Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden, The Netherlands*).

[II]*Abbreviations*: see page viii.

xyloglucan substrate which would offer endoglucanases the possibility to hydrolyze their linkage of preference.

A number of these more linear xyloglucans have been described in literature. The xyloglucan of rice seedlings [7] and immature barley plants [8] are predominantly built from XXGGG structural units; the former also contains regions with an even lower degree of substitution. Contrary to apple fruit xyloglucan, these polysaccharides are poorly water soluble. Xyloglucan of potato has a slightly higher degree of branching than that of rice seedlings or barley plants (*c.* 45%; [9]), and is soluble in water; therefore, this polysaccharide seems a better candidate to study the mode of action of endoglucanases. Potato xyloglucan lacks fucosylated sidechains as in apple, but contains Ara-(1→2)-Xyl-(1→6)-sidechains instead. The presence of Ara residues has also been observed in xyloglucans of *Nicotiana tabacum* [10] and *Phaseolus coccineus* [11].

Presently, the distribution of sidechains of potato xyloglucan is unknown. A degree of substitution of almost 50% allows structures like XGXGXG, in which case the endoglucanase is forced to cleave right next to a sidechain. However, in analogy to barley plants [8] and rice seedlings [7], clusters of unbranched Glc residues and xylosylated Glc residues (as in GXXGGXXG) seem more likely. This study investigates the branching pattern of potato xyloglucan. In addition, the different mode of action of two endoglucanases from *Trichoderma viride* (endoIV and endoV) towards this substrate will be discussed.

EXPERIMENTAL

Materials

Raw material.—Potatoes (*Solanum tuberosum* var. Bintje) were obtained at a local market. A mixture of cellodextrins (DP 1 to 6) was obtained from Merck (Darmstadt, Germany).

Enzymes.—Two endoglucanases (endoIV and endoV; EC 3.2.1.4) and an exoglucanase (exoI; EC 3.2.1.91) were purified from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme Cl, Gist-Brocades, Delft, The Netherlands) by Beldman *et al.* [12]. Pancreas α-amylase was obtained from Merck and showed no activity towards cell wall polysaccharides. A purified endogalactanase from *Aspergillus niger* was present in the laboratory of the authors [13].

Purification of POsxg

Isolation of WUS.—Potatoes (4 kg) were peeled, cut (1 cm^3) and blanched (20 min) in water (70 °C) to inactivate endogenous enzymes and gelatinize starch. The potatoes were homogenized in Waring Blendor with 3 L of 10 mM maleate buffer (pH 6.5) containing 1 mM $CaCl_2$, 10 mM NaCl and 0.01% (w/v) NaN_3. The potato mash was treated with 75 mg of α-amylase (*c.* 15,000 U) for 16 h at 40 °C. After centrifugation (15 min, 16,300 *g*), the residue was extracted twice (1 h) with 3 L of distilled water. The first extraction was performed at 40 °C, the second one at 70 °C. The α-amylase treatment and subsequent extractions were repeated four times. The residual material was freeze-dried and ground in a Fritsch pulverisette (sieve 1.0 mm, Germany) and designated as WUS.

Extraction of WUS.—Potato WUS was subjected to a sequence of alkali extraction steps (0.05 M NaOH containing 5 mM CDTA, 1 M KOH containing 1% (w/w) $NaBH_4$ and 4 M KOH

containing 1% (w/w) $NaBH_4$; 1 g of WUS per 200 mL of extractant), in a similar manner as described for apple [3]. Extracts were acidified to pH 5 with HOAc, dialyzed extensively against distilled water and freeze dried. In some cases tiny amounts of material precipitated, which were removed by centrifugation (20 min, 50,000 *g*).

Purification of the 4 M KOH extract.—Residual starch was removed by treating *c.* 125 mg of the freeze dried material with α-amylase (100 μg) in 250 mL of 50 mM NaOAc buffer (pH 6) containing 0.01% (w/v) NaN_3 at 40 °C. After 24 h a similar dose of enzyme was added and the incubation was continued for another 24 h. The incubation was stopped by heating the reaction mixture 10 min at 100 °C. The mixture was then dialyzed (2 times 12 h) against 50 mM NaOAc buffer (pH 5) containing 0.01% (w/v) NaN_3. Subsequently, the material was depectinized on a DEAE Sepharose CL-6B column (40 x 440 mm, Pharmacia, Uppsala, Sweden) in a similar manner as described for apple xyloglucan [3]. The neutral fraction was concentrated under reduced pressure, acidified to pH 4.5 with HOAc, and treated with *c.* 20 mU of endogalactanase for 48 h at 40 °C. The reaction mixture was heated for 10 min at 100 °C, dialyzed extensively against distilled water and freeze dried. The resulting material was designated POsxg.

Preparation and purification of xyloglucan oligosaccharides

Enzymic degradation of POsxg.—POsxg (50 mg) was dissolved in 5 mL of 40 mM NaOAc buffer (pH 5), containing 0.01% (w/v) of NaN_3 and 10 μg of endoV protein, and incubated for 48 h at 40 °C. The resulting digest was heated for 10 min at 100 °C to inactivate endoV, and freeze-dried. In a similar manner, POsxg was treated with endoIV (*c.* 2 μg of protein).

BioGel P-2 chromatography.—The POsxg digests were dissolved in 2 mL of distilled water and applied to a column (100 x 2.6 cm, i.d.) of BioGel P-2 (200-400 mesh, Bio-Rad, Richmond, CA, USA) at 60 °C and eluted with distilled water (18 mL h^{-1}). Fractions (2.0 mL) were assayed for total neutral sugar content. Appropriate fractions were combined. The column was calibrated using a mixture of Glc, G_2, raffinose, stachyose and Dextran T150 (Pharmacia, Uppsala, Sweden).

Preparative HPAEC of BioGel P-2 fractions.—The BioGel P-2 fractions ***5*** to ***9*** of the endoV digest and fraction ***9*** of the endoIV digest were subjected to HPAEC using a Spectra Physics P4000 pump (San José, CA, USA) equipped with a Dionex CarboPac PA-100 column (250 x 22 mm, Sunnyvale, CA, USA). Samples of 400 μL (containing 3 to 7 mg mL^{-1}) were injected with a Spectra Physics AS3000 autosampler, and eluted (25 mL min^{-1}) at 20 °C with 100 mM NaOH containing different concentrations of NaOAc. The NaOAc gradients, which were optimized for each BioGel P-2 fraction, were as follows: ***5***, 0→30 min, linear gradient of 20→80 mM NaOAc; ***6***, 0→20 min, linear gradient of 30→60 mM NaOAc; 20→40 min, linear gradient of 60→100 mM NaOAc; ***7***, 0→60 min, linear gradient of 30→80 mM NaOAc; ***8***, 0→50 min, linear gradient of 40→80 mM NaOAc; ***9***, 0→15 min, linear gradient of 50→60 mM NaOAc; 15→20 min, linear gradient of 60→70 mM NaOAc; 20→50 min, linear gradient of 70→100 mM NaOAc. After each run, the column was cleaned for 5 min by elution with 100 mM NaOH containing 1 M NaOAc, and subsequently equilibrated for 15 min with the starting eluent. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The eluate was splitted post-column (ratio 1 to 9), and the smaller current was monitored using a Dionex PED detector in the PAD mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1, 0.1 V and 0.5 s; E_2, 0.6 V and 0.1 s; E_3, -0.6 V and 0.1 s. The eluate was neutralized by on-line addition of 1 M HOAc and appropriate fractions (*c.* 5 to 12 mL) were combined.

Desalting of oligosaccharides.—In order to remove NaOAc from the HPAEC pools, the oligosaccharides were adsorbed on Bakerbond SPE C_{18} disposable extraction columns (JT Baker Inc., Phillipsburg, NJ, USA), washed extensively with distilled water, and eluted with methanol. The most prominent purified oligosaccharides were characterized by monosaccharide analysis, mass spectrometry and partial degradation with exoI (or endoV).

Chemical and enzymic treatment of oligosaccharides

Reduction of oligosaccharides.—In order to label oligosaccharide ***9.2*** at its reducing terminus, this fraction (*c.* 100 μg) was treated with 200 μL of 1.5 M ammonia containing 75 mg mL^{-1} of $NaBH_4$ for 1 h at 30 °C. The reduced oligosaccharide was desalted using a mixture of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) resins (Bio-Rad) in a ratio of *c.* 0.6 (v/v), and subsequently dried in a stream of air.

Release of oligosaccharides from POsxg in time.—POsxg (2 mg) was dissolved in 1 mL of 50 mM succinate buffer (pH 4) containing 0.01% (w/v) NaN_3 and degraded with endoIV (*c.* 140 ng) or endoV (*c.* 5 μg) during 48 h at 40 °C. Samples of 50 μL were taken during the course of degradation. After inactivation of the enzyme (10 min, 100 °C), the products were analyzed by HPAEC.

Degradation of (mixtures of) oligosaccharides by exoI or endoV.—Monosaccharide analysis and mass spectrometry revealed that several oligosaccharides contained more than one unsubstituted Glc residue. In order to investigate whether these extra residues were located on the reducing or nonreducing terminus, the oligosaccharides were treated with either exoI (active on the nonreducing end) or endoV (active on the reducing end). Approximately 10 μg of oligosaccharide (reduced ***9.2*** was also included in this series) or 100 μg of a POsxg digest of endoIV were treated (12 h, 40 °C) with exoI (*c.* 100 ng of protein), endoV (*c.* 1 μg of protein), or endoIV (*c.* 200 ng of protein) in 100 μL of a 50 mM NaOAc buffer (pH 5) containing 0.01% (w/v) NaN_3. Mixtures were heated for 10 min at 100 °C to stop the reaction and the release of Glc (or glucitol in case of reduced ***9.2***) was analyzed by HPAEC.

Analytical methods

Sugar analysis.—Uronic acids were estimated colorimetrically with an automated *m*-hydroxydiphenyl test [14] using concentrated H_2SO_4 containing 0.0125 M $Na_2B_4O_7$ for hydrolysis. The total neutral sugar content was determined colorimetrically with an automated orcinol/H_2SO_4 assay [15]. Glc was used as a standard. For determination of the neutral sugar composition various methods were used. WUS was subjected to a 72% (w/w) H_2SO_4 prehydrolysis (1 h at 30 °C) followed, after dilution with water, by a 1 M H_2SO_4 hydrolysis (3 h at 100 °C). Alkali extracts from WUS and BioGel P-2 fractions were hydrolyzed (1 h, 121 °C) using 2 M TFA. The released neutral sugars were converted into their alditol acetates and analyzed by GC as described previously [3]. Oligosaccharides, obtained after fractionation on HPAEC, were hydrolyzed using 2 M TFA as above and the sugar composition was determined by HPAEC as described by De Ruiter *et al.* [16].

Glycosyl linkage composition.—POsxg was methylated according to a modification of the Hakomori method [17] and subsequently dialyzed against water and dried by evaporation (airstream, room temperature). This procedure was repeated once. Next, the methylated xyloglucan was hydrolyzed using 2 M TFA as described above. The released (partially methylated) sugars were converted into their alditol acetates, which were quantified by GC and identified by GC-MS as described before [3]. Sodium borodeuteride was used for reduction.

Starch content.—The amount of starch which was present in WUS was determined using a test-kit obtained from Boehringer (Mannheim, Germany).

Protein content.—The protein content of WUS (N x 6.25) was determined by a semi-automated micro-Kjeldahl method. Protein content of enzyme preparations was determined according to Sedmak and Grossberg [18]. BSA was used as a standard.

Determination of mol wt of XG oligosaccharides.—The mol wt of the purified xyloglucan oligosaccharides was determined with a Finnigan MAT TSQ-70 mass spectrometer (San José, CA, USA), equipped with a 20 kV conversion-dynode and a Finnigan MAT electrospray interface as described previously [19].

Analysis of xyloglucan oligosaccharides.—Xyloglucan oligosaccharides and their degradation products were analyzed by HPAEC (CarboPac PA-100 column; Dionex) combined with PAD-

analysis as described previously [5]. Samples (20 μL) were eluted (1mL min^{-1}) with the following NaOAc gradient in 100 mM NaOH: 0→5 min, linear gradient of 0→50 mM NaOAc; 5→30 min, linear gradient of 50→100 mM NaOAc; 30→45 min, linear gradient of 100→250 mM NaOAc. After each analysis, the column was rinsed for 5 min with 1 M NaOAc in 100 mM NaOH, and equilibrated in 100 mM NaOH for 15 min.

RESULTS

Isolation and purification of xyloglucan

Successive water extractions and α-amylase treatments of the potato mash yielded a fraction (WUS) which consisted for *c.* 65% of carbohydrates and *c.* 25% of proteins; *c.* 2% of the peeled potato material was recovered as WUS. Approximately 25% (w/w) of the sugars in WUS could be attributed to starch. Several α-amylase treatments, preceded by an extraction at 70 °C, were insufficient to obtain an essentially starch-free WUS. DMSO, which is known as an effective starch extractant [9,20], was not used because some pectic polysaccharides, which were of interest to us in a different study, would be solubilized as well. The WUS was subjected to a sequence of alkali extractions; the polysaccharides present in WUS were recovered in four fractions: 0.05 M NaOH {27% (w/w)}, 1 M KOH {44% (w/w)}, 4 M KOH {8% (w/w)}, and the residue {21% (w/w)}. The sugar composition of WUS and extracts are summarized in Table I. Pectic polysaccharides accumulated in the 0.05 M NaOH extract. The relatively large amount of Glc in the 1 M KOH extract suggested that this fraction contained predominantly starch. This was confirmed by treatment with α-amylase.

Our primary interest in this study was xyloglucan; the presence of Xyl indicated that both the 1 M KOH and the 4 M KOH extract contained this polysaccharide. The 4 M KOH extract was chosen as a source for purification of xyloglucan because this extract

TABLE I. Sugar composition (mol%) of WUS, of fractions obtained by sequential extractions of WUS and of a purified potato xyloglucan.

	Rha	Ara	Xyl	Man	Gal	Glc	GalA
starting material							
WUS	tr.*	5	2	1	24	51	17
sequential extractions							
0.05 M NaOH	3	7	0	1	43	12	34
1 M KOH	1	4	2	0	16	68	9
4 M KOH	0	9	20	3	22	43	3
Residue	0	0	0	2	2	91	5
4 M KOH after several purification steps							
POsxg	0	8	25	4	9	54	0

* Traces.

TABLE II. Glycosyl linkage composition of POsxg.

Deduced linkage	Molar Ratio	Total
L-ara*f*-(1→*	8	8
D-Gal*p*-(1→	8	8
D-Xyl*p*-(1→	9	
→2)-D-Xyl*p*-(1→	12	
→4)-D-Xyl*p*-(1→	1	22
→4)-D-Glc*p*-(1→	34	
→4,6)-D-Glc*p*-(1→	20	54
→4)-D-Man*p*-(1→	6	
→4,6)-D-Man*p*-(1→	2	8

* Determined as 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, *etc.*.

contained more xyloglucan and less starch than the 1 M KOH extract. Small amounts of contaminating polysaccharides were removed subsequently by α-amylase treatment (starch), anion-exchange chromatography (pectic material), and treatment by a purified endogalactanase (galactan). The selective degradation of starch and galactan was monitored by HPAEC; no typical degradation products of xyloglucan were observed. The resulting high mol wt material was designated POsxg and comprised *c.* 1% (w/w) of WUS. The sugar composition of POsxg is given in Table I, which is in close agreement with the totals of the corresponding differently linked residues determined by methylation analysis (Table II). The 1,4,6-linked Glc is indicative for the presence of xyloglucan; *c.* 37% of the (1→4)-linked glucan backbone is branched at the C-6 position. This amount of 1,4,6-linked Glc is in good agreement with that of the sum of terminal Xyl {D-Xyl*p*-(1→} and 1,2-linked Xyl. Terminal and 1,2-linked Xyl residues occurred in similar amounts. All Ara and Gal were present as terminal residues, and are probably attached to the C-2 position of Xyl. Detection of 1,4-linked Xyl suggests the presence of a small contamination of xylan in POsxg; short sidechains as observed in the xyloglucan of *Phaseolus coccineus* might be another possibility [21]. No 1,4-linked Gal or 1,4,6-linked Gal was detected which suggests that the ß-(1→4)-galactan contamination was effectively removed by the endogalactanase treatment. Methylation analysis could not be conclusive on the removal of starch. Table II further indicates the presence of a (1→4)-linked mannan of which *c.* 25% of the Man residues {→4,6)-D-Man*p*-(1→} are branched with, most likely, Gal. As a result of this, potato xyloglucan will have more terminal Ara than Gal residues. These data are in agreement with those reported by Ring and Selvendran [9] and Ryden and Selvendran [20].

Fractionation of xyloglucan oligosaccharides obtained by endoV

The degradation of POsxg by two endoglucanases having a high xyloglucanase activity, was investigated. Only the fractionation and characterization of products obtained by

treatment with endoV was performed in detail. POsxg was completely degraded by endoV as is indicated by the elution profile of the digest on BioGel P-2 (Fig. 1); no material eluted in the void volume of the column. The assignment of fraction numbers *1* to *9* is in

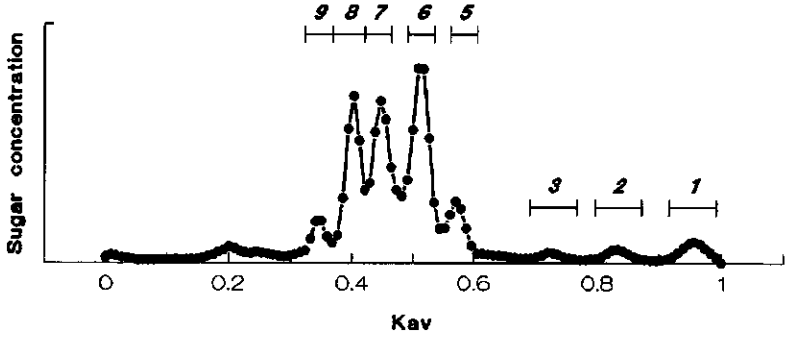

FIGURE 1. Elution pattern on BioGel P-2 of a digest of POsxg with endoV.

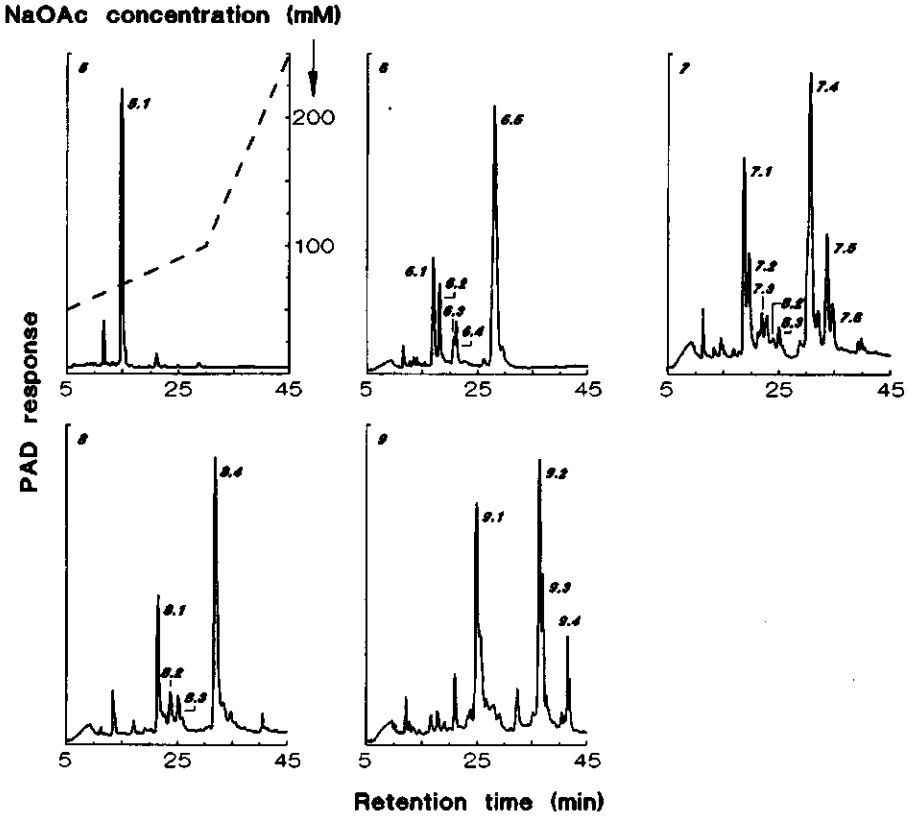

FIGURE 2. Elution profiles on HPAEC of BioGel P-2 pools *5* to *9*, obtained after treatment of POsxg with endoV. Solid lines and dashed line indicate PAD response and NaOAc gradient, respectively.

accordance with the DP of the oligosaccharides, as deduced from their elution behavior. The DP was later confirmed by mass spectrometry of the oligosaccharides. Pool *1* contained only Glc (data not shown), *2* and *3* were not analyzed; the sugar composition of *5* to *9* is given in Table III. It should be noted that the Man-containing polysaccharide of POsxg was also degraded by endoV because hardly any material was detected in the void fraction. Apparently, endoV is able to cleave this polysaccharide or it contains a residual endomannanase activity. HPAEC analysis of *5* to *9* (Fig. 2) demonstrated that each pool (except *5*) consisted of at least four products. Pools *5* to *9* were subsequently fractionated by preparative HPAEC which yielded a large number of oligosaccharides with a sugar composition as is indicated in Table III, and mol wts as summarized in Table IV. Together with the data from methylation analysis, and the known endoglucanases cleavage at the reducing side of an unsubstituted Glc residue, it can be deduced that each oligosaccharide contained two (adjacent) substituted Glc residues. The sidechains attached to these residues can have any arrangement: XX, LX, XL, LL, SX, XS, SS, LS and SL. Fraction *6.3* and *8.2* are an exception to the above, because their large Xyl content suggests the presence of three sidechains. Their structures will be discussed later.

Fractionation of a POsxg digest obtained by endoIV

POsxg was also degraded by endoIV and the resulting digest was fractionated by BioGel P-2 chromatography (data not shown). The void volume of the BioGel P-2 column contained material with similar amounts of Man, Glc and Gal; no Xyl and Ara were found. Apparently, the Man-containing polysaccharide of POsxg was not degraded by endoIV. The presence of a large amount of Glc (in the absence of Xyl) indicates that POsxg still contained some residual starch. Typically, the degradation products of endoIV seemed slightly larger than those formed by endoV (data not shown). Whereas treatment of POsxg with endoV yielded predominantly hexa-, hepta-, and octasaccharides (*6*, *7*, and *8*; see Fig. 1), the treatment with endoIV yielded mainly hepta-, and octasaccharides.

Only BioGel P-2 pool *9* from the digest obtained with endoIV was further fractionated by preparative HPAEC. This yielded an oligosaccharide with a Glc:Xyl:Gal:Ara ratio of *c.* 6:3:1:1. The mol wt of this oligosaccharide was 1386, which corresponds to a bruto formula of $Glc_5Xyl_2Gal_1Ara_1$. Because this oligosaccharide contained many Glc residues compared to those obtained from apple xyloglucan [5], it was treated with exoI or endoV. HPAEC analysis showed that in both cases *c.* 20% of the Glc was released from the oligosaccharide (data not shown); however, the oligomeric products that were formed, differed. This suggested that exoI and endoV removed one Glc residue from a different terminus. This means that $Glc_5Xyl_2Gal_1Ara_1$ corresponds to either GSLGG or GLSGG (note that endoglucanases do not catalyze GSGL↓G). The exact position of Ara and Gal residues is not known yet, and the structural elements containing these residues will therefore be indicated between brackets, *i.e.* G[LS]GG. After reduction of the oligosaccharide, it was shown that exoI released Glc, whereas endoV released glucitol. Thus, endoV removed Glc from the reducing end of the molecule (G[LS]G↓G) while exoI

TABLE III. Sugar composition (mol%) of fractions obtained after enzymic degradation of POsxg by endoV. The POsxg digest was fractionated on BioGel P-2, and these fractions were further purified by HPAEC.

	Glc	Xyl	Gal	Ara	Man
BioGel P-2 pentamer	54	40	1	3	2
5.1	61	39	0	0	0
BioGel P-2 hexamer	46	34	5	13	2
6.1	45	39	15	1	0
6.2	50	35	14	1	0
6.3	50	45	1	4	0
6.4	51	36	0	13	0
6.5	40	40	0	20	0
BioGel P-2 heptamer	51	29	8	11	1
7.1	46	39	15	0	0
7.2	55	29	13	3	0
7.3	54	30	13	3	0
7.4	57	29	2	12	0
7.5	60	27	0	12	0
7.6	56	28	1	15	0
BioGel P-2 octamer	48	26	15	9	2
8.1	45	29	26	0	0
8.2	46	42	12	0	0
8.3	52	26	10	12	0
8.4	50	25	12	13	0
BioGel P-2 nonamer	50	24	15	8	3
9.1	58	20	19	3	0
9.2	58	21	10	11	0
9.3	57	21	10	12	0
9.4	64	18	2	16	0

acted on the other end (G↓[LS]GG). This example demonstrated that treatment with exoI or endoV can be used to determine the position of unbranched Glc residues in an oligosaccharide. Incubation of G[LS]GG with endoIV also removes Glc from the reducing terminus, but this reaction was much slower than with endoV.

Further characterization of oligosaccharides by enzyme treatment

Table III and IV showed that many oligosaccharides (***7.1-7.6***, ***8.1-8.4*** and ***9.1-9.4***) contained a relatively large proportion of Glc. To investigate whether these residues were located at nonreducing terminus, these oligosaccharides were treated with exoI. In the case of no Glc release by exoI, the oligosaccharides were treated by endoV, as a positive control. The results of these incubations are shown in Table IV. Based on sugar

composition, mol wt, and incubations with exoI and/or endoV, tentative structures were proposed for the different oligosaccharides (Table IV). The order of the structural elements between brackets ([●●]) is interchangeable. However, it should be noted that the order of letter codes between brackets is not completely arbitrary. The presented sequence of the elements between the brackets do show the relation between certain oligosaccharides and their degradation products, *e.g.* degradation of ***7.4*** (G[XS]G) by exoI and ***7.6*** ([XS]GG) by endoV, both yielded ***6.5*** ([XS]G) as a product. Product ***7.5*** has a similar sugar composition as ***7.6*** ([XS]GG), but a different retention time upon HPAEC; its tentative structure might correspond to [SX]GG.

When assuming that the molar response factors are similar for oligosaccharides in one BioGel P-2 pool, it can be concluded from Fig. 2 that [XS]G (***6.5***), G[XS]G (***7.4***) and [XS]GG (***7.6***) are much more abundant than [SX]G (***6.4***; [SX]GG was not found). This suggests that Ara substituents are predominantly attached to one of the two Xyl residues. Gal residues are more equally distributed over both Xyl residues; [XL]G (***6.1***) and [LX]G (***6.2***), as well as G[XL]G (***7.1***) and G[LX]G (***7.2***) plus [LX]GG (***7.3***), are present in similar amounts.

The nature of ***6.3*** and ***8.2*** is not fully understood. The mol wt of these products corresponds to $Hexose_3Pentose_3$ and $Hexose_5Pentose_3$, respectively. The absence of Ara residues in these oligosaccharides (Table III), suggests that Xyl accounted for all three pentoses. The possibility that ***8.2*** corresponds to XLXG, a rather abundant oligosaccharide in apple xyloglucan, can be excluded because exoI removed Glc from this molecule. This means that the Glc residue at the nonreducing terminus is unbranched (G[●L]G). These data indicate that sidechains containing two Xyl residues might be present. Ryden and Selvendran [21] provided some evidence for the presence of 1,4-linked Xyl in the sidechains of xyloglucan derived from *Phaseolus coccineus*. Possibly, ***6.3*** and ***8.2*** contain similar structures in their sidechains. This might also explain the small percentage of 1,4-linked Xyl found in the methylation analysis of POsxg.

Mode of action of endoV and endoIV

The release of oligosaccharides from POsxg by endoIV or endoV was monitored in time by HPAEC analysis. An example of a typical degradation pattern of POsxg is shown in Fig. 3 for both enzymes. No G_2 or G_3 was detected in any of the digests. Products eluting between 5 and 15 min only appeared upon incubation with endoV; they probably originate from the galactomannan contamination in POsxg. The numerals in Fig. 3 correspond to those in the last column of Table IV. It can be seen that galactosylated oligosaccharides elute much faster upon HPAEC than arabinosylated oligosaccharides {compare, for instance, [XL]G (***6.1***) and [XS]G (***6.5***) or GLLGG (***9.1***) and GSSGG (***9.4***)}. Further, oligosaccharides are retarded on CarboPac PA-100 when they contain an extra unsubstituted Glc residue; the effect is most pronounced when this residue is present at the reducing terminus {compare, [LX]G (***6.2***), G[LX]G (***7.2***) and [LX]GG (***7.3***) or [XS]G (***6.5***), G[XS]G (***7.4***) and [XS]GG (***7.6***)}.

TABLE IV. Data on purified potato xyloglucan oligosaccharides obtained after enzymic degradation of POsxg by endoV.

Fraction	Mol wt*	ExoI†	EndoV‡	Tentative Structure§	Peak No.‖
5.1	768	nd.¶	nd.	XXG	1
6.1	nd.	nd.	nd.	[XL]G	2
6.2	930	nd.	nd.	[LX]G	3
6.3	900	nd.	nd.	?	6
6.4	nd.	nd.	nd.	[SX]G	6 or 7**
6.5	900	nd.	nd.	[XS]G	12
7.1	1092	+	nd.	G[XL]G	4
7.2	1092	+	nd.	G[LX]G	5
7.3	1092	-	+	[LX]GG	8
7.4	1062	+	nd.	G[XS]G	14
7.5	nd.	nd.	nd.	?	16
7.6	1062	-	+	[XS]GG	17
8.1	1254	+	nd.	GLLG	7
8.2	1224	+	-	?	10
8.3	1062	+	-	?	11
8.4	1224	+	-	G[LS]G	15
9.1	nd.	nd.	nd.	GLLGG	10 or 11**
9.2	1386	+	+	G[LS]GG	19
9.3	nd.	nd.	nd.	?	20
9.4	nd.	nd.	nd.	GSSGG	21

* Determined by electrospray mass spectrometry. † Oligosaccharides were treated with exoI to test for unsubstituted Glc on nonreducing terminus. ‡ Oligosaccharides with additional endoV treatment to test for two consecutive Glc on reducing terminus. § Tentative structure based on sugar composition, methylation analysis, mol wt, and exoI/endoV degradation patterns. Nomenclature according to Fry *et al.* [6]. Segments between brackets [●●] are interchangeable because the exact position of Ara and Gal residues has not been determined. However, treatment of, for instance, *7.6* with endoV yields *6.5*, indicating a similar position of Ara (relative to Xyl) for both oligosaccharides. ‖ Numbers refer to peaks indicated in Fig. 3. ¶ Not determined. ** Exact location not known.

The degradation of POsxg by endoV, as shown in Fig. 3, was not yet completed. Peaks indicated with "dimers" disappeared during the course of degradation, while the relative amounts of products 1 to 19 remained similar (data not shown). From Table IV it can be deduced that, if an oligosaccharide contains two adjacent Glc residues which are both substituted with a disaccharide sidechain, then this molecule contains an unsubstituted Glc residue at the nonreducing terminus (for instance, GLSG and GLSGG). Apparently, substitution of both Xyl residue hinders endoV, and cleavage of a more accessible linkage (-G↓GLSGG-) is preferred over a less accessible one (-GG↓LSGG-). Based on this observation, it was expected that endoV would show a similar behavior in the case of GGLXGG (only the Xyl residue towards the nonreducing end is substituted), *i.e.*

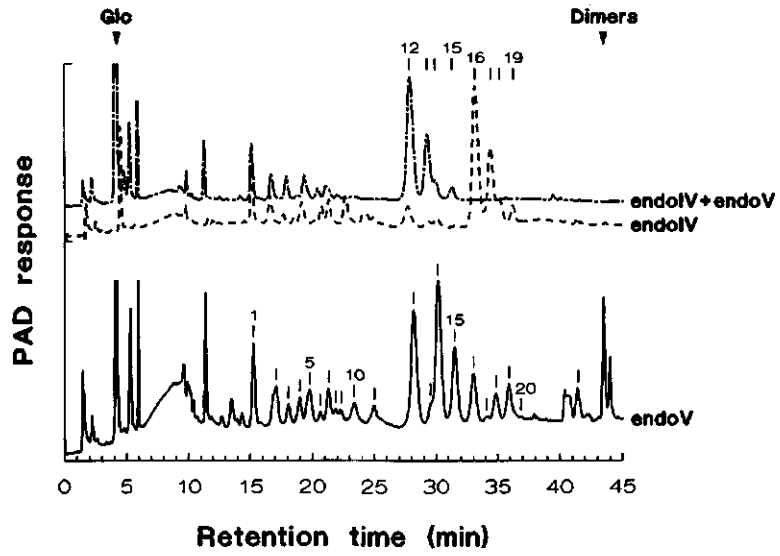

FIGURE 3. Elution profiles on HPAEC of a POsxg digest by endoV (——), endoIV (- - -), and endoIV after subsequent treatment with endoV (- . -). Peak numbers correspond to numerals and tentative structures in Table IV.

-G↓GLXGG- is preferred over -GG↓LXGG-. However, this is contradicted by finding both [LX]G and [XL]G.

The absence of "dimers" in the dashed line (- - -) of Fig. 3 suggested that the degradation of POsxg by endoIV was complete. Different products (peaks 16-19) as with endoV were accumulated. Further, only a small amount of Glc was released by endoIV. This amount increased, however, at prolonged incubation times with a concomitant shift in retention time of most other products. Upon treatment of the POsxg digest of endoIV by exoI, a few minor oligosaccharides (for instance peaks 5 and 19) disappeared and a small amount of Glc was released (data not shown). Apparently, only a small percentage of the oligosaccharides contained an unsubstituted Glc residue at the nonreducing terminus; thus, only very small amounts of G[●●]G-type of oligosaccharides are formed by endoIV. Incubation of the same digest with endoV resulted in the release of Glc and a shift of the main products of endoIV towards smaller retention times (peaks 12-15; Fig. 3, line - . -). The retention time of peak 13 is similar to that of the product of G[LS]G (*8.4*) after exoI treatment; peak 13 probably represents [LS]G.

DISCUSSION

In order to be able to study the mode of action of two endoglucanases purified from *Trichoderma viride*, the substitution pattern of potato xyloglucan was investigated. Hereto,

an alkali-extracted xyloglucan was enzymically degraded to its building units, which resulted in a very complex mixture of oligosaccharides. Purification and (partial) characterization of the degradation products demonstrated that sidechains appeared in clusters of two adjacent Glc residues. These sidechains consisted of either a terminal Xyl residue or a Xyl residue with an additional Ara or Gal substituent. Any combination of these terminal residues seemed possible; however, the exact position of these residues was not determined.

An important question was how the clusters of sidechains were distributed along the glucan backbone. This distribution could be deduced from the action pattern of endoIV on POsxg. EndoIV mainly releases [●●]GG-type of oligosaccharides and hardly any Glc from POsxg. Prolonged incubation with endoIV showed that these oligosaccharides were further degraded to [●●]G-type of products and Glc. Therefore, the relatively small amounts of [●●]G and Glc in Fig. 3 (solid line) probably originate from "trimming" of slightly larger oligosaccharides ([●●]GG). It is concluded that blocks of two adjacent unbranched Glc residues are characteristic for potato xyloglucan. Thus, [●●]GG is the major repeating unit of POsxg, which is illustrated by Fig. 4. It might be argued that methylation analysis of POsxg indicates a higher proportion of 1,4-linked Glc than is shown in Fig. 4. However, the monosaccharide composition of the BioGel P-2 void fraction of a digest, obtained after degradation of POsxg by endoIV, suggested that POsxg contained some residual starch. The data of methylation analysis were therefore not considered in the degree of branching of POsxg as indicated in Fig. 4, but only to demonstrate that Gal and Ara were present as terminal residues. It should be noted that the proposed structure is not necessarily representative for all xyloglucan in potato. The 1 M KOH extract contained some xyloglucan as well (data not shown), but the polysaccharides of this fraction were not further investigated. Compared to other xyloglucans, the structure of POsxg is rather unique. It resembles barley [8] and rice xyloglucan [7] in having clusters of two adjacent branched Glc residues; however, the number of unsubstituted Glc residues between these clusters is two for POsxg instead of three as established for barley and rice (repeating units of [●●]GG *vs.* [●●]GGG). The data for barley and rice xyloglucan leave room to speculate that their branching pattern is similar to that of potato. Further, POsxg differs from apple [4,5], sycamore, tamarind [22] and many other xyloglucans [1] in its degree of substitution ([●●]GG *vs.* X[●●]G), although the backbone length of their repeating units is similar.

The release of different oligosaccharides by endoIV and endoV demonstrates that endoglucanases can differ in their mode of action towards xyloglucans. This has never been observed before because in most studies either crude cellulase mixtures or too heavily branched xyloglucans were used. EndoIV gives predominantly a [●●]GG-type of oligosaccharides, even when both Xyl residues of the oligosaccharide are substituted. EndoV seems to cleave more at random, but the action of this enzyme is influenced when both of two adjacent xylosylated Glc units are substituted with Ara and/or Gal. In such a case, endoV releases oligosaccharides only as G[●●]G, and not as [●●]G or [●●]GG. Differences in mode of action of a similar kind have been made for the degradation of

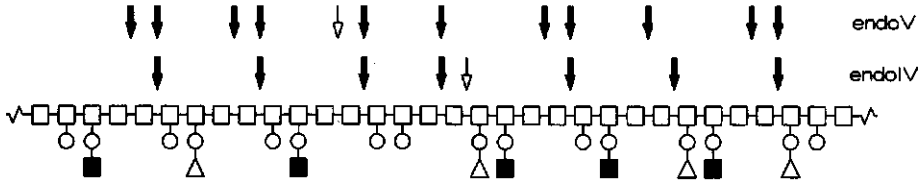

FIGURE 4. Tentative structure of POsxg based on methylation analysis, data in Table IV and degradation pattern of POsxg by endoIV. The location of Ara and Gal residues is in accordance with Table IV; it should be realized that the exact position of these residues still needs to be determined. Building units with two Ara or two Gal residues also occur; these were not indicated because they are present in very small quantities. The different mode of action of endoIV and endoV towards POsxg is illustrated by arrows. Solid and open arrow heads indicate preferred and possible cleavage sites, respectively. Note that we do not have evidence for the release of hexasaccharide XXGG. □, ß-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; △, ß-D-Gal*p*-(1→2)-; ■, α-L-Ara*f*-(1→2)-.

wheat flour arabinoxylans by two endoxylanases from *Aspergillus awamori* [23,24]. The possible mode of action of endoIV and endoV is summarized in Fig. 4. The fact that endoIV releases similar amounts of XXG as endoV, and that no indications for the presence of GXXG or XXGG were found, suggests that endoIV is also influenced by Ara and Gal substitution (see open arrows in Fig. 4). Therefore, determination of the exact location of the Gal and Ara residues is still necessary to further refine Fig. 4. The release of Glc and relatively large amounts of a [●●]G-type of oligosaccharide by endoV shows that this enzyme is better in "trimming" unsubstituted Glc residues from the reducing end of oligosaccharides ([●●]G↓G) than endoIV; in case of the latter, Glc release was very slow. This might be related to a difference in substrate-binding of both enzymes, the investigation of which is discussed in Chapter 6.

REFERENCES

[1] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[2] McCann, M.C., and Roberts, K. (1994) Changes in cell wall architecture during cell elongation. *J. Exp. Bot.* **45**, 1683-1691.

[3] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and endoglucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).

[4] Renard, C.M.G.C., Lomax, J.A., and Boon, J.J. (1992) Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and alkali-extracted xyloglucans. *Carbohydr. Res.* **232**, 303-320.

[5] Vincken, J.-P., Beldman, G., Niessen, W.M.A., and Voragen, A.G.J. (1996) Degradation of apple fruit xyloglucan by endoglucanase. *Carbohydr. Polym.*, in press (this thesis, Chapter 3).

[6] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.

[7] Kato, Y., Ito, S., Iki, K., and Matsuda, K. (1982) Xyloglucan and ß-D-glucan in cell walls of rice seedlings. *Plant Cell Physiol.* **23**, 351-364.

[8] Kato, Y., Iki, K., and Matsuda, K. (1981) Cell-wall polysaccharides of immature barley plants. II. Characterization of a xyloglucan. *Agric. Biol. Chem.* **45**, 2745-2753.

[9] Ring, S.G., and Selvendran, R.R. (1981) An arabinogalactoxyloglucan from the cell wall of *Solanum tuberosum*. *Phytochem.* **20**, 2511-2519.
[10] Mori, M., Eda, S., and Kato, K. (1980) Structural investigation of the arabinoxyloglucan from *Nicotiana tabacum*. *Carbohydr. Res.* **84**, 125-135.
[11] O'Neill, M., and Selvendran, R.R. (1985) Structural analysis of the xyloglucan from *Phaseolus coccineus* cell-walls using cellulase-derived oligosaccharides. *Carbohydr. Res.* **145**, 45-58.
[12] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases, and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.
[13] Van de Vis, J.W., Searle-van Leeuwen, M.J.F., Siliha, H.A., Kormelink, F.J.M., and Voragen, A.G.J. (1991) Purification and characterization of endo-1,4-ß-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinanases from *Aspergillus niger* in enzymic conversion of potato arabinogalactan. *Carbohydr. Polym.* **16**, 167-187.
[14] Thibault, J.-F. (1979) Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényl. *Lebensm. Wiss. Technol.* **12**, 247-251.
[15] Tollier, M.T., and Robin, J.P. (1979) Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Ann. Technol. Agric.* **28**, 1-15.
[16] De Ruiter, G.A., Schols, H.A., Voragen, A.G.J., and Rombouts, F.M. (1992) Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.* **207**, 176-185.
[17] Sandford, P.A., and Conrad, H.E. (1966) The structure of the *Aerobacter aerogenes* A3(S1) polysaccharide. I. A reexamination using improved procedures for methylation analysis. *Biochem.* **5**, 1508-1517.
[18] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.
[19] Tinke, A.P., van der Hoeven, R.A.M., Niessen, W.M.A., van der Greef, J., Vincken, J.-P., and Schols, H.A. (1993) Electrospray mass spectrometry of neutral and acidic oligosaccharides: methylated cyclodextrins and identification of unknowns derived from fruit material. *J. Chromatogr.* **647**, 279-287.
[20] Ryden, P., and Selvendran, R.R. (1990) Structural features of cell-wall polysaccharides of potato (*Solanum tuberosum*). *Carbohydr. Res.* **195**, 257-272.
[21] Ryden, P., and Selvendran, R.R. (1990) Cell-wall polysaccharides and glycoproteins of parenchymatous tissues of runner bean (*Phaseolus coccineus*). *Biochem. J.* **269**, 393-402.
[22] York, W.S., van Halbeek, H., Darvill, A.G., and Albersheim, P. (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and Fast-Atom-Bombardment Mass Spectroscopy. *Carbohydr. Res.* **200**, 9-31.
[23] Gruppen, H., Kormelink, F.J.M., and Voragen, A.G.J. (1993) Water-unextractable cell wall material from wheat flour. 3. A structural model for arabinoxylans. *J. Cereal Sci.* **18**, 111-128.
[24] Kormelink, F.J.M., Gruppen, H., Viëtor, R.J., and Voragen, A.G.J. (1993) Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans. *Carbohydr. Res.* **249**, 355-367.

CHAPTER 6

Substrate Specificity of Endoglucanases: What Determines Xyloglucanase Activity?[I]

Endoglucanases from *Trichoderma viride* differ in their activity and mode of action towards xyloglucans. In order to explain the basis for their different behavior, the number of substrate-binding sites of three endoglucanases (endoI, endoIV and endoV) were determined using bond cleavage frequencies of both normal and reduced cellodextrins and k_o/K_m. EndoIV differed from other endoglucanases described so far, in having at least nine putative binding sites. The specificities of the three endoglucanases towards various xyloglucans derived from apple fruit and potato were determined. Also, the release of oligosaccharides from these substrates in time was monitored. It was concluded that the endoglucanases prefer to bind unbranched glucosyl residues. Because most xyloglucans are composed of XXXG-type of building units, distant subsites are needed to bind xyloglucan. Having at least nine substrate-binding sites, endoIV seems to be well equipped to degrade xyloglucans which was confirmed by its high xyloglucanase activity.

Nature has equipped many fungal and bacterial species with a large set of (1→4)-ß-glucanases, sometimes containing as many as seven different enzymes [1]. From a genetic point of view, these enzymes are often rather well characterized. For instance, most of the glucanases of *Trichoderma reesei* have been sequenced and cloned. These are EGI[II] [2], EGII (formerly called EGIII) [3], EGIII [4], EGV [5], CBHI [6], and CBHII [7]. Analysis and comparison of amino acid sequences, as well as limited proteolysis [8], have revealed that most of these glucanases are built of discrete functional domains; a cellulose binding domain and a catalytic core [9]. These two domains are joined by a linker peptide, of which both length and structure greatly depend on microorganism and enzyme [9]. It has been shown for CBHI that sufficient spatial separation of the two domains is required for efficient function of the enzyme [10].

The specificity of cellulases is determined by the catalytic core, and in this domain, the different glucanases share little amino acid sequence identity in a linear alignment. However, hydrophobic cluster analysis [11-13,] suggests that the fold of proteins is better conserved than the sequence of their amino acids. Using this technique, glucanases can be grouped into 10 families (A-K, G contains only xylanases), and it is expected that the same fold will be found for each member of a family [12].

Cellulases can cleave (1→4)-ß-glycosidic linkages in a variety of substrates such as cellulose, CMC, (1→3),(1→4)-ß-D-glucan [5], and xylan [14,15]. Further, they show

---

[I]Vincken, J.-P., Beldman, G., and Voragen, A.G.J. Submitted for publication to *Carbohydr. Res.*.

[II]*Abbreviations*: BCF, bond cleavage frequency; Glc*, glucitol; G_n*, cello-alditol with chain length *n*; $G_p \downarrow G_{n-p}$*, cello-alditol with chain length *n* which gives the products G_p (with chain length *p*) and G_{n-p}* (with chain length *n-p*) after cleavage by an endoglucanase; R, gas constant (8.314 J mol^{-1} K); T, temperature (K); other abbreviations, see page viii.

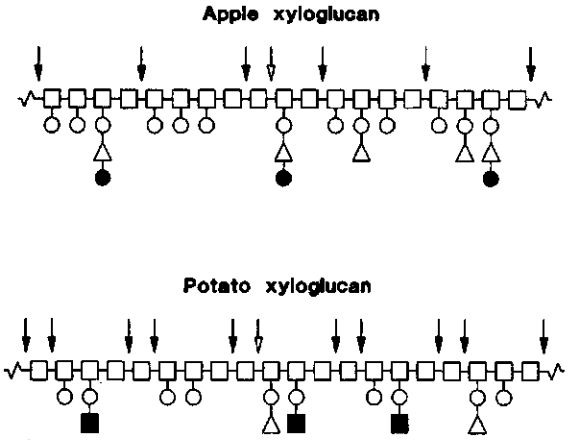

FIGURE 1. Schematic structures of APfxg and POsxg. □, ß-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; ▵, ß-D-Gal*p*-(1→2)-; ●, α-L-Fuc*p*-(1→2)-; ■, α-L-Ara*f*-(1→2)-. Sites of endoglucanase-attack are indicated by arrows. Cleavage of linkage indicated with open arrow is uncertain.

activity towards several small chromophoric glycosides. Each cellulase family demonstrates a characteristic specificity pattern on these artificial substrates [16]. Until now, relatively little attention has been paid to their activity towards xyloglucans, although this is an important polysaccharide in the primary cell wall of plants [17]. We have shown previously that the glucanases of *Trichoderma viride* (similar to *T. reesei* [18]) differ greatly in their activity towards apple xyloglucan (APfxg) [19]. The structure of APfxg is illustrated by Fig. 1. APfxg is mainly built of oligosaccharide building units having a backbone of four Glc residues. Small amounts of building units with a backbone length of only two or three Glc residues also occur [20,21]. Degradation of APfxg by endoglucanases is restricted to only a few linkages (indicated by arrows in Fig. 1); Fuc residues inhibit the action of endoglucanases to some extent [20]. Potato xyloglucan (POsxg) has a larger number of cleavage sites than APfxg (Fig. 1), and the linkage that is actually cleaved depends on the endoglucanase used [22]. These observations prompted us to study the basis of the specificity of endoglucanases in more detail.

Hydrolysis by glycosyl hydrolases often proceeds by general acid catalysis, usually promoted by Glu or Asp residues [23-25]. The fact that the same catalytic amino acids are found in hydrolases with different target substrates suggests that catalytic residues do not determine the substrate specificity of these enzymes. Despite these similarities, the stereochemistry of the products of a hydrolysis reaction can be altered. The anomeric configuration may either be inverted (single displacement) or retained (double displacement) during hydrolysis [26], but within a given enzyme family this stereoselectivity seems to be consistent [16,27]. It is not very likely that the mechanism of catalysis determines substrate specificity, because glucanases with different specificities (exo *vs.* endo) can belong to the same family as is the case with, for instance, CBHI and

EGI (family C).

Crystallography showed that the catalytic residues of CBHI from *Trichoderma reesei* are located in a relatively long substrate-binding tunnel (compared to CBHII), which can slide along a glucan chain [28]. Several loops, which are involved in building this tunnel, appear to be deleted in the EGI protein [2,28]. Similar observations were made for CBHII from *Trichoderma reesei* and EG2 from *Thermomonospora fusca*, both belonging to family B. Recently, it has been shown that deletion of a surface loop of CBH A from *Cellulomonas fimi* resulted in an enhancement of the endoglucanase activity of this enzyme [29]. These experiments suggest that exoglucanases may be ancestors of related endoglucanases [28], in which the tunnel has evolved to a more open structure (groove) to facilitate multiple-chain attack.

Although the tunnel/groove concept may explain the basis for an exo/endo type of specificity, it does not provide an answer to the question why some glucanases have xyloglucanase activity and others have little. It has been suggested that the basis of substrate specificity of glycosyl hydrolases is determined by their ability to bind different polysaccharides in their substrate-binding sites. Evidence for this was obtained by studying the interaction between various active site-directed inhibitors and ß-glucan endohydrolases [30]. Epoxyalkyl ß-oligoglucosides, varying in glycosyl chain length, linkage type and aglycon length appeared to inactivate these enzymes to a different extent. This example illustrates the key role of substrate-binding in enzyme specificity.

In order to investigate the basis for xyloglucanase activity, the number of substrate-binding sites of three endoglucanases from *Trichoderma viride* (endoI, endoIV and endoV) [31] were determined according to the method of Suganuma *et al.* [32]. Homologous cellodextrins were used for this purpose. Further, the specificity of the endoglucanases towards various xyloglucans was studied, as well as the release of oligosaccharides from these xyloglucans in time. A possible relationship between number of substrate-binding sites, specificity and mode of action of these glucanases is discussed.

EXPERIMENTAL

Materials

Substrates.—APfxg and POsxg were obtained by extraction from, respectively, apple and potato cell wall material with strong alkali (4 M KOH) and purified as described previously [19,22]. CMC (Akucell AF type 0305) was purchased from Akzo (Arnhem, The Netherlands). Avicel (type SF) was obtained from Serva (Heidelberg, Germany); GlcA and G_2 were purchased from Fluka AG (Buchs, Switzerland). Glc and cellodextrins (mixture I; Glc to G_6) were obtained from Merck (Darmstadt, Germany).

Enzymes.—Three endoglucanases (endoI, endoIV and endoV; EC 3.2.1.4) were purified to homogeneity from a commercial enzyme preparation from *Trichoderma viride* (Maxazyme Cl, Gist-Brocades, Delft, The Netherlands) by Beldman *et al.* [31].

Chemical treatment of substrates

Preparation of normal cellodextrins.—Normal cellodextrins (Glc to G_6) were prepared by

partial hydrolysis of 6 g Avicel with 15 mL 80% (w/v) H_2SO_4 according to a slightly modified procedure of Voloch *et al.* [33]. Portions of 500 mg of the hydrolysate were dissolved in 2 mL of distilled water, applied onto a column (100 x 2.6 cm, i.d.) of BioGel P-2 (200-400 mesh, Bio-Rad, Richmond, CA, USA) at 60 °C and eluted with distilled water (18 mL h^{-1}). Appropriate fractions (2.0 mL) were combined according to their neutral sugar content. The column was calibrated using a mixture of Glc, G_2 and Dextran T150 (Pharmacia, Uppsala, Sweden). The purified cellodextrins were concentrated under reduced pressure, freeze-dried and redissolved to give a stock solution of *c.* 5 mM. The concentration of cellodextrins in this solution was determined colorimetrically.

Preparation of reduced cellodextrins.—Reduced cellodextrins (Glc* to G_6^*) were prepared by treating *c.* 2.5 mmol of the purified normal cellodextrins with 500 μL of 1.5 M NH_4OH containing 75 mg mL^{-1} $NaBH_4$ for 1 h at 30 °C. Reaction mixtures were acidified by dropwise addition of glacial HOAc. Glc* and G_2^* were desalted using columns (30 x 80 mm) of Dowex 50W X8 (H^+) and AG3 X4A (OH^-) resins (Bio-Rad) in series. G_3^* to G_6^* were desalted on a Sephadex G-10 column (Pharmacia, 26 x 400 mm). All samples were concentrated under reduced pressure, dried under a stream of air and redissolved to give a stock solution of *c.* 5 mM. The concentration of reduced cellodextrins in this solution was determined colorimetrically.

Removal of Fuc from APfxg by mild acid hydrolysis.—APfxg (40 mg) was treated with 8 mL of 25 mM TFA for 90 h at 60 °C. Approximately 95% of the Fuc residues could be removed by this treatment. The defucosylated xyloglucan (referred to as APxg) was dialyzed extensively against distilled water and freeze-dried.

Incubation with endoI, endoIV, and endoV

Determination of BCFs.—BCFs were determined in 400 μL 20 mM succinate buffer (pH 4.0; 40 °C) containing 25 μM of a normal or reduced cellodextrin (appropriately diluted from stock solutions G_2 to G_6 or G_3^* to G_6^*) and 15 μM GlcA as internal standard. Incubation time and enzyme concentration were such that the evolution of a second generation of degradation products was minimized. Samples were heated for 10 min at 100 °C to inactivate the enzymes, evaporated to dryness under a stream of air, and dissolved in 100 μL of water. The individual degradation products were quantified by high-performance anion-exchange chromatography (HPAEC). Response factors for the individual oligosaccharides were accounted for.

Determination of the kinetic parameter (k_o/K_m).—For these experiments only normal cellodextrins were used. From an identical reaction mixture (4.0 mL) as described above, 400 μL aliquots were taken at various time intervals and handled as above. From the HPAEC data a $\ln([S]_o/[S])$ *vs.* time-plot could be constructed from which the kinetic parameter k_o/K_m was determined.

Turnover number of various xyloglucans.—APfxg, APxg and POsxg (500 μg) were dissolved in 100 μL of 50 mM succinate buffer (pH 4) containing 0.01% (w/v) NaN_3 as a preservative, and incubated for 1 or 12 h at 40 °C with of endoI, endoIV or endoV. Enzyme dosage was such that no substrate limitation occurred. Incubations were stopped by heating the reaction mixtures for 10 min at 100 °C. The samples were then diluted twice and the increase in reducing sugars was determined according to the procedure of Somogyi [34] using Glc for calibration. Similar experiments were done using CMC as a substrate.

Release of oligosaccharides from various xyloglucans in time.—APfxg and APxg (2 mg dissolved in 1 mL of 50 mM succinate buffer, pH 4, containing 0.01% (w/v) NaN_3) were (partially) degraded with endoI (*c.* 10 μg), endoIV (*c.* 100 ng) or endoV (*c.* 500 ng) during 24 h at 40 °C. Using this enzyme dosage, each incubation mixture contained a similar amount of xyloglucanase activity. Samples of 50 μL were taken during the course of degradation (0, 1, 2, 3, 4, 8, 12, and 24 h). Both APfxg and APxg were degraded completely by a 10-fold larger dose of endoIV (*c.* 1 μg). After inactivation of the enzyme (10 min, 100 °C), the oligosaccharides were analyzed by HPAEC. The amount of an oligosaccharide that was released in a certain time interval, was expressed as the percentage of the PAD response of this oligosaccharide in the

chromatogram of the completely degraded xyloglucan. POsxg was treated in a similar manner, but using a different enzyme dose: 5 μg, 0.140 μg and 5 μg of endoI, endoIV and endoV, respectively.

Analytical methods

Total neutral sugar content.—The total neutral sugar content was determined colorimetrically with an automated orcinol/H_2SO_4 assay [35]. Glc was used as a standard.

Neutral sugar composition.—APxg was hydrolyzed (1 h, 121 °C) using 2 M TFA. The released neutral sugars were determined by HPAEC as described by de Ruiter *et al.* [36].

Protein content.—Protein content of enzyme preparations was determined according to Sedmak and Grossberg [37]. BSA was used as a standard.

Analysis of normal and reduced cellodextrins.—Quantitative analysis of normal and reduced cellodextrins was performed with HPAEC using a Dionex Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-100 column (250 x 4 mm, Dionex, Sunnyvale, CA, USA) at 20 °C. Samples (20 μL) were injected using a SP8780 autosampler (Spectra Physics, San José, CA, USA) equipped with a Tefzel rotor seal in a 7010 Rheodyne injector valve. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The eluate (1 mL min^{-1}) was monitored using a Dionex PED detector in the PAD mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1, 0.1 V and 0.5 s; E_2, 0.6 V and 0.1 s; E_3, -0.6 V and 0.1 s.

Mixtures containing both normal and reduced cellodextrins were eluted as follows: 0→2 min, 200 mM NaOH (isocratic); 2→24 min, 0→110 mM NaOAc (linear gradient) and simultaneously 200→189 mM NaOH (linear gradient); 24→30 min, equilibration of the column in 200 mM NaOH (isocratic). After eight runs the column was washed with 1 M NaOAc in 100 mM NaOH for 10 min and equilibrated for 15 min in 200 mM NaOH. An internal standard of GlcA was required to provide accurate quantitations because of the response fluctuation of the electrochemical detector.

For analysis of mixtures containing only normal cellodextrins a different gradient was used. A complete separation of these oligosaccharides was achieved by the application of a linear gradient to 100 mM NaOH/140 mM NaOAc over 14 min, after the column had been equilibrated in 100 mM NaOH containing 40 mM NaOAc for 6 min. After eight runs the column was washed with 1 M NaOAc in 100 mM NaOH for 10 min and equilibrated for 15 min in 100 mM NaOH containing 40 mM NaOAc.

Analysis of xyloglucan oligosaccharides.—The degradation of APfxg, APxg and POsxg was monitored by HPAEC using a similar system as described above. Oligosaccharides derived from APfxg or APxg, were eluted with the following NaOAc gradient in 100 mM NaOH: 0→5 min, linear gradient of 0→30 mM NaOAc; 5→45 min, linear gradient of 30→80 mM NaOAc; 45→55 min, linear gradient of 80→200 mM NaOAc. Oligosaccharides derived from POsxg were analyzed with a different NaOAc gradient, also in 100 mM NaOH: 0→5 min, linear gradient of 0→50 mM NaOAc; 5→30 min, linear gradient of 50→100 mM NaOAc; 30→45 min, linear gradient of 100→250 mM NaOAc. After each analysis, the column was rinsed for 5 min with 1 M NaOAc in 100 mM NaOH, and equilibrated in 100 mM NaOH for 15 min. The identification of xyloglucan oligosaccharides is described elsewhere [20-22]. Nomenclature of these oligosaccharides is according to Fry *et al.* [38].

Results and Discussion

Action pattern of endoI, endoIV, and endoV on homologous cellodextrins

BCFs of normal and reduced cellodextrins.—The individual normal cellodextrins were

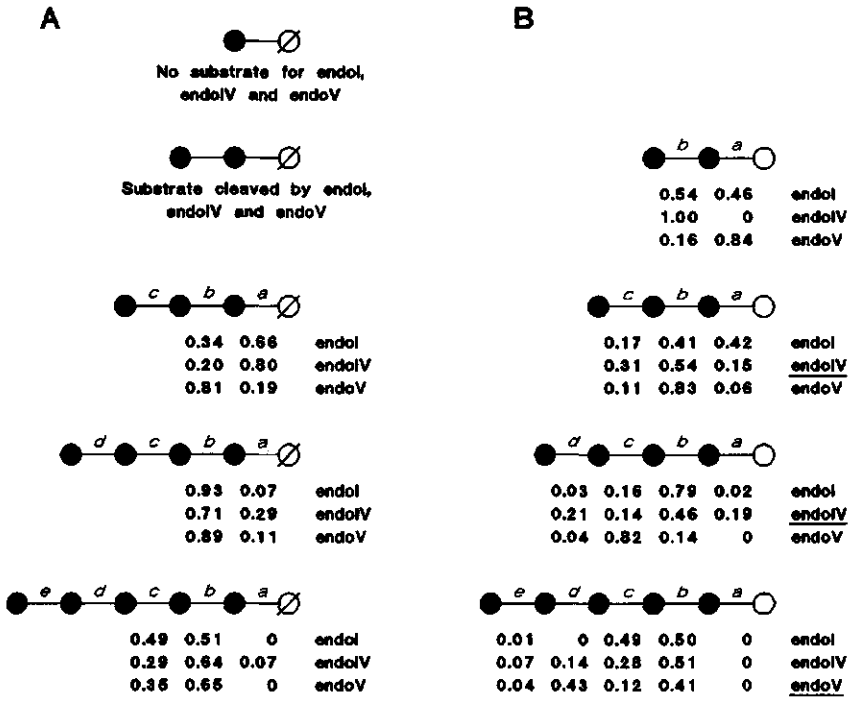

FIGURE 2. BCFs of normal (A, G_2 to G_6) and reduced cellodextrins (B, G_3^* to G_6^*) by three endoglucanases of *Trichoderma viride*. In case of asymmetric cleavage of normal cellodextrins (not $G_2\downarrow G_2$ and $G_3\downarrow G_3$) the BCFs are the sum of the cleavages of the indicated linkage and their "in reverse" on the nonreducing side. For instance, BCFs denoted under linkage *a* of G_5, is the sum of BCF of linkage *a* and that of *d*. Enzymes are underlined if their mode of action is influenced by reduction of the cellodextrin. Concentration of cellodextrins was 25 μM in 20 mM succinate buffer (pH 4.0), containing 15 μM GlcA; 40 °C. ●, nonreducing Glc residue; ø, reducing Glc residue; ○, Glc residue labelled by reduction with $NaBH_4$; *a* to *e*, indicate different linkages starting from the (former) reducing terminus.

incubated with the three endoglucanases at low substrate concentrations (25 μM), and analyzed by HPAEC. Under the conditions used, no evidence for multi-substrate reaction mechanisms (transglycosylation or condensation) was found. In preliminary studies only endoV showed transferase activity at increased substrate concentrations (1 mM) of G_4, G_5 and G_6.

BCFs were calculated from the peak areas of the degradation products of purified oligosaccharides using their molar response factors. Incubation time was such that all products were the result of one cleavage of the original substrate (molar amount of products formed is *c.* twice the decrease in molar amount of the original material); in other words, no "second generation" of products was present. None of the endoglucanases had the ability to hydrolyze G_2. The three endoglucanases showed a different mode of action towards normal cellodextrins (Fig. 2A). It should be noted that only the BCFs of

symmetrical cleavages in Fig. 2A are real. In all other cases the BCFs are distributed over two bonds. To quantify all cleavages other than $G_2 \downarrow G_2$ and $G_3 \downarrow G_3$, the cellodextrins have to be labelled.

Among the methods currently in use to label oligosaccharides for determination of BCFs, radio-labelling is probably the most elegant method because it does not alter the substrate significantly. Degradation products are then separated using TLC and quantified with a scintillation counter [15,32,39,40]. A disadvantage of this method is its inability to quantify unlabelled products. This quantitation is important to verify that no second generation of degradation products is formed. The sum of molar amounts of all labelled products should be equal to that of all unlabelled products. Also, nucleophilic competition between water and methanol has been reported to allow unequivocal assessment of cleavage sites [41]. Other methods involve ring-opening by reduction [1,42,43], or coupling to chromophoric groups such as 4-methyl-umbelliferyl [16,41,42].

In our study, reduced cellodextrins, combined with HPAEC for product analysis, were used to deduce BCFs in case of asymmetric cleavage. Our procedure to analyze normal and reduced cellodextrins simultaneously is very similar to that of Bray and Clarke [43], differing slightly in NaOAc gradient and in internal standard (GlcA instead of Ara). A satisfactory separation could be achieved as is shown in Fig. 3. Under the conditions used, the CarboPac PA-100 column achieves a superior separation compared to other techniques [44,45], and does not require derivatization of the oligosaccharides. BCFs of reduced cellodextrins were determined in a similar manner as decribed for the normal ones. Fig. 2B demonstrates different cleavage patterns for the three endoglucanases. EndoI and

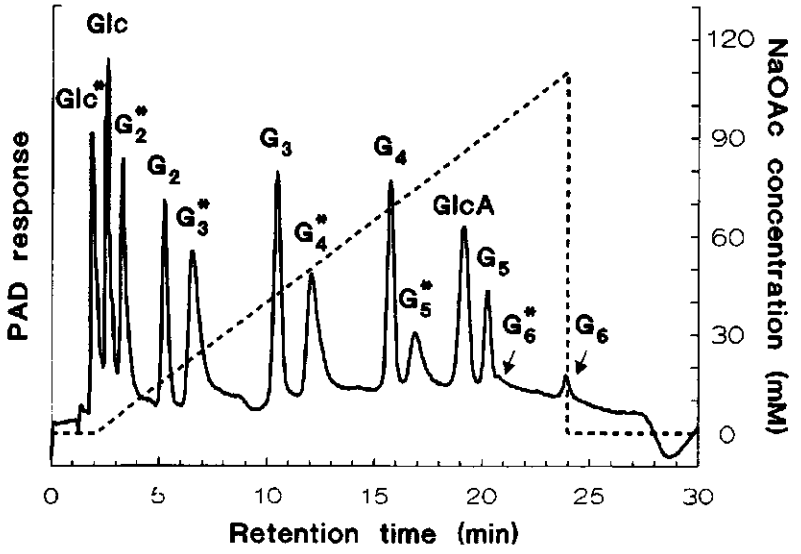

FIGURE 3. Elution pattern of normal and reduced cellodextrins upon HPAEC. Symbols: —, PAD response; - - -, NaOAc gradient. Elution times of normal (Glc to G_6) and reduced cellodextrins (Glc* to G_6*) are indicated.

endoV seemed to act in a reverse fashion in that the former enzyme preferentially released G_2^* whereas the latter releases G_2. Sites of attack were more evenly distributed over the alditols in case of endoIV; however, there was some preference to form G_2^*.

Until now, it was not known to what extent reduction affects the mode of action of the endoglucanases of *Trichoderma viride*. Comparison of the BCFs of G_4^* to G_6^* and G_4 to G_6 should indicate if reduction really affects the interaction between Glc residues and subsites. For instance, the BCF of linkage "*b*" of G_4^* should be equal to that of linkage "*b*" of G_4 (symmetrical cleavage). Also, the sum of BCFs of linkage "*a*" and "*c*" of G_4^* should be similar to that indicated underneath linkage "*a*" of G_4 (asymmetrical cleavage). When deviations larger than 0.10 (corresponding to two times the experimental error) occurred, reduction was thought to influence the mode of action of the enzyme. These cases are underlined in Fig. 2B. From Fig. 2 it must be concluded that the effect of reduction on BCFs is largely enzyme dependent. EndoIV was affected to a larger extent than endoV, whereas endoI was not affected. The chain length of the oligosaccharides also seemed to be important in this respect, although no clear correlation was found. Reduction of the oligosaccharides did not simply shift the location of cleavage towards linkage "*e*". Instead, the effects on BCFs were rather unpredictable. Similar observations were done for the endoglucanases of *Penicillium pinophilum* [42]. Here, enzyme action on certain substrates was completely abolished after reduction. On the other hand, Schou *et al.* [1] reported for the cellulases of *Humicola insolens* that the open alditol unit at the end of reduced oligosaccharides did not alter their hydrolysis patterns much.

Determination of the kinetic parameter k_0/K_m.—By monitoring rate of substrate loss in time for G_3 to G_6, $\ln([S]_0/[S])$ *vs.* time-plots could be constructed for the three endoglucanases (data not shown). All plots proved to be linear for a limited period of time. Under conditions of first order kinetics ($[S] \ll K_m$), the slope of these lines equals the kinetic parameter V/K_m from which k_0/K_m can be deduced by dividing by the enzyme concentration [32]. Kinetic parameters are summarized in Table I. The cellodextrins were degraded faster with increasing chain length, except for endoI (optimal substrate is G_5). This increase in k_0/K_m was most spectacularly with endoIV and most likely its maximum turnover number has not yet been reached. Unfortunately, no oligosacharides larger than G_6 could be used due to their poor solubility.

TABLE I. Kinetic parameter k_0/K_m ($min^{-1}\ M^{-1}$) of three endoglucanases of *Trichoderma viride* for normal cellodextrins (G_3 to G_6). The kinetic parameter was evaluated from the slope of the linear part of $\ln([S]_0/[S])$ *vs.* time-plots.

Enzyme	G_3	G_4	G_5	G_6
EndoI	$2*10^{+5}$	$1*10^{+7}$	$11*10^{+7}$	$9*10^{+7}$
EndoIV	$\ll 1*10^{+3}$	$0.081*10^{+7}$	$4*10^{+7}$	$21*10^{+7}$
EndoV	$35*10^{+5}$	$5*10^{+7}$	$7*10^{+7}$	$8*10^{+7}$

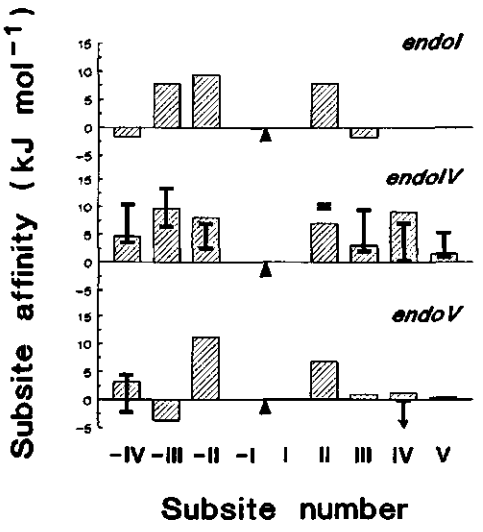

FIGURE 4. Histogram of subsite interaction energy with Glc residues of normal cellodextrins for three endoglucanases of *Trichoderma viride*. Subsite affinities were determined according to the method of Suganuma *et al.* [32] using BCFs of reduced cellodextrins (shaded bars). Vertical lines in bars (I) indicate the lower and upper limit values of subsite affinities obtained after reevaluation (for details, see text). Subsite affinities of "-I" and "I" were not determined. Lines with arrow head indicate that values are off-scale. ▲, indicates the catalytic group.

Subsite mapping of endoI, endoIV and endoV.—The first step in enzymic hydrolysis of polysaccharides can be envisaged as binding of several glycosyl residues with an array of subsites. Each subsite interacts with one sugar residue, independent of vicinal glycosyl residues. Homologous cellodextrins have often been used as tools to elucidate the subsite structure of ß-glucanases [1,15,39-42,46]. According to Suganuma *et al.* [32] the number of subsites, and their corresponding affinities, can be deduced conveniently from BCFs combined with the kinetic parameter k_0/K_m, provided certain requirements are met. Parameters should be determined under conditions of first order kinetics ($[S]_0 \ll K_m$) to simplify the Michaelis-Menten equation, and to prevent bi-substrate processes, such as transglycosylation and condensation reactions. Further, BCFs should be evaluated with first generation degradation products. Subsite affinity is defined as the decrease in free energy upon interaction of an enzyme subsite with a glycosyl residue. The interaction energy of one subsite can then be calculated by subtracting the two appropriate free energies for binding of an oligosaccharide with a chain length of *n* and *n-1*.

BCFs of G_3^* to G_6^* and the k_0/K_m values of their reducing homologues, G_3 to G_6 (see Fig. 2 and Table I, respectively) meet the requirements of the method of Suganuma *et al.* [32]. Subsite affinities for Glc residues were calculated for ten hypothetical subsites of endoI, endoIV and endoV as is shown in Fig. 4. The two subsites adjacent to the catalytic group can not be evaluated by this procedure (non-productive binding of one of the oligosaccharides [32]). Presumably, the sum of subsite affinity "-I" and that of "I" is negative for the three endoglucanases used in this study because G_2 is not cleaved by these enzymes. Further, it should be noted that small BCFs were not considered in these calculations because they are liable to relatively large experimental errors. The number of subsites clearly differed for the three endoglucanases. EndoIV had the largest number of subsites (at least 9; "-IV"→"V"), whereas endoI had only 5 subsites ("-III"→"II"). EndoV

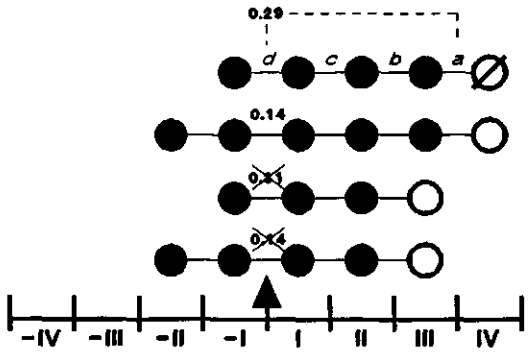

FIGURE 5. An example of reevaluation of subsite affinities of subsite "-II" of endoIV. Different binding modes of normal and/or reduced cellodextrins for (re)evaluation of this subsite are indicated. Numbers indicate the BCF for that particular linkage. ×, unreliable BCF (see Fig. 3); – – –, the BCF is distributed over the two indicated linkages *a* and *d*; other symbols, as in Fig. 2, and 4.

has 7 substrate-binding sites ("-IV"→"III") although the affinity of "III" is rather low. Typically, the negative affinity of subsite "-III" was flanked by positive affinities of subsite "-IV" and "-II". It should be mentioned that due to the limited solubility of G_7 and larger cellodextrins more distant subsites like "-V" and "VI" can not be evaluated. However, their existence can not be excluded.

As already mentioned, normal and reduced cellodextrins can be positioned differently in the substrate-binding sites of endoIV and endoV. Therefore, all subsite affinities calculated with unreliable BCFs of alditols (see Fig. 2) were reevaluated. Recalculation leads to lower and upper limit values of subsite affinities rather than absolute values. Subsite "-II" of endoIV will be used as an example to illustrate our approach. The affinity of this subsite was calculated using G_4^* and G_5^* (Fig. 5). However, since reduction influenced the BCFs of both cellodextrins, the subsite affinity of "-II" was also calculated using G_5 and G_6^*, although the BCFs of these oligosaccharides are smaller. In this case, 29 percent of all cleavages of G_5 gave Glc and G_4. The exact location of cleavage {linkage number "*a*" ($G_4 \downarrow G$) or "*d*" ($G \downarrow G_4$)} is, however, unknown. Assuming that only linkage number "*d*" is split (BCF=0.29), recalculation of this subsite affinity yields $RT*\ln\{(0.14*2.1*10^{+8})/(0.29*3.9*10^{+7})\} = 2.5$ kJ mol^{-1}. However, the other extreme case that linkage number "*d*" is not split at all (BCF=0) is equally probable. Here, recalculating the subsite affinity does not allow any conclusion (ln0). Therefore, a BCF of 0.05 was assumed instead, yielding a subsite affinity of 7.0 kJ mol^{-1}. The value of 0.05 corresponds to the experimental error. Other substrate-binding sites were reevaluated in a similar manner. For endoIV the result remains unchanged, as is shown by the lines (I) in Fig. 4. This enzyme has a long array of substrate-binding sites (at least nine). The positive affinity of subsite "-IV" of endoV should be approached with some caution. Our experiments can not be conclusive on the number of subsites of endoV.

The nomenclature of our endoglucanases deviates from the one generally adopted in literature. In order to compare our data with those in literature, we have tentatively characterized endoI, endoIV and endoV as being EGII [2,41], EGIII [4], and EGI [3,15], respectively. The presumed homology is based on similarities in mol wt, iso-electric point [31], adsorption behavior [47], the ability to degrade xylan [14] and transferase activity.

Our results on the number of subsites of endoI and endoV are in agreement with those of EGII [41] and EGI [15,39], respectively. Unfortunately, the k_0/K_m for G_6 of EGI was not determined by these authors, and consequently a putative binding site "-IV" could not be evaluated. Our results suggest a reasonable probability of a positive affinity of subsite "-IV", but this should be further substantiated using another method for labelling of cellodextrins instead of reduction. Until now no information was available on the number of subsites of endoIV (EGIII). With at least 9 subsites, this enzyme clearly differs from all other endo-(1→4)-ß-glucanases from *Trichoderma* described sofar.

Activity of endoI, endoIV, and endoV towards xyloglucan

Turnover number of various xyloglucans.—The activities of the three endoglucanases towards different substrates are summarized in Table II. Although the activity towards CMC was in the same order of magnitude for all three enzymes, their specificity for xyloglucans differed considerably. EndoIV and, to a lesser extent, endoV are far better xyloglucanases than endoI is. Despite its high degree of backbone branching, xyloglucan appeared to be a better substrate for endoIV than CMC was. In a previous study it was shown that Fuc residues hinder the degradation of xyloglucan by endoglucanases [20]. Table II shows that removal of these residues (APxg) enhances the action of all three endoglucanases, especially that of endoI. The presence of extra unsubstituted Glc residues, as is the case in POsxg (see Fig. 1), facilitated cleavage by the endoglucanases. The activity of endoI and endoV were affected to a larger extent than that of endoIV. The activity of endoI on POsxg is relatively low which suggests that endoI requires larger stretches than two unbranched Glc residues to achieve an activity comparable to that of endoIV or endoV.

For comparison, the activities of the three endoglucanases towards Avicel crystalline cellulose [31] and oat spelts xylan [14] were included in Table II. The low activity of endoIV towards Avicel is at least partly explained by its poor binding to cellulose. Typically, the endoglucanases with a high xyloglucanase activity are also active towards xylan. We do not have an explanation for this observation. The two polysaccharides differ considerably in their degree of backbone branching as well as in their conformation [48,49].

TABLE II. Turnover number (min^{-1}) of various ß-(1→4)-glucans and oat spelts xylan by three endoglucanases from *Trichoderma viride*. Activity towards Avicel and xylan is indicated qualitatively: −, no detectable activity; ±, very low activity; +, low activity; ++, high activity.

	CMC	APfxg	APxg	POsxg	Avicel*	xylan†
endoI	1518	9	32	36	+	−
endoIV	1139	2017	2237	2390	±	++
endoV	1049	600	781	1149	+	++

* Determined by Beldman *et al.* [31]. † Determined by Beldman *et al.* [14].

Based on degradation patterns of small chromophoric substrates, Claeyssens and Henrissat [16] showed that substrate specificity within one cellulase family is consistent. EndoIV, which was tentatively classified as low mol wt endoglucanase or EGIII, belongs to cellulase family H [50] which has three more representatives until now: CelS from *Erwinia carotovora* [51], FI-CMCase from *Aspergillus aculeatus* [52], and EGIII from *Humicola insolens* [1]. It is expected that all members of family H possess xyloglucanase activity; for the endoglucanase from *Aspergillus aculeatus* this has already been confirmed [53]. EndoV appears to be the most versatile endoglucanase, having activity on various natural substrates as cellulose, xylan and xyloglucan. EndoV is probably similar to EGI (as discussed before), which belongs to family C. Only one other endoglucanase has been reported to belong to this family [1]. Its xyloglucanase activity, however, has not been determined.

Release of oligosaccharides from various xyloglucans.—In order to refine our ideas on the binding of xyloglucan molecules into the subsites of the endoglucanases, the release of oligosaccharides from various xyloglucans was studied in time. Because of its heterogeneity, APfxg is a very suitable substrate for monitoring the release of different oligosaccharides. If an oligosaccharide accumulates rapidly, then this fragment is thought to interact well with the subsites of the enzyme. It is assumed that the oligosaccharides are distributed in a random fashion over the xyloglucan molecule and consequently two cleavages in the backbone are required to release oligosaccharide building units. The amount of each oligosaccharide was determined by HPAEC at various time intervals as is shown Fig. 6. HPAEC did not achieve separation of XXG and GLG; therefore, one line representing the sum of these two oligosaccharides is shown. It should be realized that the abundance of the individual oligosaccharides in apple xyloglucan is different.

Fig. 6 demonstrates that the rate at which the individual oligosaccharides are released from xyloglucan, is determined by the structure of the oligosaccharide as well as by the endoglucanase being used. The most important trends are summarized below. The sidechain configuration of the penultimate Glc residue of an oligosaccharide building unit determines the rate of release of this oligosaccharide to a large extent. Fuc-containing oligosaccharides accumulate generally slower than other oligosaccharides. The relatively fast release of GFG by endoI forms an exception. Removal of Fuc can have a large effect on the release of some oligosaccharides (compare FG and LG by endoI). EndoI and endoV are more affected by Fuc residues than endoIV, which is in accordance with Table II. Also, Gal residues probably play a role considering the faster release of XLXG compared to XXLG, and XXLG compared to XLLG. The rate of release of oligosaccharides is further determined by their backbone length. EndoIV prefers oligosaccharides with a backbone of four Glc residues (XXXG and XLXG) whereas endoI prefers two or three Glc residues (LG, XXG and GFG). EndoV holds an intermediate position in this. The preference to release short oligosaccharides can be overruled by Fuc substitution (compare FG and LG by endoI).

The results on the release of oligosaccharides from POsxg can not be discussed in further detail because the exact position of Ara and Gal residues is still unknown. EndoIV

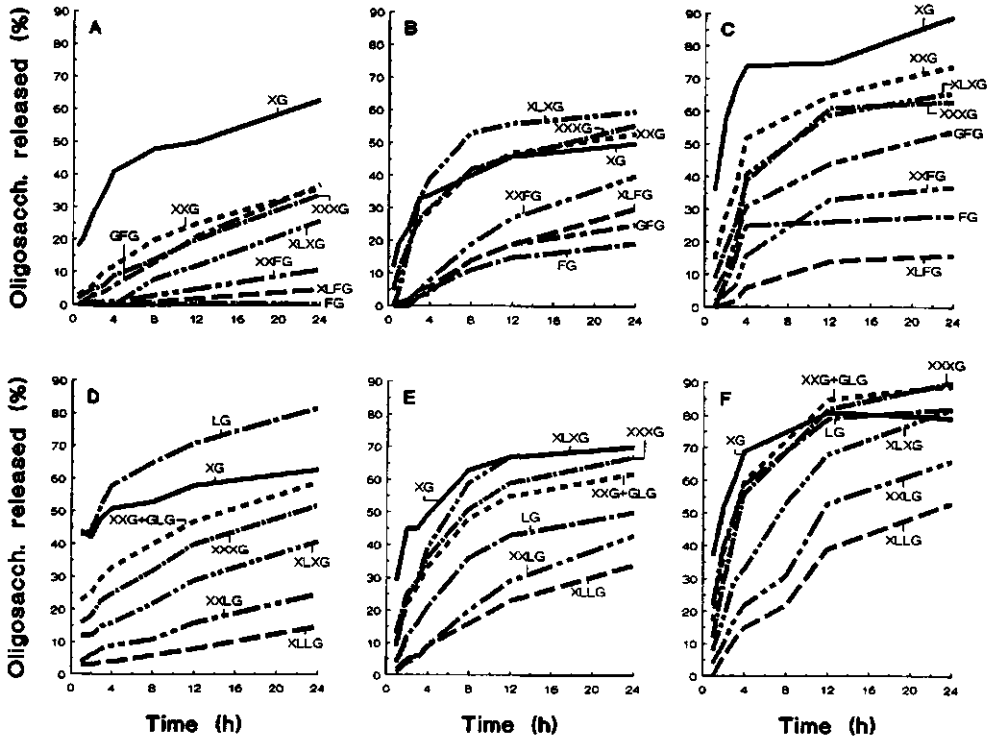

FIGURE 6. Release of oligosaccharides from APfxg (A-C) and APxg (D-F) in time by the action of endoI (A, D), endoIV (B, E), and endoV (C, F). The amount of a certain oligosaccharide that was released, is expressed as the percentage of the PAD response of this oligosaccharide in the chromatogram of APfxg or APxg which was completely degraded by endoIV. Different xyloglucan oligosaccharides are indicated according to the nomenclature of Fry *et al.* [38].

released predominantly XXGG-type of oligosaccharides whereas endoV also produced a GXXG-type [22]. In addition, substantial amounts of Glc and XXG-type of oligosaccharides accumulated in the POsxg digest obtained by endoV. The latter probably originate from further cleavage ("trimming") of the XXGG-type of oligosaccharides. From this it was concluded that endoV is better in "trimming" G elements from xyloglucan oligosaccharides than endoIV [22]. Degradation patterns of POsxg by endoI were very similar to those obtained with endoV (data not shown).

What determines xyloglucanase activity?

The time course studies demonstrated a striking difference between endoI and endoIV. In particular, endoI preferred to release shorter oligosaccharides than endoIV. In order to explain this difference, the subsite models were combined with the data on the release of oligosaccharides. Two typical examples are shown in Fig. 7 in which the subsite maps of the endoglucanases (Fig. 4) have been simplified, and only positive and negative affinities

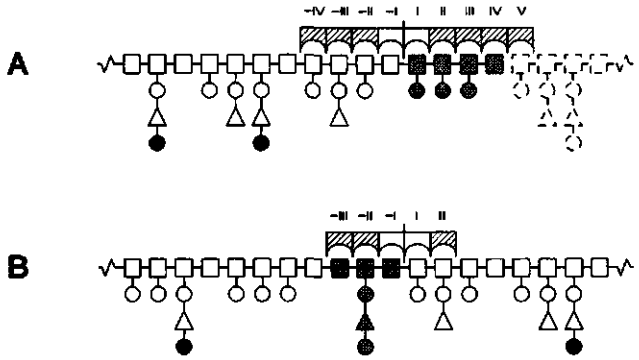

FIGURE 7. Schematic representation of the interaction of APfxg and endoIV (A) or endoI (B). Assuming a random distribution of oligosaccharides over xyloglucan, two linkages should be cleaved to release the shaded oligosaccharide. Only in case of endoIV the second cleavage is shown. Dashed fragments indicate the part of the xyloglucan molecule which is removed after the first cleavage. Shaded substrate-binding sites bind to xyloglucan (positive subsite affinity), open subsites (negative subsite affinity) do not bind to xyloglucan. Other symbols as in Fig. 1.

are indicated. The figure shows clearly that during cleavage by endoIV, there is always an unbranched Glc residue which interacts with a subsite having a positive affinity. Especially, the distant subsite "IV" is important in this respect (Fig. 7A). EndoI does not possess a subsite like "IV", and consequently prefers to release the shorter oligosaccharides such as GFG and LG. An explanation for this might be the favorable interaction of subsite "-III" with unbranched Glc residues (Fig. 7B).

The data discussed above suggest that binding of unbranched Glc residues plays an important role in the cleavage of a xyloglucan molecule. Since APfxg is built predominantly from XXXG, XLXG, XXFG and XLFG [20,21], it may be expected that APfxg is faster degraded by endoIV than by endoI. This is in accordance with the data in Table II. Because most xyloglucans are composed of XXXG-type of building units [17], it is tempting to generalize our results to the statement that "xyloglucanase activity requires a large number of substrate-binding sites". In agreement with our data, there is some evidence that an endoglucanase from *Pisum sativum*, showing xyloglucanase activity next to CMCase, has at least six substrate-binding sites [54,55].

This study does not provide an explanation for the high xyloglucanase of endoV. It can not be excluded that endoV has a distant subsite "-V", which would have a similar effect as subsite "IV" of endoIV. In principle, the affinity of subsite "-V" could be determined with the BCFs of $G_4\downarrow G$ and $G_5\downarrow G$. However, the corresponding BCF values are zero, and a different set of BCFs, such as $G_4\downarrow G_2$ and $G_5\downarrow G_2$, would be needed. Because G_7 is insoluble in water, the latter BCF can not be determined. An indication for a subsite "-V" might be the release of GXXG-type of oligosaccharides (G element of POsxg binds in subsite "-V"). Another possibility might be that endoV has a much broader substrate-binding groove than endoI, which may facilitate interaction with xyloglucan molecules.

The different mode of action of endoIV on POsxg, compared to endoI or endoV, can

only partly be explained by our results. Interaction of subsites with unbranched Glc residues does not explain the cleavage patterns shown in Fig. 1. The absence of strong distant subsites like "III" and "IV" in endoI and endoV does explain why these enzymes are better than endoIV in "trimming" oligosaccharides and releasing Glc. The action of endoglucanases on POsxg is probably determined by the position of Gal and Ara residues. The slower release of XXLG compared to XLXG from APxg suggests that Gal residues next to a G element in xyloglucan hamper the action of endoIV. This might explain why endoIV prefers the release of XXGG-type of oligosaccharides (Fig. 1). The release of XXG from POsxg by endoIV [22] is in agreement with this. Contrary to endoIV, the release of XLXG by endoI and endoV is generally slower than that of XXXG, which indicates that the action of endoI and endoV is more influenced by substitution than that of endoIV. This might explain the release of a mixture of XXGG-type and GXXG-type of oligosaccharides from POsxg by endoI or endoV, depending on whether or not the Xyl residue on the nonreducing side is substituted.

REFERENCES

[1] Schou, C., Rasmussen, G., Kaltoft, M.-B., Henrissat, B., and Schülein, M. (1993) Stereochemistry, specificity and kinetics of the hydrolysis of reduced cellodextrins by nine cellulases. *Eur. J. Biochem.* **217**, 947-953.

[2] Penttilä, M., Lehtovaara, P., Nevalainen, H., Bhikhabhai, R., and Knowles, J. (1986) Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene* **45**, 253-263.

[3] Saloheimo, M., Lehtovaara, P., Penttilä, M., Teeri, T.T., Ståhlberg, J., Johansson, G., Pettersson, G., Claeyssens, M., Tomme, P., and Knowles, J.K.C. (1988) EGIII, a new endoglucanase from *Trichoderma reesei*: characterization of both gene and enzyme. *Gene* **63**, 11-21.

[4] Ward, M., Wu, S., Dauberman, J., Weiss, G., Larenas, E., Bower, B., Rey, M., Clarkson, K., and Bott, R. (1993) Cloning, sequence and preliminary structural analysis of a small, high pI endoglucanase (EGIII) from *Trichoderma reesei*. In Proceedings of the Second TRICEL Symposium on Trichoderma reesei *Cellulases and other Hydrolases* (Suominen, P., and Reinikainen T., eds., Foundation for Biotechnical and Industrial Fermentation Research 8, Helsinki, Finland), 153-158.

[5] Saloheimo, A., Henrissat, B., Hoffrén, A.-M., Teleman, O., and Penttilä, M. (1994) A novel, small endoglucanase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast. *Mol. Microbiol.* **13**, 219-228.

[6] Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., and Innis, M. (1983) Molecular cloning of exo-cellobiohydrolase I derived from *Trichoderma reesei* strain L27. *Bio/Technol.* **1**, 691-696.

[7] Teeri, T.T., Lehtovaara, P., Kauppinen, S., Salovuori, I., and Knowles, J. (1987) Homologous domains in *Trichoderma reesei* cellulolytic enzymes: gene sequence and expression of cellobiohydrolase II. *Gene* **51**, 43-52.

[8] Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. (1988) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* **170**, 575-581.

[9] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., and Warren, R.A.J. (1991) Domains of microbial ß-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**, 303-315.

[10] Srisodsuk, M., Reinikainen, T., Penttilä, M., and Teeri, T.T. (1993) Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. *J. Biol. Chem.* **268**, 20756-20761.

[11] Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L., and Mornon, J.-P. (1989) Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81**, 83-95.

[12] Henrissat, B., and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781-788.
[13] Woodcock, S., Mornon, J.-P., and Henrissat, B. (1992) Detection of secondary structure elements in proteins by hydrophobic cluster analysis. *Protein Eng.* **5**, 629-635.
[14] Beldman, G., Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F., and Pilnik, W. (1988) Specific and nonspecific glucanases from *Trichoderma viride*. *Biotechnol. Bioeng.* **31**, 160-167.
[15] Biely, P., Vršanská, M., and Claeyssens, M. (1991) The endo-1,4-ß-glucanase I from *Trichoderma reesei*. Action on ß-1,4-oligomers and polymers derived from D-glucose and D-xylose. *Eur. J. Biochem.* **200**, 157-163.
[16] Claeyssens, M., and Henrissat, B. (1992) Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. *Protein Sci.* **1**, 1293-1297.
[17] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.
[18] Montenecourt, B.S. (1983) *Trichoderma reesei* cellulases. *Trends Biotechnol.* **1**, 156-161.
[19] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and endoglucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).
[20] Vincken, J.-P., Beldman, G., Niessen, W.M.A., and Voragen, A.G.J. (1996) Degradation of apple fruit xyloglucan by endoglucanase. *Carbohydr. Polym.*, in press (this thesis, Chapter 3).
[21] Spronk, B.A., Rademaker, G.J., Haverkamp, J., Thomas-Oates, J.E., Vincken, J.-P., Voragen, A.G.J., Kamerling, J.P., and Vliegenthart, J.F.G. Dimers of a GFG hexasaccharide occur in apple fruit xyloglucan. To be submitted (this thesis, Chapter 4).
[22] Vincken, J.-P., Wijsman, A.J.M., Beldman, G., Niessen, W.M.A., and Voragen, A.G.J. (1996) Potato xyloglucan is bult from XXGG-type building units. Accepted for publication in *Carbohydr. Res.* (this thesis, Chapter 5).
[23] Baird, S.D., Hefford, M.A., Johnson, D.A., Sung, W.L., Yaguchi, M., and Seligy, V.L. (1990) The GLU residue in the conserved ASN-GLU-PRO sequence of two highly divergent endo-ß-1,4-glucanases is essential for enzymatic activity. *Biochem. Biophys. Res. Commun.* **169**, 1035-1039.
[24] Svensson, B., and Søgaard, M. (1993) Mutational analysis of glycosylase function. *J. Biotechnol.* **29**, 1-37.
[25] Chen, L., Fincher, G.B., and Høj, P.B. (1993) Evolution of polysaccharide hydrolase substrate specificity. Catalytic amino acids are conserved in barley 1,3-1,4- and 1,3-ß-glucanases. *J. Biol. Chem.* **268**, 13318-13326.
[26] Sinnott, M.L. (1990) Catalytic mechanisms of enzymic glycosyl transfer. *Chem. Rev.* **90**, 1171-1202.
[27] Gebler, J.C., Gilkes, N.R., Claeyssens, M., Wilson, D.B., Béguin, P., Wakarchuk, W.W., Kilburn, D.G., Miller, R.C., Warren, R.A.J., and Withers, S.G. (1992) Stereoselective hydrolysis catalyzed by related ß-1,4-glucanases and ß-1,4-xylanases. *J. Biol. Chem.* **267**, 12559-12561.
[28] Divne, C., Ståhlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J.K.C., Teeri, T.T., and Jones, T.A. (1994) The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. *Sci.* **265**, 524-528.
[29] Meinke, A., Damude, H.G., Tomme, P., Kwan, E., Kilburn, D.G., Miller, R.C., Warren, R.A.J., and Gilkes, N.R. (1995) Enhancement of the endo-ß-1,4-glucanase activity of an exocellobiohydrolase by deletion of a surface loop. *J. Biol. Chem.* **270**, 4383-4386.
[30] Høj, P.B., Rodriguez, E.B., Stick, R.V., and Stone, B.A. (1989) Differences in active site structure in a family of ß-glucan endohydrolases deduced from the kinetics of inactivation by epoxyalkyl ß-oligoglucosides. *J. Biol. Chem.* **264**, 4939-4947.
[31] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases, and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.
[32] Suganuma, T., Matsuno, R., Ohnishi, M., and Hiromi, K. (1978) A study of the mechanism of action of Taka-amylase A on linear oligosaccharides by product analysis and computer simulation. *J. Biochem.* **84**, 293-316.
[33] Voloch, M., Ladisch, M.R., Cantarella, M., and Tsao, G.T. (1984) Preparation of cellodextrins using sulfuric acid. *Biotechnol. Bioeng.* **26**, 557-559.
[34] Somogyi, N. (1952) Notes on sugar determination. *J. Biol. Chem.* **195**, 19-23.
[35] Tollier, M.T., and Robin, J.P. (1979) Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Ann. Technol. Agric.* **28**, 1-15.
[36] De Ruiter, G.A., Schols, H.A., Voragen, A.G.J., and Rombouts, F.M. (1992) Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.* **207**, 176-185.
[37] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.

[38] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.

[39] Claeyssens, M., van Tilbeurgh, H., Kamerling, J.P., Berg, J., Vršanská, M., and Biely, P. (1990) Studies of the cellulolytic system of the filamentous fungus *Trichoderma reesei* QM 9414. *Biochem. J.* **270**, 251-256.

[40] Vršanská, M., and Biely, P. (1992) The cellobiohydrolase I from *Trichoderma reesei* QM 9414: action on cello-oligosaccharides. *Carbohydr. Res.* **227**, 19-27.

[41] Macarron, R., Acebal, C., Castillon, M.P., Dominguez, J.M., de la Mata, I., Pettersson, G., Tomme, P., and Claeyssens, M. (1993) Mode of action of endoglucanase III from *Trichoderma reesei*. *Biochem. J.* **289**, 867-873.

[42] Bhat, K.M., Hay, A.J., Claeyssens, M., and Wood, T.M. (1990) Study of the mode of action and site-specificity of the endo-(1→4)-β-D-glucanases of the fungus *Penicillium pinophilum* with normal, 1-^{3}H-labelled, reduced and chromogenic cello-oligosaccharides. *Biochem. J.* **266**, 371-378.

[43] Bray, M.R., and Clarke, A.J. (1992) Action pattern of xylo-oligosaccharide hydrolysis by *Schizophyllum commune* xylanase A. *Eur. J. Biochem.* **204**, 191-196.

[44] Gum, E.K., and Brown, R.D. (1977) Two alternative HPLC separation methods for reduced and normal cellooligosaccharides. *Anal. Biochem.* **82**, 372-375.

[45] Björkqvist, B. (1981) Separation and determination of phenyl isocyanate-derivatized carbohydrates and sugar alcohols by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr.* **218**, 65-71.

[46] Claeyssens, M., van Tilbeurgh, H., Tomme, P., Wood, T.M., and McRae, S.I. (1989) Fungal cellulase systems. Comparison of specificities of the cellobiohydrolases isolated from *Penicillium pinophilum* and *Trichoderma reesei*. *Biochem. J.* **261**, 819-825.

[47] Beldman, G., Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F., and Pilnik, W. (1987) Adsorption and kinetic behaviours of purified endoglucanases and exoglucanases from *Trichoderma viride*. *Biotechnol. Bioeng.* **30**, 251-257.

[48] Ogawa, K., Hayashi, T., and Okamura, K. (1990) Conformational analysis of xyloglucans. *Int. J. Biol. Macromol.* **12**, 218-222.

[49] Atkins, E.D.T. (1992) Three-dimensional structure, interactions and properties of xylans. In *Xylans and xylanases* (Visser, J., Beldman, G., Kusters-van Someren, M.A., and Voragen, A.G.J., eds., Elsevier Science Publishers B.V., Amsterdam, The Netherlands), 39-50.

[50] Tõrrõnen, A., Kubicek, C.P., and Henrissat, H. (1993) Amino acid sequence similarities between low molecular weight endo-1,4-β-xylanases and family H cellulases revealed by clustering analysis. *FEBS Lett.* **321**, 135-139.

[51] Saarilahti, H.T., Henrissat, B., and Palva, E.T. (1990) CelS: a novel endoglucanase identified from *Erwinia carotovora* subsp. *carotovora*. *Gene* **90**, 9-14.

[52] Ooi, T., Shinmyo, A., Okada, H., Hara, S., Ikenaka, T., Murao, S., and Arai, M. (1990) Cloning and sequence analysis of a cDNA for cellulase (FI-CMCase) from *Aspergillus aculeatus*. *Curr. Genet.* **18**, 217-222.

[53] Dalbøge, H., Andersen, L.N., Kofod, L.V., Kauppinen, M.S., and Christgau, S. (1994) An enzyme with endoglucanase activity. *World Patent*, WO 94/14953.

[54] Maclachlan, G., and Wong, Y.-S. (1979) Two pea cellulases display the same catalytic mechanism despite major differences in physical properties. *Adv. Chem. Ser.* **181**, 347-360.

[55] Hayashi, T., Wong, Y.-S., and Maclachlan, G. (1984) Pea xyloglucan and cellulose. II. Hydrolysis by pea endo-1,4-β-glucanases. *Plant Physiol.* **75**, 605-610.

CHAPTER 7

Fractionation of Xyloglucan Fragments and Their Interaction with Cellulose[1]

Tamarind seed xyloglucan was partially degraded with a purified endoglucanase (endoV) from *Trichoderma viride*. Analysis by high-performance anion-exchange chromatography showed that this digest was composed of fragments consisting of 1 to 10 repeating building units ($[xg]_1$-$[xg]_{10}$). To study the adsorption of xyloglucan fragments to cellulose in detail, this digest was fractionated on BioGel P-6. Fragments were separated satisfactorily up to 5 repeating building units ($[xg]_5$). The galactose substitution of the fragments increased with increasing molecular weight. The BioGel P-6 pools, as well as polymeric xyloglucan ($[xg]_\infty$), were tested for their ability to interact with Avicel crystalline cellulose. Quantitative binding to cellulose occurred for sequences consisting of (at least) 4 repeating building units. The adsorption of $[xg]_4$ to Avicel was very high relative to that of $[xg]_\infty$. The dimensions of these fragments were such that they could also penetrate the smaller pores of cellulose. Apparently, the effective surface area for the polymers is much smaller. Adsorption isotherms of $[xg]_\infty$ and $[xg]_4$ showed a pattern that is typical for polydisperse systems. However, the mechanisms underlying these patterns were different. At high xyloglucan concentrations, this polydispersity resulted in preferential adsorption of the larger molecules in the case of $[xg]_\infty$ and a more extensive colonization of the smaller pores of cellulose in the case of $[xg]_4$. The pH influenced the interaction between xyloglucan (fragments) and cellulose to only a small extent.

Cellulose and xyloglucan are important structural components of the primary cell wall of plants [1]. Both polysaccharides are composed of a ß-(1→4)-glucan backbone. In cellulose these chains associate laterally by hydrogen bonding to form microfibrils, but self-association of xyloglucan molecules is prohibited by a large number of sidechains [2]. Although cellulose and xyloglucan are assembled at different locations, an intimate interaction between these two polysaccharides exists *in vivo*. In the primary cell wall, cellulose microfibrils are extensively coated with xyloglucans [2], which prevent their aggregation into even larger cellulose-complexes [3,4]; only small amounts of "naked" cellulose occur [5]. Strong chaotropic reagents are needed to swell cellulose and solubilize xyloglucan from cell wall material [6], which suggests that binding of xyloglucan to cellulose is mediated by hydrogen bonds [1,5]. Recently, two endogenous proteins (expansins) were found that are thought to interfere in the noncovalent binding between cellulose and xyloglucan, thereby inducing cell wall extension [1,7].

The xyloglucan-binding capacity of cellulose in native cell walls surpasses by many times that found in *in vitro* binding experiments [8,9]. In addition to these studies on the

---

[1]Vincken, J.-P., de Keizer*, A., Beldman, G., and Voragen, A.G.J. (1995) *Plant Physiol.* **108**, 1579-1585
(* *Department of Physical and Colloid Chemistry, Wageningen Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands*).

interaction of $[xg]_\infty$[II] and cellulose, Valent and Albersheim [10] showed that xyloglucan oligosaccharides (XXXG and XXFG; for nomenclature see [11]) do not bind to cellulose in aqueous conditions. Recently, Hayashi *et al.* [12] demonstrated that a minimum length of at least five consecutive Glc residues is required to enable binding of xyloglucan molecules to cellulose. Cello-oligosaccharides of similar backbone length were found to bind to a larger extent under the same conditions. Structural aspects might also be important. Based on computer simulations, Levy *et al.* [13] demonstrated that fucosylated side chains can facilitate the adoption of a flat backbone conformation. They speculated that these straightened regions trigger the binding of xyloglucan to cellulose. *In vitro* modification of xyloglucans by a ß-galactosidase increased self-association of the backbone, ultimately leading to gel formation [14]. Hisamatsu *et al.* [15] reported the existence of endoglucanase-resistant fragments that contained a Glc residue with additional branching at the C-2 position. These structural elements were hypothesized to destabilize xyloglucan-cellulose interactions. The examples indicate that, in addition to size, the side chain configuration also might play an important role in the adsorption behavior of xyloglucans.

Efficient enzymic degradation of cell wall embedded cellulose requires a preceding solubilization of the xyloglucan coating [16]. Although the data of Hayashi *et al.* [12] demonstrated that the adsorption equilibrium shifts towards more bound material with increasing chain length, these data do not predict to what extent xyloglucans have to be degraded before "lift-off" from cellulose microfibrils occurs. In other words, what is the minimum length of a xyloglucan molecule for quantitative binding to cellulose? The present paper reports the fractionation of such molecules and compares their *in vitro* adsorption on cellulose with that of polymeric xyloglucan.

EXPERIMENTAL

Materials

Chemicals.—Avicel crystalline cellulose (type SF) was purchased from Serva (Heidelberg, Germany). Cotton linters cellulose powder was obtained from Fluka Chemie (Buchs, Switzerland). Tamarind seed xyloglucan was kindly provided by Dainippon Pharmaceutical (Osaka, Japan). Standards of xyloglucan oligosaccharides (XXXG, XLXG, XXLG, and XLLG) were obtained by enzymic degradation of apple fruit xyloglucan and subsequent fractionation by BioGel P-2 and HPAEC. Their characterization is described in Chapters 3 and 4.

Enzymes.—EndoV (EC 3.2.1.4) was purified to homogeneity from a commercial preparation from *Trichoderma viride* (Maxazyme CI, Gist-Brocades, Delft, The Netherlands) by Beldman *et al.* [17].

Preparation of xyloglucan fragments

Xyloglucan fragments were prepared by partial degradation of 250 mg of tamarind seed

---

[II] *Abbreviations:* Γ, adsorbed amount; other abbreviations, see page viii.

xyloglucan with 5 μg (*c.* 53 mU) of endoV in 25 mL of a 25 mM NaOAc buffer pH 5.0 (40 °C, 9 h). The degradation was monitored using HPSEC. When the mol wt distribution was such that mainly dimers, trimers, and tetramers of xyloglucan oligosaccharides were present, the incubation was stopped by pouring the mixture into 475 mL of boiling water, followed by 10 min of heating (100 °C). The digest was concentrated under reduced pressure to a final volume of 4 mL. Two mL were then applied to a column (100 x 2.6 cm, i.d.) of BioGel P-6 (200-400 mesh, Bio-Rad, Richmond, CA, USA) at 60 °C and eluted (20 mL h^{-1}) with distilled water. Fractions (2.6 mL) were assayed for total neutral sugar content. Appropriate fractions were combined and designated as *I*, *II*, *III*, *IV*, *V*, and *VI*. The column was calibrated using a mixture of Dextran T150 (Pharmacia, Uppsala, Sweden) and Glc (Kav = 0 and 1, respectively). Chromatography of a completely digested (endoV) tamarind seed xyloglucan was conducted in a similar way on BioGel P-2.

Adsorption of Xyloglucan (Fragments) to Cellulose

In a series of experiments some aspects of the interaction between xyloglucan and cellulose were studied. The length of xyloglucan molecules, the pH, and the origin of cellulose were parameters that were varied. Also, the oligosaccharide (building unit) composition of bound xyloglucan was investigated. All incubations were done in 1 mL of 25 mM buffer (specified for the individual experiments) containing 10 mM $CaCl_2$ and appropriate amounts of cellulose and xyloglucan (fragments). After 6 h of incubation at 40 °C (head-over-tail mixing) the samples were centrifuged (1 min, 20,000 *g*), and the amount of unbound material in the supernatants was quantified by either HPSEC or determination of total neutral sugar content. The adsorption was calculated from the difference in soluble material before and after incubation with cellulose.

To determine their critical length for quantitative binding to cellulose, 1 mg of an unfractionated xyloglucan digest was incubated with Avicel (50 mg) in NaOAc buffer (pH 5.8) and analyzed by HPSEC. In another experiment, Avicel (25 mg) was incubated with the same buffer containing 250 μg of purified xyloglucan fragments (BioGel P-6 pools *I-VI*) or $[xg]_\infty$ in triplicate, and analyzed in a similar way.

Adsorption isotherms were obtained by treating 50 mg of Avicel or cotton linters in NaOAc buffer (pH 5.8) with varying concentrations of $[xg]_\infty$ (25-1000 μg). In a similar way, pools *IV* and *VI* were mixed with 1 or 5 mg of Avicel. The unbound material was quantified by total neutral sugar analysis. To study the effect of the pH on the interaction of xyloglucan and cellulose, adsorption isotherms of $[xg]_\infty$ to Avicel were also made using NaOAc buffers of different pH levels (5.0 and 4.0) and a citrate buffer of pH 3.0 under conditions similar to those described above. Additionally, the adsorption of pool *VI* (500 μg) to Avicel (5 mg) was investigated at different pH levels using the four buffers described previously.

The oligosaccharide (building unit) composition of material that was bound to cellulose was determined as follows. Cellulose (1 mg) was incubated with NaOAc buffer (pH 5.8) containing 200 μg of pool *IV* as described above. After centrifugation the supernatant was removed and the pellet (cellulose with bound xyloglucan fragments) was resuspended in 1 mL of a similar buffer. This suspension was then treated with 50 μg of endoV for 24 h (head-over-tail mixing; 40 °C). The parental material of pool *IV* was treated with endoV in a similar way. The resulting oligosaccharides were analyzed by HPAEC.

Analytical Methods

Total neutral sugar content.—The total neutral sugar content was determined colorimetrically with an automated orcinol/H_2SO_4 assay [18]. Glc was used as a standard.

Neutral sugar composition.—Tamarind seed xyloglucan and fragments thereof were hydrolyzed using 2 M TFA; crystalline cellulose was pretreated with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C. The released neutral sugars were converted to their alditol acetates and analyzed by GC as described previously [16].

Protein content.—The protein content of enzyme preparations was determined according to Sedmak and Grossberg [19]. BSA was used as a standard.

Analysis of Xyloglucan Fragments.—Quantitative analyses of xyloglucan fragments was performed by HPSEC as described previously [16]. In addition, samples were analyzed by HPAEC using a Dionex (Sunnyvale, CA, USA) Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-100 column (250 x 4 mm, 20 °C). Samples (20 μL) were injected using a SP 8780 autosampler (Spectra Physics, San José, CA, USA) equipped with a Tefzel rotor seal in a 7010 Rheodyne injector valve. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The eluate (1 mL min^{-1}) was monitored using a Dionex PED detector in the PAD mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1, 0.1 V and 0.5 s; E_2, 0.6 V and 0.1 s; E_3, −0.6 V and 0.1 s. The xyloglucan fragments were analyzed by application of the following gradient: 0→8 min, linear gradient of 80→170 mM NaOAc in 100 mM NaOH; 8→30 min, linear gradient of 170→220 mM NaOAc in 100 mM NaOH. After each analysis the column was rinsed for 5 min with 1 M NaOAc in 100 mM NaOH and equilibrated in 80 mM NaOAc in 100 mM NaOH for 15 min.

RESULTS

Fractionation of Xyloglucan Fragments

Tamarind seed xyloglucan has been shown to consist of four repeating building units: XXXG (*c.* 13 mol%), XLXG (*c.* 9 mol%), XXLG (*c.* 28 mol%) and XLLG (*c.* 50 mol%) [20]. In our study, tamarind seed xyloglucan was completely degraded by endoV and the resulting oligosaccharides were fractionated and quantified by BioGel P-2 chromatography (data not shown). The fractions were analyzed by HPAEC and the retention times of the four products were compared with those of standards derived from apple fruit xyloglucan. Our results were in close agreement with those reported by York *et al.* [20]. This collection of four oligosaccharides will be further referred to as $[xg]_1$.

The degradation of tamarind seed xyloglucan by endoV was monitored by HPSEC, and continued until a pattern as shown in Fig. 1A (solid line) was obtained. The major part of this digest is composed of molecules that were later shown to contain one, two or three repeating building units ($[xg]_1$, $[xg]_2$ and $[xg]_3$, respectively). Elution of material with a retention time less 29.5 min indicated that larger molecules were also present; however, no complete separation of these fragments was obtained using HPSEC. When cellulose was added to this digest, very few molecules larger than $[xg]_2$ were found in the supernatants after 6 h of incubation and subsequent centrifugation (Fig. 1A, dashed line). This demonstrated that chain length largely determines the adsorption behavior of xyloglucan molecules. Subsequently, the xyloglucan digest was fractionated on BioGel P-6, and six pools were obtained as indicated in Fig. 1B. The Kav values of pools *I* and *II* were similar to those reported by Hayashi and Maclachlan [5] for pea xyloglucan oligosaccharides ($[xg]_1$) and dimers ($[xg]_2$) thereof, respectively. When it was assumed that pools *III* to *V* correspond to $[xg]_3$ to $[xg]_5$, respectively, a linear relationship between Log(DP) and Kav was obtained in the range $[xg]_1$ to $[xg]_5$. Fractions *I* to *III* were

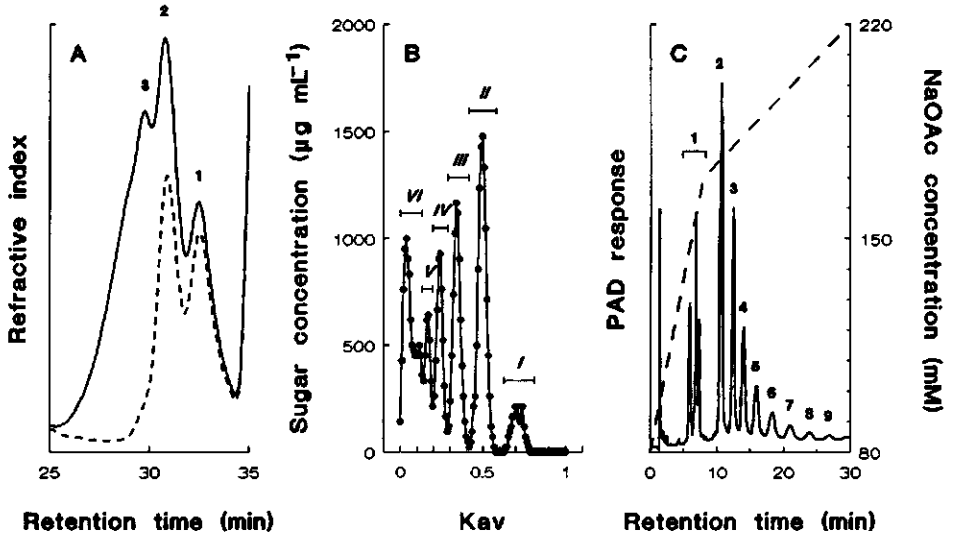

FIGURE 1. Elution profiles of a digest of tamarind seed xyloglucan analyzed by HPSEC (A), BioGel P-6 chromatography (B), and HPAEC (C). A, The degradation of xyloglucan by endoV was monitored using HPSEC, and the enzyme was inactivated when the profile shown as a solid line was obtained. Incubation {25 mM NaOAc buffer (pH 5.8) containing 10 mM $CaCl_2$} of this mixture of xyloglucan fragments (1 mg) with Avicel crystalline cellulose (50 mg) for 6 h yielded the profile shown as a dashed line (---). B, The partially digested tamarind seed xyloglucan was fractionated using BioGel P-6 (for experimental details, see text). C, The same sample was also analyzed by HPAEC (—, PAD response; - - -, NaOAC-gradient). Digits 1 to 9 indicate the number of repeating building units ($[xg]_1$ to $[xg]_9$). Roman numerals *I* to *VI* are used to designate BioGel P-6 pools.

analyzed by HPSEC and corresponded to peaks 1 to 3 (Fig. 1A), respectively.

The same digest was also analyzed using HPAEC. Fig. 1C shows that this method gives a much higher resolution than HPSEC and BioGel P-6; the sample is composed of 10 products. Pools *I* to *IV* corresponded with $[xg]_1$ to $[xg]_4$, respectively, in the pattern of Fig. 1C. Pool *V* contained primarily $[xg]_5$ and some slight contamination of $[xg]_6$. Typically, pool *VI* did not contain only products larger than $[xg]_5$; relatively large amounts of smaller fragments, including $[xg]_1$, were also present. Even when taking into consideration the decreasing PAD response for fragments with increasing mol wts, *c.* 35% (w/w) of pool *VI* consisted of $[xg]_{n<6}$. Self-association with larger xyloglucan fragments might be an explanation for this phenomenon. Fig. 1C suggests that xyloglucan molecules consisting of at least 10 repeating building units ($[xg]_{10}$) can be separated.

Both $[xg]_1$ and $[xg]_2$ showed multiple signals upon HPAEC (Fig. 1C) which demonstrated that these were heterogeneous fractions. Fraction *I* ($[xg]_1$) contained primarily XXXG and XXLG building units, but also some XLXG and XLLG (Fig. 2A). $[xg]_2$ probably contains any combination of the above forms of $[xg]_1$ oligosaccharides

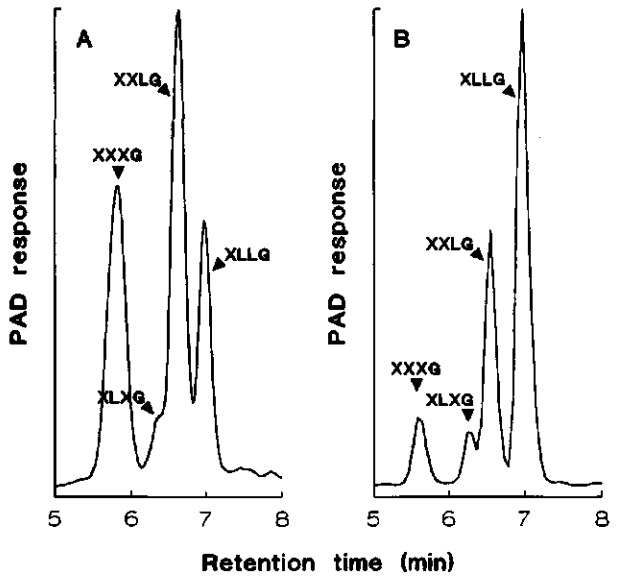

FIGURE 2. HPAEC elution profiles of BioGel P-6 pool *I* ($[xg]_1$, A) and an endoV-digest of BioGel P-6 pool *IV* ($[xg]_4$, B).

which renders 16 possibilities. For $[xg]_3$ and $[xg]_4$, the number of possibilities increases dramatically. Incomplete resolution of the different compounds within one population by HPAEC results in an apparent homogeneity of populations larger than $[xg]_2$.

Sugar analysis of the BioGel P-6 pools showed that the fragments were gradually enriched in Gal with increasing mol wt (data not shown). This suggested that larger fragments were composed of different building units than the smaller molecules. To verify this, the fragments of pool *IV* were degraded to their building units with endoV. Fig. 2B demonstrates that XLLG is the major building unit of $[xg]_4$, in contrast to $[xg]_1$, where XXXG and XXLG are the most abundant oligosaccharides (Fig. 2A).

Adsorption of Xyloglucan Fragments of Different Length to Cellulose

The combination of the results of Fig. 1, A and B, suggested that fractionation on BioGel P-6 enabled the purification of fragments of critical length in relation to adsorption on cellulose. Therefore, pools *I* to *VI*, as well as $[xg]_\infty$, were tested for their ability to interact with cellulose. This is shown in Fig. 3. The $[xg]_1$ oligosaccharides did not bind to cellulose in aqueous conditions, which is in accordance with the results of Valent and Albersheim [10]. Binding of only a small amount of $[xg]_2$ and a larger amount of $[xg]_3$ occurred, which is in agreement with results reported by Hayashi *et al.* [12]. Apparently, $[xg]_5$ molecules possessed the minimum length required for quantitative binding to cellulose under the conditions used. It should be noted that $[xg]_{6<n<11}$ is a part of pool *VI*, and $[xg]_1$ and $[xg]_2$, which are also present in this fraction, did not adsorb. Typically, the adsorption of $[xg]_\infty$ was much lower compared to the adsorption of $[xg]_5$. Obviously, the effective specific surface area is much smaller for the polysaccharides.

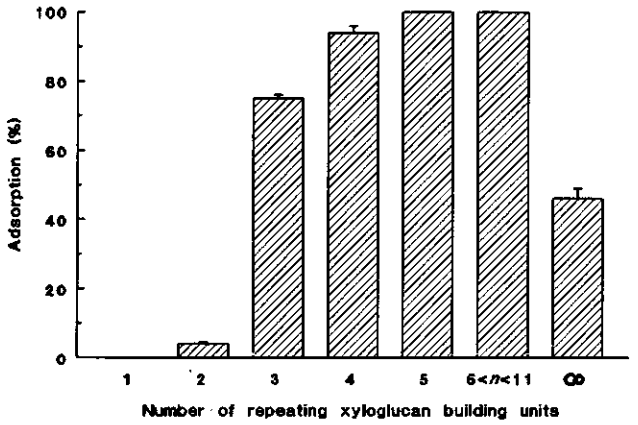

FIGURE 3. Adsorption of xyloglucan fragments of varying DP to Avicel crystalline cellulose. Various xyloglucan fragments (250 μg) were incubated (40 °C) with 1 mL of 25 mM NaOAc buffer (pH 5.8) containing 10 mM $CaCl_2$ and 25 mg of cellulose. After 6 h of incubation the samples were centrifuged (1 min, 20,000 *g*), and the supernatants were analyzed by HPSEC. The adsorbed amount was calculated from the ratio of the peak areas before and after incubation. Error bars indicate deviations of the average of three measurements.

The above findings indicated that, relative to $[xg]_\infty$, large amounts of $[xg]_4$ and pool *VI* were needed to saturate the cellulose surface. This was further substantiated by the adsorption isotherms in Fig. 4, A and B. Initially, all xyloglucan bound to cellulose which is illustrated by the steep part of the isotherms. At higher xyloglucan concentrations, Γ increased less sharply and tended to a constant value. A difference in adsorption behavior between $[xg]_4$ and pool *VI* became prominent here, because only for pool *VI* was this "plateau value" realized. In accordance with Fig. 3, the adsorption of $[xg]_\infty$ was much smaller relative to, for instance, that of $[xg]_4$.

The origin of the cellulose was shown to influence the adsorption behavior of $[xg]_\infty$ (Fig. 4A; compare ▵ to ■). The adsorption of $[xg]_\infty$ to cotton linters cellulose was much lower than that to Avicel. Both cellulose preparations seemed to contain a small amount of hemicellulosic and/or pectic material. Apart from Glc (*c.* 92 mol%), minor amounts of uronic acid (*c.* 4 mol%), Xyl (*c.* 2 mol%), Man and Rha (each *c.* 1 mol%) were found. Apparently, cellulose had some ionizable groups and these might have affected the adsorption of xyloglucan to cellulose. Therefore, the adsorption of $[xg]_\infty$ and pool *VI* were studied as a function of pH. Fig. 4A shows that adsorption of $[xg]_\infty$ decreased from pH 5.8 to pH 4.0. The xyloglucan adsorption at pH 3.0 did not follow this tendency; the adsorption at pH 3.0 was similar to that at pH 5.0. This might be due to different buffer ions; another explanation might be protonation of xyloglucan at low pH values, but this was not further substantiated. Similar effects were observed for the fragments of pool *VI*.

The possible heterogeneity of the $[xg]_4$ population was discussed above. To investigate whether some fragments in this population adsorbed preferentially to cellulose, the

oligosaccharide composition of adsorbed $[xg]_4$ was determined. To this end, $[xg]_4$, which had previously been adsorbed on Avicel, was degraded by endoV after the unbound $[xg]_4$ had been removed by centrifugation. Analysis of the resulting oligosaccharides by HPAEC showed that the adsorbed $[xg]_4$ had a higher portion of the XXXG and XXLG building units compared to a digest of the total population $[xg]_4$ (data not shown). This experiment demonstrated that the adsorbed $[xg]_4$ material was enriched in smaller building units and that subtle differences in molecular structure of $[xg]_4$ influence their adsorption: substitution with Gal probably decreases the ability of fragments to penetrate the smaller pores in cellulose.

DISCUSSION

The present paper discusses several chromatographic techniques aimed at fractionation and analysis of large xyloglucan fragments. HPSEC was used for monitoring the degradation of $[xg]_\infty$ and allowed simple quantification of xyloglucan by the refractive index. Rather pure fragments of up to five repeating building units could be obtained by fractionation of a partially degraded tamarind seed xyloglucan using BioGel P-6 chromatography. HPAEC served merely as an analytical tool to check the purity of BioGel P-6 pools and the oligosaccharide composition of certain fragments. However, from the results presented here, it might be anticipated that this technique could be used successfully for preparative fractionation of fragments (much) larger than $[xg]_5$. This was not further substantiated since a sequence of three or four xyloglucan building units appeared to be of critical length for interaction with cellulose.

The observation that polymeric xyloglucan binds to cellulose whereas its individual building units ($[xg]_1$) do not suggests that the interaction between xyloglucan and cellulose is a reversible process. Adsorption of $[xg]_\infty$ to cellulose can be envisaged as is indicated in Fig. 5 [21]. A number of building units ("trains") will interact with the cellulose surface. The so-called "loops" and "tails" stick out into solution. Our results suggest that binding of $[xg]_\infty$ involves at least five building units; however, it is unknown whether these should occur contiguously. In principle, adsorption of xyloglucan to cellulose is a cooperative effect. For instance, it is thought that $[xg]_\infty$ can bind equally well by a train of six building units as by three trains of two building units.

The first part of the adsorption isotherm represents a situation in which all xyloglucan molecules are bound to the cellulose surface: Γ increases dramatically with increasing $[xg]_\infty$ concentration. The steepness of this part of the isotherm emphasizes the high-affinity character of xyloglucan adsorption on cellulose. Finally, this surface is saturated with xyloglucan, and for homodisperse systems Γ will reach a constant value. However, HPSEC analysis of $[xg]_\infty$ (data not shown) showed a rather broad mol wt distribution, which indicates that we are dealing with a polydisperse system. When the supply of $[xg]_\infty$ exceeds the binding capacity of the cellulose, preferential adsorption of larger molecules

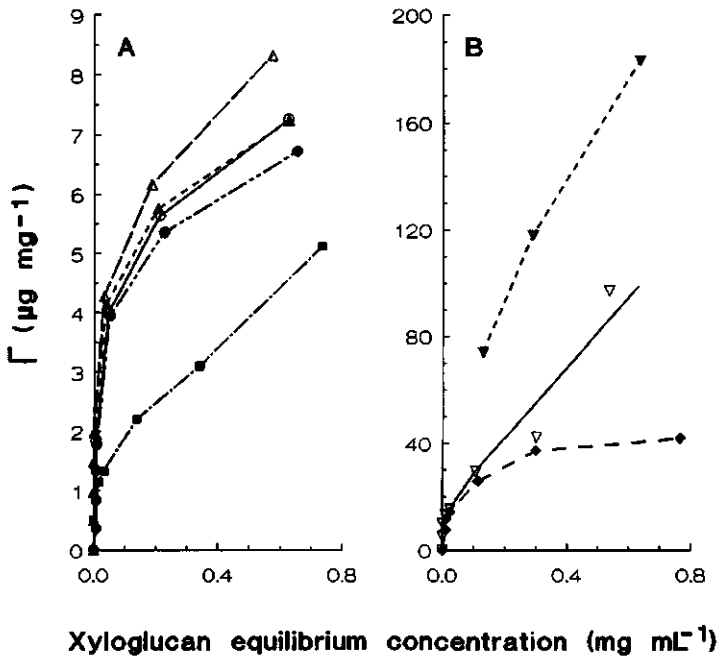

FIGURE 4. Adsorption isotherms of polymeric xyloglucan $[xg]_\infty$ (A) and two BioGel P-6 fractions (B) under various conditions. A: ○, Avicel (50 mg), pH 3.0; ●, Avicel (50 mg), pH 4.0; ▲, Avicel (50 mg), pH 5.0; △, Avicel (50 mg), pH 5.8; ■, cotton linters (50 mg), pH 5.8. B: ▽, pool *IV* ($[xg]_4$), Avicel (5 mg), pH 5.8; ▼, pool *IV* ($[xg]_4$), Avicel (1 mg), pH 5.8; ◆, pool *VI* ($[xg]_{6<n<11}$), Avicel (5 mg), pH 5.8. After 6 h of incubation the samples were centrifuged (1 min, 20,000 *g*), and the supernatants were analyzed for their neutral sugar content, from which the adsorbed amount was calculated.

occurs because their binding is more favorable in terms of entropy compared to smaller molecules. As a result of this, none of the isotherms (Fig. 4A) reach their plateau value instantaneously; rather, the isotherms level off until, finally, the cellulose surface is covered with the largest molecules. Thus, with increasing supply of $[xg]_\infty$, loops and tails will contribute more and more to the adsorbed amount, although the coverage (train density) of the cellulose remains the same.

Adsorption isotherms show that the origin of cellulose influences the amount of xyloglucan that can adsorb. Similar observations were made by Hayashi *et al.* [8]. The lower adsorption of $[xg]_\infty$ to cotton linters cellulose compared to Avicel suggests a larger effective surface area for the latter. This seems to be in accordance with the higher crystallinity index for cotton linters reported by Hoshino *et al.* [22]. Stone and Scallan [23] demonstrated that cellulose is a porous matrix in which small pores contribute the major part of the total surface area of the cellulose. The molecules of $[xg]_\infty$ are too large to reach the smaller pores. The stiffness of the glucan chain in solution, which is partially due to its extensive branching [24], probably reinforces their poor penetration.

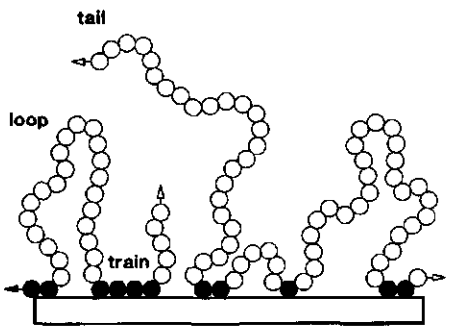

FIGURE 5. Schematic representation of the interaction of polymeric xyloglucan ($[xg]_\infty$) and cellulose. ○, Xyloglucan oligosaccharide ($[xg]_1$) in so-called loops and tails; ●, xyloglucan oligosaccharides that interact with cellulose (trains); □, cellulose.

Apparently, the surface of many of these small pores can be colonized by $[xg]_4$, considering the much higher adsorption (*c.* 10-fold) of these fragments compared to $[xg]_\infty$.

Maclachlan *et al.* [25] showed that an important part of tamarind seed xyloglucan is composed of XXLG→XXLG→XLLG fragments that were found to be reasonably enzyme resistant. Additionally, substitution with Gal at the penultimate position of an oligosaccharide seems to hinder the release of such oligosaccharides by endoV (see Chapter 6). These observations suggest that the degradation of xyloglucan is not completely *random*, but rather that cleavage occurs at linkages adjacent to an XXXG building unit. This explains why $[xg]_1$ contains predominantly XXXG and XXLG and why larger fragments, such as $[xg]_4$, are enriched in Gal-containing oligosaccharides. Theoretically, $[xg]_4$ can adopt 256 different permutations, but this number of possibilities is narrowed down to some extent due to the mode of action of endoV. All $[xg]_4$ structures containing only XXXG and/or XLXG are very improbable; mol wts will range from *c.* 4.8 to 5.5 kDa. These results demonstrate that $[xg]_4$, like $[xg]_\infty$, is a polydisperse population, but on a miniature scale.

The polydispersity of $[xg]_4$ finds expression in its adsorption isotherm, but because these molecules are too small to form loops and tails, a mechanism different from that described for $[xg]_\infty$ must underlie this pattern. The Γ of $[xg]_4$ seems to depend on the amount of Avicel (Fig. 4B), which suggests that not only the dimensions of $[xg]_4$ but also the pore size distribution of cellulose plays an important role here. At low $[xg]_4$ concentrations, the surface of the smallest pores of cellulose is probably only partly covered with xyloglucan due to the limited supply of the smaller molecules (built mainly from XXXG and XXLG) within the $[xg]_4$ population. At higher concentrations more of these smaller molecules are present, and the smaller pores of cellulose can be further colonized. Our observation suggesting that relatively more small molecules are adsorbed is in accordance with this. The adsorption isotherm of pool *VI* was the only one achieving a constant value under the conditions used. This value was much lower than that found for $[xg]_4$, which emphasized that the smaller pores contibute an important part to the total surface area of cellulose.

Fig. 4A further suggests that the pore size can be manipulated to a small extent by changing the pH. Both cellulose preparations contained *c.* 8 mol% of sugar residues other

than Glc. The Xyl and part of the uronic acid probably originate from 4-*O*-methyl-glucuronoxylans, which are known to be closely associated with cellulose just like xyloglucans [26]. Depending on the pH, the carboxyl groups (pKa of *c.* 4.5) of these glucuronoxylans can introduce a negative charge to the cellulose. Repulsion of these ionized groups is probably attended by an enlargement of the cellulose pores. A larger portion of the cellulose surface would be accessible for xyloglucan molecules, which might explain why more $[xg]_\infty$ is adsorbed at increased pH.

We have shown previously that enzymic degradation of cell-wall-embedded cellulose is enhanced by stripping the xyloglucan coating [16]. The present paper demonstrates that $[xg]_5$ adsorbs quantitatively to cellulose. This means that xyloglucans have to be degraded extensively (preferably to $[xg]_2$) before cellulose becomes accessible to a cellulolytic enzyme combination. It should be realized that this study was done with commercially available Avicel cellulose and tamarind seed xyloglucan instead of apple fruit cellulose and xyloglucan. It was shown by solid-state ^{13}C NMR that Avicel and apple cellulose are not alike [27]; further, the presence of Fuc in apple fruit xyloglucan might influence the binding of these molecules to cellulose [13]. Apart from this, we would like to put forward some thoughts on possible implications of these results in cell wall processes. First, the DP of xyloglucans has never been considered an important factor in the assembly of the cellulose-xyloglucan complex. It could very well be that the secretory vesicles (budded off from Golgi) contain a large collection of xyloglucan molecules (ranging, for instance, from $[xg]_4$ to $[xg]_\infty$). Smaller molecules might occupy the space between cellulose-synthesizing terminal complexes [28] more easily than larger ones and serve as a kind of fixture to which larger molecules can be attached by, for instance, transglycosylation [29]. This might explain the much higher xyloglucan levels in native cellulose-xyloglucan complexes than in reconstituted ones [8]. Second, Coimbra *et al.* [30] reported a pectic fraction that was enmeshed in the cellulose microfibrils of olive. Such a pectin (after removal of methyl groups) could influence the pore size of cellulose in a way similar to a glucuronoxylan. Ricard and Noat [31] postulated a role for pectin methyl esterase in locally modulating the cell wall pH for "growth enzymes". Swelling of cellulose might also be triggered by these enzymes. Finally, and probably most importantly, this work provides a method for manufacturing, purifying, and analyzing a homologous series of xyloglucan fragments. These defined fragments and cellulose can be used to build model systems for testing a number of hypotheses that have recently been put forward: facilitated binding of fucosylated fragments [13], prohibited binding of endoglucanase-resistant fragments [15], and disruption of noncovalent interactions by expansins [7].

REFERENCES

[1] McCann, M.C., and Roberts, K. (1994) Changes in cell wall architecture during cell elongation. *J. Exp. Bot.* **45**, 1683-1691.

[2] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[3] McCann, M.C., Wells, B., and Roberts, K. (1990) Direct visualization of cross-links in the primary plant cell wall. *J. Cell Sci.* **96**, 323-334.

[4] Acebes, J.L., Lorences, E.P., Revilla, G., and Zarra, I. (1993) Pine xyloglucan. Occurrence, localization and interaction with cellulose. *Physiol. Plant.* **89**, 417-422.

[5] Hayashi, T., and Maclachlan, G. (1984) Pea xyloglucan and cellulose. I. Macromolecular organization. *Plant Physiol.* **75**, 596-604.

[6] Edelmann, H.G., and Fry, S.C. (1992) Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells. *Carbohydr. Res.* **228**, 423-431.

[7] McQueen-Mason, S., and Cosgrove, D.J. (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc. Natl. Acad. Sci. USA* **91**, 6574-6578.

[8] Hayashi, T., Marsden, M.P.F., and Delmer, D.P. (1987) Pea xyloglucan and cellulose. V. Xyloglucan-cellulose interactions *in vitro* and *in vivo*. *Plant Physiol.* **83**, 384-389.

[9] Baba, K., Sone, Y., Misaki, A., and Hayashi, T. (1994) Localization of xyloglucan in the macromolecular complex composed of xyloglucan and cellulose in pea stems. *Plant Cell Physiol.* **35**, 439-444.

[10] Valent, B.S., and Albersheim, P. (1974) The structure of plant cell walls. V. On the binding of xyloglucan to cellulose fibers. *Plant Physiol.* **54**, 105-108.

[11] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.

[12] Hayashi, T., Takeda, T., Ogawa, K., and Mitsuishi, Y. (1994) Effects of the degree of polymerization on the binding of xyloglucans to cellulose. *Plant Cell Physiol.* **35**, 893-899.

[13] Levy, S., York, W.S., Stuike-Prill, R., Meyer, B., and Staehelin, L.A. (1991) Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *Plant J.* **1**, 195-215.

[14] Reid, J.S.G., Edwards, M., and Dea, I.C.M. (1988) Enzymatic modification of natural seed gums. In *Gums and Stabilizers for the Food Industry* (Phillips, G.O., Wedlock, D.J., and Williams, P.A., eds., IRL Press, Washington), 391-398.

[15] Hisamatsu, M., York, W.S., Darvill, A.G., and Albersheim, P. (1992) Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues. *Carbohydr. Res.* **227**, 45-71.

[16] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and glucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).

[17] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.

[18] Tollier, M.T., and Robin, J.P. (1979) Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Ann. Technol. Agric.* **28**, 1-15.

[19] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.

[20] York, W.S., van Halbeek, H., Darvill, A.G., and Albersheim, P. (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and Fast-Atom-Bombardment Mass Spectrometry. *Carbohydr. Res.* **200**, 9-31.

[21] Fleer, G.J., Cohen Stuart, M.A., Scheutjens, J.M.H.M., Cosgrove, T., and Vincent, B. (1993) *Polymers at Interfaces* (Chapman & Hall, London).

[22] Hoshino, E., Sasaki, Y., Okazaki, M., Nisizawa, K., and Kanda, T. (1993) Mode of action of exo- and endo-type cellulases from *Irpex lacteus* in the hydrolysis of cellulose with different crystallinities. *J. Biochem.* **114**, 230-235.

[23] Stone, J.E., and Scallan, A.M. (1968) A structural model for the cell wall of water-swollen wood pulp fibers based on their accessibility to macromolecules. *Cellulose Chem. Technol.* **2**, 343-358.

[24] Gidley, M.J., Lillford, P.J., Rowlands, D.W., Lang, P., Dentini, M., Crescenzi, V., Edwards, M., Fanutti, C., and Reid, J.S.G. (1991) Structure and solution properties of tamarind-seed polysaccharide. *Carbohydr. Res.* **214**, 299-314.

[25] Maclachlan, G., Levy, B., and Farkas, V. (1992) Acceptor requirements for GDP-fucose:xyloglucan 1,2-α-L-fucosyltransferase activity solubilized from pea epicotyl membranes. *Arch. Biochem. Biophys.* **294**, 200-205.
[26] Sjöström, E. (1989) The origin of charge on cellulosic fibers. *Nordic Pulp Paper Res. J.* **4**, 90-93.
[27] Newman, R.H., Ha, M.-A., and Melton, L.D. (1994) Solid-state ^{13}C NMR investigation of molecular ordering in the cellulose of apple cell walls. *J. Agric. Food Chem.* **42**, 1402-1406.
[28] Delmer, D.P., and Stone, B.A. (1988) Biosynthesis of plant cell walls. In *The Biochemistry of Plants* (Priess, J., ed., Academic Press, New York) **14**, 373-420.
[29] Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828.
[30] Coimbra, M.A., Waldron, K.W., and Selvendran, R.R. (1994) Isolation and characterization of cell wall polymers from olive pulp (*Olea europaea* L.). *Carbohydr. Res.* **252**, 245-262.
[31] Ricard, J., and Noat, G. (1986) Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. 1. A theory of the ionic control of a complex multi-enzyme system. *Eur. J. Biochem.* **155**, 183-190.

CHAPTER 8

Fungal and Plant Xyloglucanases May Act in Concert During Liquefaction of Apples[I]

Efficient enzymic degradation of cellulose in isolated cell wall material of apples requires a preceding removal of its xyloglucan coating. In this study, raw and blanched apple fruit tissues were treated with pectin lyase and various (combinations of purified) cellulases. These experiments confirmed that xyloglucanase activity is important for cellulose degradation in apple fruit tissue. Apart from this, it was observed that raw apple material disintegrated faster than blanched. Typically, xyloglucan oligosaccharides XXXG and XXFG were released in different amounts from raw apple material compared to blanched material. The endogenous enzyme, xyloglucan endotransglycosylase (XET), is probably responsible for these phenomena. It is hypothesized that XET activity accelerates disintegration of apple tissue once its depolymerizing mode is triggered by xyloglucan oligosaccharides released by exogenous endoglucanases.

The complexity of the primary cell wall of dicotyledons was extensively reviewed by several authors [1,2]. In these models, a cellulose-xyloglucan network, which determines the strength of the cell wall, is embedded in a matrix of pectic polysaccharides, which are thought to be responsible for cell wall porosity. Immunogold localization revealed that xyloglucans were almost exclusively located around the microfibrils [3]. Hardly any labelling was found in the middle lamella region. By coating cellulose extensively, xyloglucans prevent association of cellulose microfibrils into larger bundles [4-6]. It has become a well established fact that xyloglucans are sufficiently long to tether different microfibrils [4,5,7-9]. Breakage of these cross links may destroy the coherence of the cell wall.

Endogenous enzymes play an important role in the development of cell walls in fruit tissues. In some cases their presence can even be exploited in processing of fruits. It appears that various enzymes are induced at different times during maturation. In apple fruit, the occurrence of ß-galactosidase [10-12], α-arabinofuranosidase [12], endo-PG[II] [13], exo-PG [14], two forms of PME [15], and a cellulase with activity towards CMC [16] have been demonstrated. These enzymes are involved in the modification of cell wall polysaccharides, and the mode of action towards their corresponding substrates is rather well documented; however, their mutual relationship with respect to developmental processes is still poorly understood.

A potential enzyme application in apple juice manufacturing is the liquefaction process which aims at a complete degradation of cell walls in apple pulp [17]. As a result of this,

---

[I]Vincken, J.-P., van den Broek, L.A.M., van der Lei, D.D., Beldman, G., and Voragen, A.G.J. Submitted for publication to *J. Sci. Food Agric.*.

[II]*Abbreviations:* see page viii.

cells will collapse which enables recovery of apple juice without an energetically unfavorable pressing operation. For this process commercial enzyme preparations can be applied which contain a broad spectrum of enzyme activities. Currently, much effort is directed towards identifying all essential enzymes in these preparations in order to design more specific, tailor-made mixtures for the liquefaction of apple pulp. Enhancement of the enzymic degradation of cell wall embedded cellulose by a xyloglucanase purified from *Trichoderma viride* [18], suggests that xyloglucanase is such a key activity. Also, the same enzyme acted synergistically with pectic enzymes like PL [19]. Both observations were done using cell wall material isolated from apple fruit. Although this material is presumably intact in chemical terms, it is inevitable that (part of) the cell wall architecture has lost its authenticity during isolation. This, together with the presence of endogenous enzymes, suggests that extrapolation of the degradation of "model substrates" to the liquefaction of apple fruit tissue should be done with caution. The objective of this study is to investigate the degradation of xyloglucan in the complex matrix of apple fruit tissue.

EXPERIMENTAL

Materials

Raw materials and substrates.—Apples (*Malus malus* L, Rosaceae, var. Golden Delicious) were obtained locally. After peeling and removing the core, the apples were kept on ice (*c.* 20 min) until shredded in a food processor (Braun MX32, Frankfurt, Germany, 5 mm blade). The shredded apples were either blanched (20 min, 70 °C) to inactivate endogenous enzymes or stored (*c.* 30 min at 4 °C) in 200 mM sodium succinate buffer (pH 4.0) containing 0.1% (w/v) ascorbic acid to prevent enzymic browning. Liquid to apple ratio was 2:1 in both cases.

Avicel crystalline cellulose (type SF) was purchased from Serva (Heidelberg, Germany). CMC (Akucell AF type 0305) was obtained from Akzo (Arnhem, The Netherlands). Tamarind seed xyloglucan was kindly provided by Dainippon Pharmaceutical (Osaka, Japan). G_2 was obtained from Merck (Darmstadt, Germany). MHR and saponified MHR from apple were obtained from Schols *et al.* [20]. Polygalacturonic acid and pectin (degree of methylation 65%) were purchased respectively from ICN (Cleveland, OH, USA) and Obipektin (Bischofszell, Switzerland). Potato arabinogalactan was prepared by van de Vis *et al.* [21]. Larchwood arabinogalactan (stractan) and branched sugar beet arabinan were obtained respectively from St. Regis Paper Company (Tacoma, WA, USA) and Koch and Light (Colnbrook, Bucks, UK). Cell wall material (WUS) from apple was prepared as described earlier [18].

Enzymes.—Three endoglucanases (endoI, endoIV, and endoV; EC 3.2.1.4) and CBH (previously referred to as exoIII; EC 3.2.1.91) were purified to homogeneity from a commercial preparation derived from *Trichoderma viride* (Maxazyme CI, Gist-Brocades, Delft, The Netherlands) as described by Beldman *et al.* [22]. The characteristics of these enzymes are summarized in Table I. A preparation derived from *Disporotrichum dimorphosporum* (Xyl-5000) was also obtained from Gist-Brocades. PL (EC 4.2.2.10) was purified to apparent homogeneity from a commercial preparation derived from *Aspergillus niger* (Ultrazym 100, Ciba-Geigy, now available from Novo Ferment AG, Basel, Switzerland) by van Houdenhoven [23].

Preparation of xyloglucan fragments

Xyloglucan oligosaccharides were obtained by treating 50 mg of tamarind seed xyloglucan with a crude *Trichoderma* cellulase preparation (100 μg of protein) in 5 mL of a 50 mM NaOAc buffer

TABLE I. Summary of the properties of some glucanases of *Trichoderma viride*. −, no detectable activity; ±, very low activity; +, low activity; ++, high activity.

Enzyme	CMCase activity	Xyloglucanase activity	Activity towards Avicel†	Adsorption on Avicel‡
endoI	++*	±*	+	yes
endoIV	++*	++*	±	no
endoV	++*	++*	+	yes
CBH	−	−	+	yes

* Vincken *et al.* [27]. † Beldman *et al.* [22]. ‡ Beldman *et al.* [30].

(pH 5.0; 40 °C; 24 h). The sample was heated for 30 min at 100 °C. The digest was concentrated under reduced pressure until dry, dissolved in 2 mL of distilled water. The crude oligosaccharide mixture was deproteinated by BioGel P-2 [24]. Hepta to decamer fractions were combined, and further referred to as $[xg]_1$. Xyloglucan fragments of four repeating building units ($[xg]_4$) were obtained by partial degradation of tamarind seed xyloglucan as described earlier [25].

Extraction of enzymes from apple fruit tissue

Approximately 20 g of shredded apple material and 1.5 g of PVPP were homogenized for 1 min using an Ultra-Turrax T25 (8,000 rotations min^{-1}, Janke & Kunkel, Staufen, Germany) in 60 mL of a 0.25 M sodium phosphate buffer (pH 6.2; 4 °C) containing 0.4 M NaCl and 10 mM DTT. The homogenate was centrifuged (10 min, 2,000 *g*) and the supernatant dialyzed extensively against 25 mM NaOAc (pH 5.8). Finally, the enzyme extract was concentrated ten times by ultrafiltration using a Diaflo PM10 membrane (Amicon, Lexington, MA, USA).

Enzymic degradation of various substrates

Enzymic degradation of apple fruit tissue.—Apple fruit tissue (2 g; blanched or raw; pieces of 10 x 3 x 2 mm) were incubated in 3 mL 200 mM sodium succinate buffer (pH 4.0) containing 0.1% (w/v) ascorbic acid and an appropriate amount of enzyme, at 40 °C for 16 h (unless stated otherwise) under continuous shaking (150 rpm min^{-1}). In a first series, the apple material was degraded with the commercial enzyme preparations Maxazyme and Xyl-5000. Enzyme dosage was such that the Avicelase activity was 10 mU for all incubations, while the concomitant xyloglucanase activities were 200 and 100 mU for Maxazyme and Xyl-5000, respectively. When indicated, the xyloglucanase level of the Xyl-5000 incubations was increased by adding 14 μg (protein) of endoV (100 mU). A second series was performed with mixtures of purified glucanases. By combining endoI and CBH at a mass ratio of 3.5:1, a "low xyloglucanase" mixture was obtained. A "high xyloglucanase" mixture was made using endoV instead of endoI. By combining these two mixtures in an appropriate way, five more mixtures with intermediate xyloglucanase activity were prepared. Enzyme dosage was such that each incubation contained an equal amount of G_2 releasing activity (0.26 mU), but a varying amount of xyloglucanase activity (5 to 40 mU). A similar experiment was performed with a doubled amount of the mixtures and a longer incubation period (40 h). PL (50 mU) was added to certain incubations (as indicated in Results) to degrade pectic material. After visual evaluation of tissue disintegration, 1 mL was removed from the incubation mixture, heated for 10 min at 100 °C to inactivate the enzymes, centrifuged (2 min, 20,000 *g*), and the degradation products in the supernatant were analyzed by HPAEC.

Degradation of xyloglucan in WUS by enzyme extracts from apple fruit.—Apple WUS (20 mg) was suspended in 1.5 mL of 100 mM NaOAc buffer (pH 5.8) containing 10 mM $CaCl_2$ [26], 0.01% (w/v) NaN_3, 1.2 μg endoV (9 mU xyloglucanase), and varying amounts of the enzyme

extracts. Samples were incubated for 10 h at 40 °C using head-over-tail mixing, heated for 10 min at 100 °C to inactivate the enzymes, and centrifuged (1 min, 20,000 *g*). Supernatants were analyzed by HPAEC for xyloglucan degradation products.

Degradation of xyloglucan fragments by enzyme extracts from apple fruit.—Immobilized $[xg]_4$ (250 μg, suspension) as well as free (unadsorbed) $[xg]_4$ (250 μg) or polymeric xyloglucan (250 μg) were incubated separately in 1 mL of 25 mM NaOAc (pH 5.8) containing 10 mM $CaCl_2$, 0.01% (w/v) NaN_3 and 100 μL of a freshly prepared enzyme extract. In some cases (as indicated in Results), the incubation mixtures contained an additional 250 μg of $[xg]_1$. After 2 h or 24 h (40 °C, head-over-tail mixing) the samples were centrifuged (1 min, 20,000 *g*), and the supernatants heated for 10 min at 100 °C to inactivate the enzymes. Xyloglucan degradation products were then analyzed by HPSEC and HPAEC. The immobilized $[xg]_4$ was prepared as follows: purified $[xg]_4$ (250 μg) was adsorbed on Avicel crystalline cellulose (25 mg), according to Vincken *et al.* [25] in 1 mL of 25 mM NaOAc (pH 5.8) containing 10 mM $CaCl_2$ and 0.01% (w/v) NaN_3; after 6 h of incubation (40 °C, head-over-tail mixing) all $[xg]_4$ was adsorbed; the suspensions were centrifuged (1 min, 20,000 *g*), and the supernatants were removed.

Analytical methods

Analysis of cellulose and xyloglucan degradation products.—Cellulose and xyloglucan degradation products were quantified by HPAEC using a Dionex Bio-LC GPM-II quaternary gradient module equipped with a Dionex CarboPac PA-100 column (250 x 4 mm, 20 °C, Dionex, Sunnyvale, CA, USA). Samples (20 μL) were injected using a SP8780 autosampler (Spectra Physics, San José, CA, USA) equipped with a Tefzel rotor seal in a 7010 Rheodyne injector valve. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The eluate (1 mL min^{-1}) was monitored using a Dionex PED detector in the PAD mode. A reference Ag/AgCl electrode was used with a working gold electrode with the following pulse potentials and durations: E_1, 0.1 V and 0.5 s; E_2, 0.6 V and 0.1 s; E_3, -0.6 V and 0.1 s.

G_2 release after treatment of apple fruit tissue was quantified by application of the following gradient: 0→12 min, linear gradient of 25→85 mM NaOH, 12→25 min, linear gradient of 0→100 mM NaOAc in 85 mM NaOH. After each analysis the column was rinsed for 5 min with 1 M NaOAc in 100 mM NaOH, and equilibrated in 25 mM NaOH for 15 min. Degradation products of Avicel by mixtures of purified glucanases were determined according to Vincken *et al.* [27]. Xyloglucan oligosaccharides and larger fragments were analyzed as described before [24,25].

HPSEC was performed as described previously [18] with a different series of BioGel TSK columns (60XL, 40XL, 30XL).

Determination of enzyme activities.—Activity towards Avicel crystalline cellulose, as well as xyloglucanase and CMCase activity were determined by measuring the increase of reducing end groups [28]. Glc was used as a standard. Cellobiase activity was quantified by analyzing the decrease of the G_2 concentration by HPAEC as described by Vincken *et al.* [27]. One milliunit is defined as the amount of enzyme that creates one nmol of reducing end groups per minute (pH 4; 40 °C). Activity towards polygalacturonic acid, pectin, saponified MHR, branched arabinan, potato arabinogalactan and stractan were determined qualitatively by comparing the mol wt distribution of the enzyme-treated and the untreated sample using HPSEC. Activities were determined in 50 mM sodium succinate buffer (pH 4.0; glucanase) or 50 mM NaOAc buffer (pH 5.0; others) after 24 h of incubation at 40 °C. The protein content of enzyme preparations was determined according to Sedmak and Grossberg [29]. BSA was used as a standard.

RESULTS

Liquefaction of apple fruit tissue by various cellulase preparations

The activity spectrum of Maxazyme and Xyl-5000 was determined with focus on different glucanases (Table II). Although CMCase activity was in the same order of magnitude for both preparations, Maxazyme had the highest activity towards both Avicel crystalline cellulose and xyloglucan. When compared to Xyl-5000, Maxazyme contains only minor amounts of pectinase, arabinanase and other side activities which are important for the degradation of apple cell wall polysaccharides.

TABLE II. An overview of enzyme activities in two commercial preparations. Glucanase activities were determined quantitatively (mU mg^{-1}). Other activities were determined qualitatively by HPSEC analysis. −, no detectable activity; +, low activity; ++, high activity.

Activity towards	Maxazyme	Xyl-5000
CMC	14300	15700
Xyloglucan	9900	600
Avicel	502	60
G_2	500	100
Polygalacturonic acid	−	++
Pectin (65% esterified)	+	++
Saponified MHR	−	++
Sugar beet arabinan	+	++
Potato arabinogalactan	−	+
Stractan	−	−

The performance of Maxazyme and Xyl-5000 in liquefaction trials was evaluated visually, as well as by determining the extent of cell wall degradation. The conversion of cellulose, which is deeply buried in the cell wall, to cellodextrins is considered to be indicative for this degradation. Beldman *et al.* [30] showed that degradation of Avicel crystalline cellulose by the different endoglucanases of *Trichoderma viride* gave different ratios of Glc, G_2, and G_3 as reaction products. G_2 is generally the major product, *esp.* when the endoglucanases are used in combination with CBH (data not shown). When apple WUS instead of Avicel crystalline cellulose was treated with a combination of endoglucanase(s) and CBH, only G_2 (no Glc and G_3) was released as a cellulose degradation product [18]. Treatment of apple tissue with mixtures of purified glucanases gave results which were comparable to those obtained with WUS (data not shown). No G_3 was detected in any of the reaction mixtures. Glc could not be determined because it naturally occurs in apple tissue in large quantities. In the liquefaction experiments described below, the release of G_2 was used as a measure for the degradation of cell wall

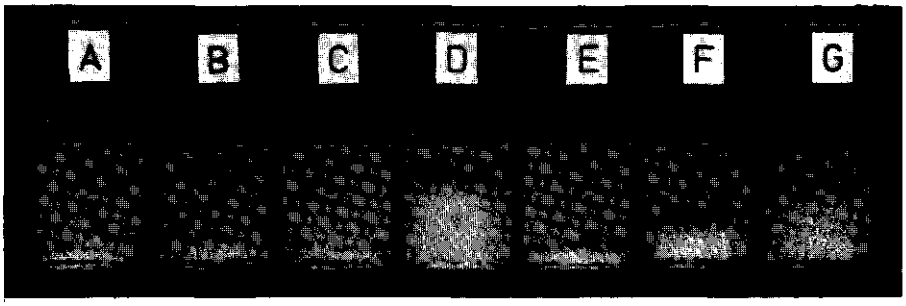

FIGURE 1. Liquefaction of raw apple fruit tissue by PL and (upgraded) commercial enzyme preparations. Raw apple fruit tissue was incubated in 200 mM sodium succinate buffer pH 4.0 for 16 h at 40 °C, and 150 rpm min^{-1}. **A**, no enzyme added; **B**, Maxazyme (10 mU of Avicelase activity, 200 mU xyloglucanase); **C**, 50 mU PL; **D**, combination of "B" and "C"; **E**, Xyl-5000 (10 mU of Avicelase activity, 100 mU xyloglucanase); **F**, combination of "C" and "E"; **G**, as "F" plus 100 mU endoV.

embedded cellulose.

Maxazyme alone is unable to liquefy raw apple fruit tissue to a large extent within 16 h (evaluated visually; Fig. 1). PL is known to effectively solubilize pectins from cell walls [19]. Supplementation with a minimum amount of 50 mU PL was required to enable Maxazyme to completely liquefy the material under the same conditions. Therefore, PL was added in this concentration to all incubations to ensure that the cellulose-xyloglucan network was accessible for the glucanases. No complete liquefaction could be achieved by a combination of PL and Xyl-5000 although the Avicelase activity was similar as in experiments with Maxazyme. However, the xyloglucanase activity was a factor 2 lower, and this might indicate that xyloglucanase activity is an important factor in liquefaction. Therefore, the xyloglucanase activity of the Xyl-5000 preparation was increased to the level of Maxazyme by addition of an appropriate amount of endoV. Fig. 1 shows that the extent of disintegration in this case is comparable to the one obtained by a combination of PL plus Maxazyme. The G_2 release upon addition of endoV increased 1.6 times but was still *c.* 5 times lower when compared to an incubation with PL plus Maxazyme. Addition of a similar amount of endoI protein instead of endoV had no effect on the G_2 release as well as the extent of disintegration (data not shown). The experiments described above were repeated with apple fruit of different maturity. The extent of disintegration, as determined visually, seemed to depend on the physiological state of the apple fruit, but the G_2 release was similar for all stages of maturity (data not shown). From this it can be concluded that the G_2 release and the disintegration of apple tissue do not necessarily correlate.

The G_2 release from blanched apple tissue was studied with mixtures of purified glucanases. By combining endoI and/or endoV with CBH, it was possible to treat the blanched material with enzyme cocktails containing equal amounts of G_2 releasing activity, but having a different xyloglucanase activity. PL (50 mU) was included in all mixtures for reasons mentioned above. Fig. 2 shows that more G_2 is released with increasing

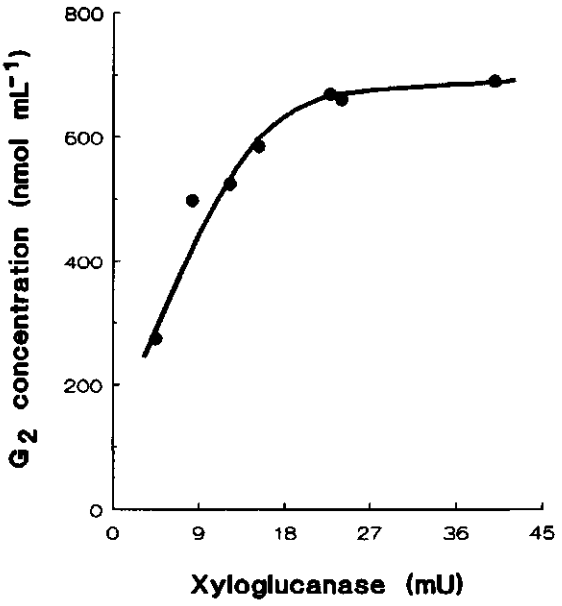

FIGURE 2. Release of G_2 from apple fruit tissue by mixtures of purified glucanases. Blanched apple fruit tissue was incubated (200 mM sodium succinate buffer pH 4.0, 16 h, 40 °C, 150 rpm min^{-1}) with PL in combination with various glucanase mixtures which were equal in Avicelase activity (0.26 mU) but different in their xyloglucanase activity (5 to 40 mU). G_2 was quantified by HPAEC.

xyloglucanase activity, until the xyloglucanase activity reached a level of *c.* 25 mU. Apparently, "naked" surface area is the limiting factor in cellulose degradation. The xyloglucanase activity removes the xyloglucan coating from cellulose, thereby improving the accessibility of the cellulose surface for *esp.* CBH. At xyloglucanase levels larger than *c.* 25 mU, the G_2 release is probably limited by the amount of CBH. The extent of disintegration of the apple fruit tissue in these experiments was comparable to sample "C" in Fig. 1, in which the apple material seems hardly degraded. The experiments were repeated with a doubled dose of glucanases as well as with an increased incubation time (40 h). Incubations with low xyloglucanase activity still showed some pieces of tissue, whereas the apple material in incubations with high xyloglucanase activity was completely liquefied (data not shown).

An interesting observation was that raw apple material disintegrated to a larger extent with mixtures of purified glucanases than their blanched analogues from the same apple batch. In some cases the liquefied raw apple material had an appearance comparable to sample "F" or "G" in Fig. 1; however, the G_2 release was similar to that from the blanched samples. Apparently, the extent of disintegration of raw tissue (as determined visually) does not always correlate to G_2 release. Substrate modification as a result of blanching [31] might be an explanation for this phenomenon; however, a role for endogenous enzymes in the enhanced tissue disintegration seems also possible.

Incubations with an enzyme extract from apple fruit

The enzyme extract from apple fruit was assayed for enzymes with the ability to degrade xylogucan. A 2 h treatment of tamarind seed xyloglucan with an enzyme extract

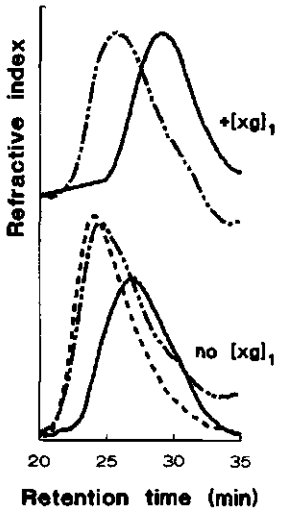

FIGURE 3. HPSEC elution patterns (BioGel TSK 60XL/40XL/30XL series) of polymeric xyloglucan before and after treatment with an enzyme extract derived from apple fruit tissue. The xyloglucan was degraded in presence and absence of $[xg]_1$. Broken lines, -..- lines, and solid lines correspond to patterns obtained after 0, 2, and 24 h of incubation, respectively.

from apple had almost no effect on the mol wt distribution of this polysaccharide. Prolonged incubation (24 h) showed that the mol wt distribution shifted to a lower value (Fig. 3). These data suggest that small amounts of a xyloglucanase activity are present in the extract. The shift in mol wt was more profound when $[xg]_1$ was added to the reaction mixture. Apparently, the presence of $[xg]_1$ triggered the depolymerization of xyloglucan (Fig. 3). Similar experiments were done with $[xg]_4$ instead of polymeric xyloglucan (data not shown). HPSEC analysis showed small but reproducible differences in mol wt distribution of $[xg]_4$ upon treatment with the enzyme extract when compared to a blank. From this it was concluded that the enzyme extract had a much higher impact on the mol wt distribution of polymeric xyloglucan than on that of xyloglucan fragments.

The effect of the enzyme extract on $[xg]_4$ is better visualized by HPAEC as is shown in Fig. 4. Small (but reproducible) amounts of larger products than the original material were formed ($[xg]_6$ and $[xg]_7$), which demonstrated that the enzyme extract contained transglycosylase activity. Typically, minor amounts of $[xg]_1$ and $[xg]_5$ were found. Upon incubation of $[xg]_4$ with the enzyme extract, in the presence of a similar amount of $[xg]_1$, small amounts of $[xg]_4$ reacted. This was attended with a small increase of the amount of $[xg]_2$ and $[xg]_3$, and much smaller quantities of the fragments $[xg]_6$ and $[xg]_7$ as above (data not shown). In this case, $[xg]_1$ competes as an acceptor substrate with (donor substrate) $[xg]_4$.

The ability of the enzyme extract to create "naked" cellulose microfibrils was also investigated. Hereto, Avicel crystalline cellulose was first coated with $[xg]_4$, which is the smallest xyloglucan fragment that binds quantitatively to cellulose [25]. The enzyme extract was unable to remove large amounts of $[xg]_4$ from Avicel, only minor amounts of $[xg]_2$ were solubilized (data not shown). This release was not enhanced by addition of $[xg]_1$.

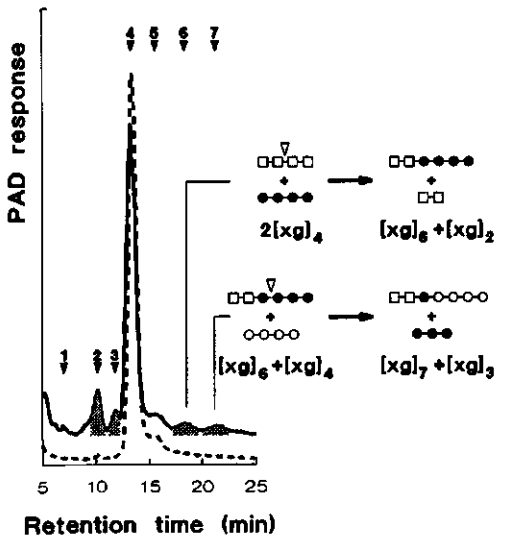

FIGURE 4. HPAEC elution patterns of $[xg]_4$ before and after treatment with an enzyme extract derived from apple fruit tissue in the absence of $[xg]_1$. Number of repeating building units is indicated at the top. □, ●, ○ represent $[xg]_1$ fragments of the donor substrate, first and second acceptor substrate, respectively. Shaded areas indicate the products formed upon transglycosylation. Broken lines, and solid lines correspond to patterns obtained after 0, and 24 h of incubation, respectively.

Xyloglucan degradation in blanched and raw apple tissue

After degradation of apple tissue by PL and mixtures of purified glucanases, the xyloglucan degradation products were analyzed by HPAEC. All liquefaction experiments with blanched apple material yielded similar products as when an alkali-extracted apple xyloglucan was degraded by endoV. This pattern is shown in Fig. 5A. The HPAEC profiles obtained after degradation of blanched apple tissue differed from Fig. 5A in that XXFG and XLFG (for nomenclature see [32]) were often present in smaller amounts. This is probably due to the fact that not all apple material was degraded, and the slow release of these fucosylated oligosaccharides by endoglucanase [24].

The degradation of raw apple fruit tissue with a similar enzyme mixture as the one used for liquefaction of blanched material showed different elution patterns for every other apple batch; however, these incubations demonstrated a consistency in having a smaller XXXG (peak no. 4) to XXFG (peak no. 5) ratio (Fig. 5B and 5C). XLFG was present in only minor quantities. The appearance of relatively large amounts of smaller xyloglucan oligosaccharides (Fig. 5B) might indicate the presence of endogenous glycosidases in this apple batch, which have modified the xyloglucan oligosaccharides either during maturation (and storage) of the apple or during the liquefaction experiment. The nature of these glycosidases was not further investigated. The XXXG to XXFG ratio in Fig. 5C is relatively low compared to that in Fig. 5B. In addition, the smaller xyloglucan oligosaccharides are less abundant than in Fig. 5B, which suggests that an endogenous enzyme other than glycosidase(s) is responsible for the pattern of Fig. 5C.

To verify the effect of endogenous enzymes on the XXXG:XXFG ratio, apple WUS was treated with endoV (to generate xyloglucan oligosaccharides) and various amounts of the enzyme extract. HPAEC-analysis showed that the xyloglucan oligosaccharide

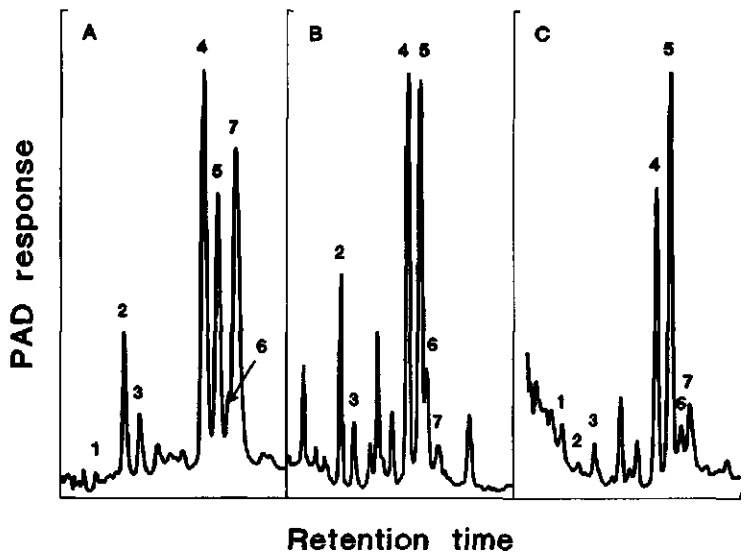

FIGURE 5. Representative HPAEC elution patterns of xyloglucan oligosaccharides obtained after degradation of an alkali-extracted apple xyloglucan (A) and of different batches of raw apple fruit tissue (B and C) with the same mixture of purified glucanases (Avicelase activity 0.26 mU; xyloglucanase activity 40 mU). Apple fruit tissue was incubated in 200 mM sodium succinate buffer pH 4.0 for 16 h at 40 °C, and 150 rpm min^{-1}. 1, FG; 2, GFG; 3, XXG; 4, XXXG; 5, XXFG; 6, XLXG; 7, XLFG.

composition altered with increasing amounts of the extract: XXFG becomes relatively more abundant than XXXG. This is illustrated in Table III. In theory the decreasing amount of XXXG might be explained by the concerted action of a ß-glucosidase and an α-xylosidase in the enzyme extract. However, this does not seem very likely, because no increased amounts of oligosaccharides with a retention time smaller than XXXG were observed. Another possibility is that the XXXG:XXFG ratio decreases as a result of the action of a ß-galactosidase, catalyzing the conversion of XLFG to XXFG. The amount of XXFG remained more or less the same for the various amounts of the enzyme extract used, which shows that such a conversion does not take place. HPAEC analysis showed that no oligosaccharides were present in the enzyme extract.

DISCUSSION

In absence of pectinase no appreciable liquefaction of apple tissue was observed. Apparently, a pectinase is necessary to increase the accessibility of the cellulose-xyloglucan network for the glucanases. Contrary to cell wall material (WUS) isolated from apples [18], intact apple tissue still contains all water-soluble pectins, and therefore

TABLE III. The influence of addition of various amounts of endogenous enzymes from apple on the release of xyloglucan oligosaccharides from apple WUS by endoV (1.2 μg) expressed as the ratio of peak areas of XXXG:XXFG.

Enzyme combination	XXXG:XXFG
endoV	3.0
endoV plus 20 μL extract	2.6
endoV plus 50 μL extract	2.2
endoV plus 100 μL extract	1.9
endoV plus 200 μL extract	1.3

addition of a pectinase, in this case PL, is relatively more important in tissue degradation. This is in accordance with Baron-Epel *et al.* [33] who demonstrated that pectins play an important role in controlling the porosity in soybean cells. A mild pectinase treatment enlarged the channels in these cell walls.

We have shown previously [18] that effective degradation of cell wall embedded cellulose involves the concerted action of three enzymes: endoI, endoIV and CBH. The xyloglucanase activity (endoIV) enhances the accessibility of cellulose for true cellulolytic enzymes (endoI and CBH). From Table I it can be concluded that a two-enzyme system of endoV plus CBH might be equally effective since endoV combines the positive aspects of endoI and endoIV. Fig. 2 clearly demonstrates that a minimum level of xyloglucanase activity is needed to degrade cell wall embedded cellulose in apple fruit tissue effectively. This confirms that previous observations with isolated apple cell walls [18] are also applicable to tissue. However, the degradation of tissue is not as straight forward as it seems. If the liquefaction of raw apple material and blanched tissue is compared, the more extensive disintegration of the former suggests that endogenous enzymes act cooperatively with the exogenous glucanases.

Recently, a new type of wall-loosening enzyme, XET, has been discovered in various plant tissues [26,34-36]. By breaking and reconnecting load-bearing bonds, XET enables cell wall flexibility without loss of strength, a prerequisite to explain cell expansion [8]. In the presence of suitable xyloglucan oligosaccharides, however, XET actually catalyzes the depolymerization of xyloglucan. This PS→OS endotransglycosylation is thought to play a key role in fruit ripening [37-39]. The enzyme did not show any activity towards other ß-(1→4)-glucans like cellulose and CMC [26]. The reduction in mol wt of tamarind seed xyloglucan upon treatment with the enzyme extract from apple was much larger in the presence of $[xg]_1$ than in its absence (Fig. 3). This demonstrates that XET is also present in apple.

The absence of $[xg]_1$ and $[xg]_5$ (Fig. 4) might hold some clues for the mode of action of XET. Most likely, $[xg]_4$ is cleaved in the middle giving the first generation products $[xg]_2$ and $[xg]_6$. Nishitani and Tominaga [35] showed that XET activity decreases with decreasing mol wt of the donor substrate. Therefore, $[xg]_6$ will be a better substrate for

XET than $[xg]_4$, although it is present in only small amounts. A mid-chain cleavage of $[xg]_6$ gives a second generation of products $[xg]_3$ and $[xg]_7$ ($[xg]_4$ is the most abundant acceptor substrate). This reaction scheme is summarized in Fig. 4.

It has been shown previously that XXXG is the most abundant oligosaccharide as building unit of apple xyloglucan [24]. Further, XXXG accumulates much faster than, for instance, XXFG upon degradation of apple xyloglucan by different endoglucanases [27]. This means that XXXG should be more abundant than XXFG at any stage during the liquefaction of apples. Fig. 5C, and to a lesser extent 5B, show that this is not the case. Typically, XXXG is a better acceptor substrate for XET than XXFG [26], and is therefore continuously used for transfer reactions. The oligosaccharide fingerprints suggest that the XET level depends on the maturity of the apple fruit. In agreement with our results, Redgwell and Fry [37] have demonstrated that the level of XET activity in kiwi is ripening-related.

The above indicates that XET plays an active role during the liquefaction of apple fruit tissue. The varying level of XET might (at least partly) explain why apple material from different batches, treated with a similar glucanase mixture, vary so much in their extent of disintegration. The interplay of exogenous and endogenous enzymes in the degradation of cellulose-xyloglucan complex during the liquefaction process is hypothesized in Fig. 6, and will be discussed below. Although removal of pectin is important to improve the accessibility of the complex, its degradation by PL is omitted from the schemes.

Stage 1.—In absence of xyloglucan oligosaccharides, XET catalyzes a PS→PS endotransglycosylation, which can result in a rearrangement of xyloglucan molecules.

Stage 2.—Addition of endoV and CBH (and PL) starts the liquefaction process. The mol wt of the enzymes probably restricts them from penetrating the tissue. Xyloglucan is degraded by endoV at the outer surface of the tissue. Efficient degradation of cellulose by CBH is delayed because further removal of the xyloglucan coating by endoV is required [18]. XET continues as above.

Stage 3.—Oligosaccharides are much smaller than the enzymes and it is expected that they can diffuse into the tissue. Their concentration will become higher than that of polymeric xyloglucan. As a result of this, oligosaccharides will become the most important acceptor substrate of XET. The depolymerizing mode of XET is triggered; the PS→PS endotransglycosylation slowly shifts towards a PS→OS type of endotransglycosylation. EndoV continues to generate xyloglucan oligosaccharides and "naked" cellulose. CBH starts releasing G_2.

Stage 4.—As a result of the PS→OS endotransglycosylation, the cross links between cellulose microfibrils are cut, and the tissue starts loosing its coherence. EndoV and CBH continue as above.

Stage 5.—The loss of coherence leads to a complete disintegration of apple tissue. EndoV and CBH do not suffer from diffusion limitation anymore. EndoV will convert all coated cellulose to "naked" cellulose (endoV prefers xyloglucan over cellulose as a substrate; see Table I). With decreasing length of donor substrates XET activity declines and becomes negligible for $[xg]_8$ [35]. Xyloglucan fragments larger than $[xg]_4$ bind

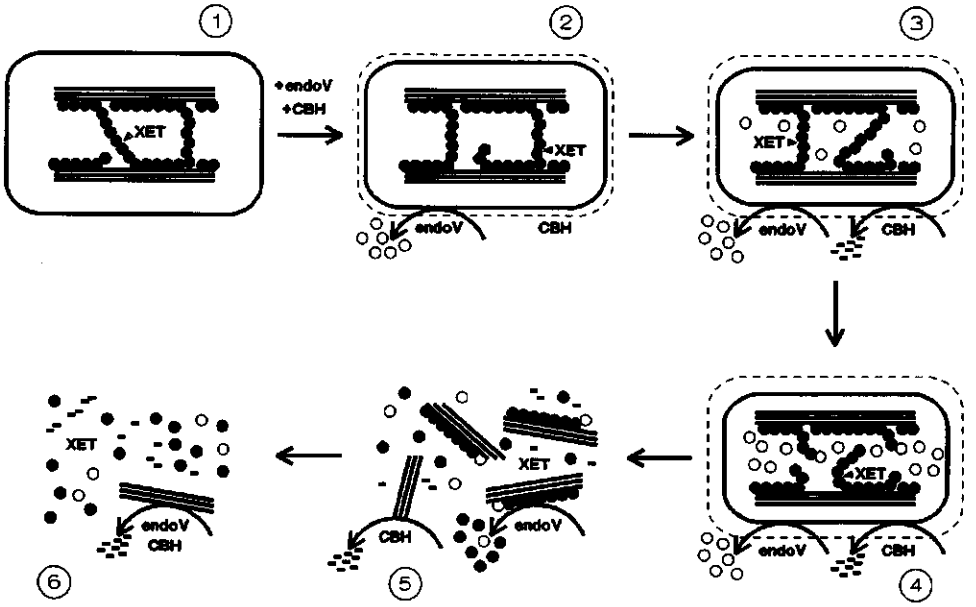

FIGURE 6. Schematic representation to explain the synergism between fungal cellulases (endoV and CBH) and XET during degradation apple fruit tissue. All incubations contained PL but this enzyme was not included in the schemes. The six stages are explained in the text. Solid rectangle, the border of a piece of apple; dashed rectangle, the original dimension of the apple piece; three parallel solid lines, a cellulose microfibril; ▬, G_2; ● and ○, xyloglucan oligosaccharides ($[xg]_1$).

quantitatively to cellulose [25]. Therefore, XET will not play a major role in removing the xyloglucan coating from cellulose.

Stage 6.—The remaining "naked" cellulose is degraded by the concerted action of endoV and CBH in the final stage of the liquefaction process.

Our results could be of importance to fruit juice manufactures, who observe that maturity and storage conditions influence liquefaction. The presence of XET *in situ* might be an important factor to explain why some apples are easier to liquefy than others. Apples having a high XET level will be degraded from both the inside and the outside. Apple is a climacteric fruit which suggests that, in analogy to kiwi [37], XET activity in pre-climacteric fruit may be induced by ethylene treatment. Future research is aimed at obtaining apples with different XET-levels in order to substantiate the role of XET during liquefaction further.

REFERENCES

[1] McCann, M.C., and Roberts, K. (1991) Architecture of the primary cell wall. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed., Academic Press, New York), 109-129.

[2] Carpita, N.C., and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**, 1-30.
[3] Moore, P.J., and Staehelin, L.A. (1988) Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta* **174**, 433-445.
[4] Hayashi, T., and Maclachlan, G. (1984) Pea xyloglucan and cellulose. I. Macromolecular organization. *Plant Physiol.* **75**, 596-604.
[5] McCann, M.C., Wells, B., and Roberts, K. (1990) Direct visualization of cross-links in the primary plant cell wall. *J. Cell Sci.* **96**, 323-334.
[6] Acebes, J.L., Lorences, E.P., Revilla, G., and Zarra, I. (1993) Pine xyloglucan. Occurrence, localization and interaction with cellulose. *Physiol. Plant.* **89**, 417-422.
[7] McCann, M.C., Wells, B., and Roberts, K. (1992) Complexity in the spatial localization and length distribution of plant cell-wall matrix polysaccharides. *J. Microscopy* **166**, 123-136.
[8] Fry, S.C., Smith, R.C., Hetherington, P.R., and Potter, I. (1992) Endotransglycosylation of xyloglucan: a role in cell wall yielding? *Cur. Top. Plant Biochem. Physiol.* **11**, 42-62.
[9] Baba, K., Sone, Y., Misaki, A., and Hayashi, T. (1994) Localization of xyloglucan in the macromolecular complex composed of xyloglucan and cellulose in pea stems. *Plant Cell Physiol.* **35**, 439-444.
[10] Bartley, I.M. (1974) ß-Galactosidase activity in ripening apples. *Phytochem.* **13**, 2107-2111.
[11] Dick, A.J., Opoku-Gyamfua, A., and DeMarco, A.C. (1990) Glycosidases of apple fruit: a multi-functional ß-galactosidase. *Physiol. Plant.* **80**, 250-256.
[12] Yoshioka, H., Kashimura, Y., and Kaneko, K. (1995) Beta-D-galactosidase and alpha-L-arabinofuranosidase activities during the softening of apples. *J. Jap. Soc. Hort. Sci.* **63**, 871-878.
[13] Wu, Q., Szakacs-Dobozi, M., Hemmat, M., and Hrazdina, G. (1993) Endopolygalacturonase in apples (*Malus domestica*) and its expression during fruit ripening. *Plant Physiol.* **102**, 219-225.
[14] Bartley, I.M. (1978) Exo-polygalacturonase of apple. *Phytochem.* **17**, 213-216.
[15] Castaldo, D., Quagliuolo, l., Servillo, L., Balestrieri, C., and Giovane, A. (1989) Isolation and characterization of pectin methylesterase from apple fruit. *J. Food Sci.* **54**, 653-655, 673.
[16] Abeles, F.B., and Takeda, F. (1990) Cellulase activity and ethylene in ripening strawberry and apple fruits. *Scientia Hortic.* **42**, 269-275.
[17] Pilnik, W. (1988) From traditional ideas to modern fruit juice winning processes. In *International Congress of Fruit Juices* (Orlando, Florida) 159-179.
[18] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and glucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).
[19] Renard, C.M.G.C., Searle-van Leeuwen, M.J.F., Voragen, A.G.J., Thibault, J.-F., and Pilnik, W. (1991) Studies on apple protopectin. II. Apple cell wall degradation by pure polysaccharidases and their combinations. *Carbohydr. Polym.* **14**, 295-314.
[20] Schols, H.A., Posthumus, M.A., and Voragen, A.G.J. (1990) Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydr. Res.* **206**, 117-129.
[21] Van de Vis, J.W., Searle-van Leeuwen, M.J.F., Siliha, H.A., Kormelink, F.J.M., and Voragen, A.G.J. (1991) Purification and characterization of endo-1,4-ß-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: Use in combination with arabinanases from *Aspergillus niger* in enzymic conversion of potato arabinogalactan. *Carbohydr. Polym.* **16**, 167-187.
[22] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.
[23] Van Houdenhoven, F.E.A. (1975) Studies on pectin lyase. PhD Thesis, Wageningen Agricultural University, The Netherlands.
[24] Vincken, J.-P., Beldman, G., Niessen, W.M.A., and Voragen, A.G.J. (1996) Degradation of apple fruit xyloglucan by endoglucanase. *Carbohydr. Polym.*, in press (this thesis, Chapter 3).
[25] Vincken, J.-P., de Keizer, A., Beldman, G., and Voragen, A.G.J. (1995) Fractionation of xyloglucan fragments and their interaction with cellulose. *Plant Physiol.* **108**, 1579-1585 (this thesis, Chapter 7).
[26] Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828.
[27] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. Substrate specificity of endoglucanases. What determines xyloglucanase activity? Submitted for publication to *Carbohydr. Res.* (this thesis, Chapter 6).
[28] Somogyi, N. (1952) Notes on sugar determination. *J. Biol. Chem.* **195**, 19-23.
[29] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.

[30] Beldman, G., Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F., and Pilnik, W. (1987) Adsorption and kinetic behaviours of purified endoglucanases and exoglucanases from *Trichoderma viride*. *Biotechnol. Bioeng.* **30**, 251-257.

[31] Plat, D., Ben-Shalom, N., Levi, A., Reid, D., and Goldschmidt, E.E. (1988) Degradation of pectic substances in carrots by heat treatment. *J. Agric. Food Chem.* **36**, 362-365.

[32] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.

[33] Baron-Epel, O., Gharyal, P.K., and Schindler, M. (1988) Pectins as mediators of wall porosity in soybean cells. *Planta* **175**, 389-395.

[34] Smith, R.C., and Fry, S.C. (1991) Endotransglycosylation of xyloglucans in plant cell suspension cultures. *Biochem. J.* **279**, 529-535.

[35] Nishitani, K., and Tominaga, R. (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J. Biol. Chem.* **267**, 21058-21064.

[36] Fanutti, C., Gidley, M.J., and Reid, J.S.G. (1993) Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1→4)-ß-D-glucanase) from the cotyledons of germinated nasturtium seeds. *Plant J.* **3**, 691-700.

[37] Redgwell, R.J., and Fry, S.C. (1993) Xyloglucan endotransglycosylase activity increases during kiwifruit (*Actinidia deliciosa*) ripening: implications for fruit softening. *Plant Physiol.* **103**, 1399-1406.

[38] Cutillas-Iturralde, A., Zarra, I., Fry, S.C., and Lorences, E.P. (1994) Implication of persimmon fruit hemicellulose metabolism in the softening process. Importance of xyloglucan endotransglycosylase. *Physiol. Plant.* **91**, 169-176.

[39] Maclachlan, G., and Brady, C. (1994) Endo-1,4-ß-glucanase, xyloglucanase and xyloglucan endo-transglycosylase activities versus potential substrates in ripening tomatoes. *Plant Physiol.* **105**, 965-974.

CHAPTER 9

Xyloglucan Endotransglycosylase Activity in Apples Is Ripening-Related: Implications for Fruit Juice Processing[I]

In order to assess the role of endogenous enzymes during apple juice manufacturing by enzymic liquefaction, apples of different maturity were assayed for various enzyme activities. Xyloglucan endotransglycosylase (XET) activity increased dramatically from early September to mid October, and decreased slowly during cold storage. It was shown that XET is probably the most important, endo-acting activity in apple. The apples of different maturity were also treated by pectin lyase and various concentrations of cellulase. It appeared that less cellulase was needed for disintegration of apples with a high level of XET activity. This suggests that XET facilitates the liquefaction process. Under certain conditions, "high XET" apples could be transformed into a cloudy apple juice. This cloud is composed of predominantly cellulose and xyloglucan, and may be regarded as a dispersion of cellulose particles which is stabilized by xyloglucan molecules.

Cellulose and xyloglucan are important structural polysaccharides in the primary cell wall of plants. The xyloglucans associate intimately with cellulose and the length of xyloglucan molecules is such, that many cellulose microfibrils can be crosslinked [1,2]. The resulting network determines the strength of the cell wall to a large extent. Breakage of these crosslinks by cellulase preparations may destroy the coherence of the cell wall.

We have been investigating the enzymic degradation of apple cell walls (liquefaction) as an alternative for pressing in apple juice manufacturing. These studies have pointed out that, next to pectinases, cellulases play an important role in this process [3,4]. The cellulase preparation from *Trichoderma viride* contains all enzyme activities that are required for a complete conversion of cellulose-xyloglucan networks. Cellulose degradation is facilitated when its xyloglucan coating is removed by certain endoglucanases [5,6]. The xyloglucans have to be degraded extensively before they are released from the cellulose surface [7].

Next to fungal enzymes, endogenous enzymes also seem to play a role in xyloglucan breakdown. In a previous paper it was shown that XET[II] is active during liquefaction of apple tissue [8]. XET can cleave polymeric xyloglucan and transfer part of this molecule to the nonreducing end of either polymeric (PS→PS) or oligomeric (PS→OS) xyloglucan fragments [9,10]. The latter is attended with a strong decrease in mol wt. We have postulated that fungal glucanases and XET act in concert during liquefaction of apple

---

[I]Vincken, J.-P., Zabotina*, O.A., Beldman, G., and Voragen, A.G.J. To be submitted (* *Institute of Biology of the Russian Academy of Sciences, P.O. Box 30, 420503, Kazan, Russia*).

[II]*Abbreviations:* see page viii.

tissue whereby endoglucanases generate oligomeric acceptor substrates for XET. Consequently, XET will start catalyzing a PS→OS endotransglycosylation. In this situation, xyloglucan crosslinks can be split and the cellulose-xyloglucan network collapses. It is thought that such a mechanism promotes the disintegration of apple tissue during liquefaction [8]. In order to test this hypothesis, apples with a different XET level were required. Therefore, XET activity was monitored during apple development, and apples of different stages were subjected to a simple liquefaction test. The present paper provides new evidence to confirm our hypothesis. Some implications for fruit juice processing are discussed.

EXPERIMENTAL

Materials

Apples (*Malus malus* L., *cv.* Jonagold) were picked at time intervals of *c.* two weeks, starting at the end of June. Peel and core were removed. Part of the apples was directly treated as described under "liquefaction experiments". The remaining material was cut in small pieces (cubes of *c.* 125 mm^3), frozen in liquid nitrogen and stored at -20 °C. Apples were harvested finally at October 15 and subsequently stored at 4 °C. During the period in cold storage apples were sampled with longer time intervals (1-2 months), and treated as described above for the freshly picked ones.

A crude cellulase preparation (Maxazyme), endoV, CBH and PL were similar to those described previously [8]. Xyloglucan oligosaccharides ($[xg]_1$) were prepared as described before [8]. Methyl esterified pectin (DM of 65%), branched pectic polysaccharides (MHR), CMC, tamarind xyloglucan and galactan (potato) were obtained as described previously [8]. An apple arabinan was provided by Röhm GmbH (Darmstadt, Germany).

Labelling of xyloglucan oligosaccharides

The oligosaccharides of $[xg]_1$ were tagged with 2-aminopyridine by reductive amination [11-13]. Three g of 2-aminopyridine was dissolved in 3 mL of water, and the pH was adjusted to 7 with *c.* 2 mL of glacial HOAc. Subsequently, 100 mg of sodium cyanoborohydride and 100 mg of $[xg]_1$ were added to 5 mL of this solution, and the mixture was left for 18 h at 80 °C. After cooling to room temperature, the 2-aminopyridine and the labelled oligosaccharides (referred to as pa-$[xg]_1$) were bound on Dowex 50W H^+ (50 g; Sigma). After 1 h, the mixture was poured onto a glass filter and the underivatized material and sodium borate were removed by washing with 1.5 L of water. After this, 2-aminopyridine and pa-$[xg]_1$ were eluted by 1.5 L of 0.2 M ammonia. This solution was evaporated to dryness. The pa-$[xg]_1$ were washed in 25 mL of dichloromethane:methanol (4:1). After centrifugation (10 min, 4,000 *g*) the supernatant containing the 2-aminopyridine was removed. This procedure was repeated once. The pa-$[xg]_1$ were dissolved in water and freeze-dried. Approximately 10% of $[xg]_1$ was recovered as pa-$[xg]_1$.

Determination of activity of endogenous enzymes

Quantification of XET activity.—Frozen apple pieces (2 g), 150 mg of PVPP and 4 mL of a 0.25 M sodium phosphate buffer (pH 6.2) containing 0.4 M NaCl and 0.01% (w/v) NaN_3, were homogenized for 15 s at 4 °C, using an ultra-turrax (TP 18-10, Ika Werk, Staufen, Germany). After 1 h of extraction at 4 °C (with periodically shaking), the suspensions were centrifuged (10 min, 2,000 *g*). All extractions were performed in triplicates. The pH in all extracts was adjusted to pH 5.8 by combining 1 mL of extract with 110 μL of water, containing an appropriate

amount of HOAc. XET activity was determined at various stages of development by incubating 70 μL of a substrate stock solution (containing 4 mg mL^{-1} polymeric tamarind xyloglucan and 400 μg mL^{-1} pa-$[xg]_1$) with 70 μL of the extract (pH 5.8) for 5 h at 40 °C. Enzymes were inactivated by heating for 10 min at 100 °C. Incorporation of label in high mol wt material was quantified using HPSEC (BioGel TSK 40XL, 30XL, and 20XL columns in series) combined with a refractive index detector (see [5]) as well as with a Spectroflow 773 UV detector (310 nm; Kratos, Westwood, NJ, USA).

Qualitative assay of other "endo" activities.—In order to determine whether XET is the most important "endo" activity in apples, three extracts (mid September, mid October and end February) were tested at three different pH values (4.0, 5.0 and 5.8) for enzyme activities other than XET. The following substrates were used: methyl esterified pectin, MHR, CMC, tamarind xyloglucan, apple arabinan and potato galactan. Incubation procedures were similar as described above. All samples were analyzed qualitatively by HPSEC to investigate if mol wt distributions had shifted upon incubation. Similar experiments were carried out with tamarind xyloglucan in the presence of $[xg]_1$.

Liquefaction experiments

Fresh apple material (1 g; cubes of *c.* 125 mm^3) was incubated at 40 °C (150 rotations min^{-1}) with 3 mL of 0.2 M NaOAc buffer (pH 4) containing 1% (w/v) ascorbic acid, 0.01% (w/v) NaN_3, 50 mU PL and 9 different concentrations of Maxazyme {220 μg (corresponding to 110 mU Avicelase activity and 2200 mU xyloglucanase activity), 73 μg, 24 μg, 8 μg, 3 μg, 1 μg, 300 ng, 100 ng and 33 ng of protein}. Incubations without enzyme addition and with 50 mU PL alone were included in these series. Protein content was determined according to Sedmak and Grossberg [14] using BSA as a standard. After 15 h of incubation, the minimum amount of cellulase to achieve complete disintegration of apple tissue was determined visually.

Isolation and characterization of cloud material

Within the liquefaction series of November 28, cloud material was most abundantly present when apple tissue was treated with 1 μg of Maxazyme. After removing undegraded apple pieces, the suspension containing the cloud material was centrifuged (10 min, 4,000 *g*). The supernatant was removed, and the cloud material was washed with 5 mL of water, and subsequently centrifuged. This procedure was repeated 5 times, and after this the cloud material was freeze-dried. The sugar composition of this material was determined as described elsewhere [5].

Cloud material (2 mg) was suspended in 1.5 mL of NaOAc buffer (pH 5) containing 0.01% (w/v) NaN_3, 5 μg of endoV and 25 μg of CBH. After 72 h of incubation at 40 °C, an aliquot of 50 μL was analyzed using HPAEC as described previously [15]. The incubation was continued for another 24 h after addition of 110 μg of Maxazyme. Subsequently, enzymes were inactivated by heating for 10 min at 100 °C. An aliquot of 50 μL was analyzed using HPAEC.

RESULTS

XET activity during apple development

In the period of June 1994 to October 1994 apples were sampled from the tree *c.* twice a month. The apples continued to grow until mid September and they turned color (green→red) around the end of this month. For each batch of apples, XET activity was determined according to a slightly modified procedure described by Nishitani and Tominaga [10,16]. The PS→OS endotransglycosylation is clearly illustrated by Fig. 1. The incorporation of labelled xyloglucan oligosaccharides in larger molecules is attended with

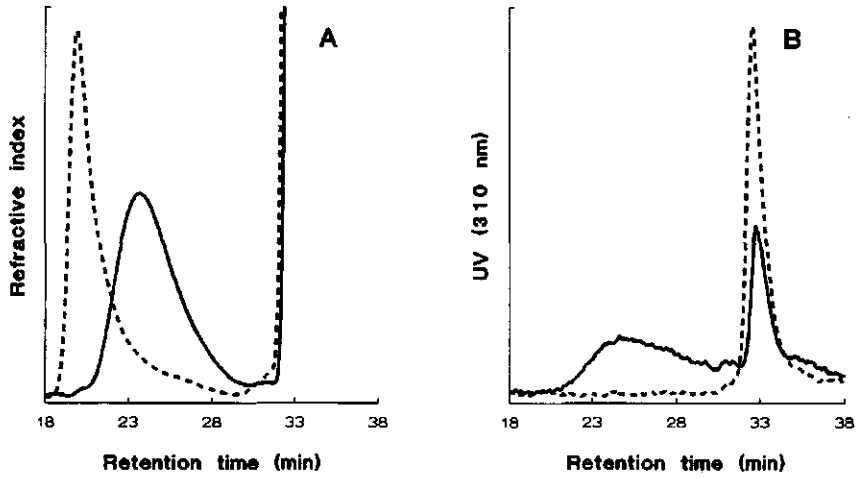

FIGURE 1. Quantitative determination of XET activity using HPSEC. Samples were analyzed simultaneously for refractive index (A) and UV signal (B). Dashed lines represent the situation at the start of the incubation; solid lines show the pattern after 5 h of incubation. For details see text.

a decrease in mol wt of polymeric xyloglucan. Under the conditions used, the endotransglycosylation proceeded linearly in time until *c.* 40% of label was transferred (data not shown).

XET level of apples was determined at various stages of development. In order to ensure that no acceptor substrate limitation occured, incubation times were chosen such (5 h) that less than 40% of label was transferred. No XET activity was detected in any of the samples from the end of June until mid August. After this, XET activity increased linearly in time until a constant value was reached in mid October (Fig. 2). During cold storage, the XET level eventually decreased slowly.

Other enzyme activities during apple development

Three enzyme extracts (mid September, mid October and February) were tested for their ability to depolymerize a number of polysaccharides which are representative for those encountered in apple (except CMC). Experiments were carried out at three different pH values: pH 4.0, this pH has been used in liquefaction experiments; pH 5.0, intermediate value; pH 5.8, the pH optimum of XET [9]. All samples were analyzed for a shift in mol wt distribution compared to a blank (data not shown). Although some activity was detected towards CMC and xyloglucan (no $[xg]_1$ added), none of these polysaccharides were depolymerized to a similar extent as xyloglucan in the presence of $[xg]_1$. A minor shift in mol wt distribution was found upon incubation of galactan and arabinan, whereas pectin and MHR were not degraded at all. These experiments demonstrated that XET is (by far) the major endo-acting enzyme activity in apple. With

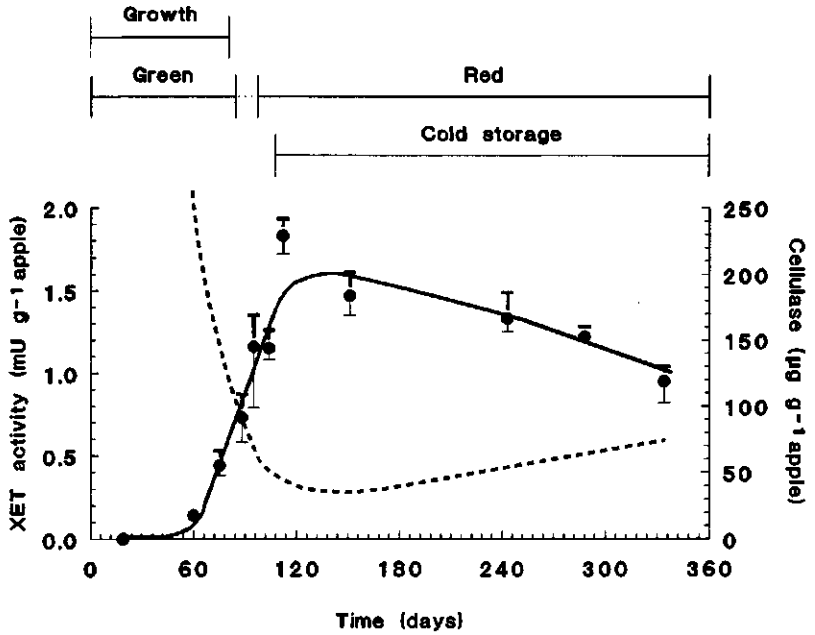

FIGURE 2. Development of XET activity in apples in relation to their liquefaction behavior. XET activity is indicated by the solid line. ●, average of three independent XET measurements; error bars display maximum deviation of these measurements. The amount of cellulase required for complete disintegration of apple is shown by the dashed line. Day 1 corresponds to the 1st of July. For details, see text.

respect to liquefaction of apples, it is interesting to note that XET still showed considerable activity at pH 4.0 (data not shown).

Liquefaction experiments

In order to investigate if apples with a high level of XET activity disintegrated faster than those with a low level, apple pieces of each stage of maturity were treated with different amounts of Maxazyme. PL was added in non-limiting amounts to all incubations. The smallest amount of cellulase that completely disintegrated apple tissue, was determined visually. Fig. 2 shows that the amount of cellulase needed is inversely proportional to the XET level of apples from the same batch. This suggests that XET accelerates the disintegration of apple tissue.

During these liquefaction experiments an interesting observation was done. A cloud was formed during liquefaction of apples from October 12, October 20 and November 28 (and to a lesser extent February 28) with intermediate doses of cellulase; this cloud was unaltered after several days at room temperature. The larger doses of cellulase (24, 73, and 220 µg) did not yield any cloud material. Apparently, the cloud material could be degraded by the cellulase preparation. With smaller amounts of cellulase (33 and 100 ng)

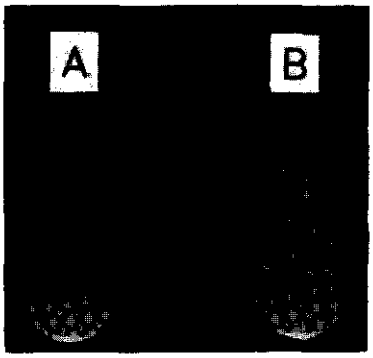

FIGURE 3. Cloud material derived from November 28 apples after treatment with PL (A) and with PL plus 1 μg of cellulase (B). The cloud material was washed extensively with distilled water.

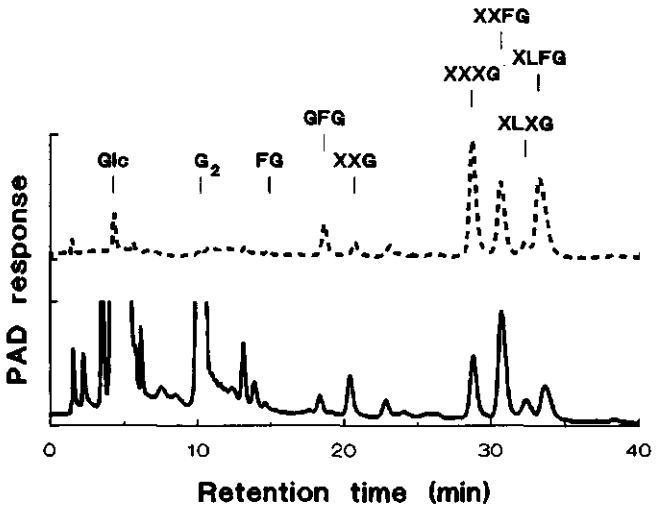

FIGURE 4. HPAEC elution profile showing the oligosaccharide building units of cloud material (solid line). The oligosaccharide composition of an alkali-extracted apple xyloglucan is shown for comparison (dashed line). Xyloglucan oligosaccharides are denoted above their corresponding peaks according to the nomenclature of Fry *et al.* [17].

the apple material was hardly degraded and no cloud was formed. The cloud material was isolated quantitatively from the November 28 apples (1 μg of cellulase), which yielded *c.* 4 mg of material from 1 g of apple fruit tissue (dry weight). The cloud material has an appearance as is shown in Fig. 3. The particles do not precipitate further; this situation can be maintained for several weeks at 4 °C. The presence of large amounts of Glc and Xyl in this material (Table I) suggests that the cloud was built predominantly from a cellulose-xyloglucan network {*c.* 75% (w/w)}. This was further substantiated by enzymic degradation (endoV and CBH) of the cloud material which yielded G_2 and xyloglucan oligosaccharides (Fig. 4). The peaks eluting around 5 min were also present in the blank, which did not contain any other compounds than these (data not shown). In order to investigate whether the cellulose-xyloglucan network was completely degraded, the

TABLE I. Sugar composition (mol%) of cloud in "juice" obtained by liquefaction of "high XET" apples with PL and a small amount of cellulase.

Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
1	1	8	11	5	7	61	6

reaction mixture obtained with endoV plus CBH was subsequently treated with Maxazyme. This yielded a similar pattern as the solid line in Fig. 4 with the difference that G_2 was further degraded to Glc (data not shown). From this it was concluded that, with respect to xyloglucan, the cloud material is mainly composed of XXXG (for nomenclature, see [17]) and XXFG building units. Typically, relatively small amounts of GFG, XXXG and XLFG were found, when compared to the degradation products of an alkali-extracted apple xyloglucan (Fig. 4) [15,18]. The presence of GalA, Rha and Ara showed that the cloud material also contains pectic polysaccharides. It is speculated that these polysaccharides are similar to modified hairy regions [19], a rhamnogalacturonan backbone having arbinan sidechains.

DISCUSSION

In the present study we have used a different apple variety compared to previous investigations. However, sugar composition of cell wall material is in close agreement with that of Golden Delicious apples, and also the oligosaccharide composition of alkali-extracted xyloglucan was very similar (data not shown). Therefore, we believe that the results described in this paper are also applicable to Golden delicious apples.

The onset of ripening of climacteric fruits is characterized by a number of events which start more or less at the same time. The level of ethylene in the full-grown fruit increases, which is attended with an increase in respiration (climacterium), turning color and synthesis of proteins [20-22]. The level of XET in apples closely corresponds to this general pattern. This shows that XET does probably not play a role during fruit growth, but that it is mainly ripening-related. Similar observations have been made for kiwi fruit (also climacteric) [23]. In these experiments the ripening process was mimicked by supplying exogenous ethylene to unripe kiwifruit. Their results suggested that XET is involved in fruit softening.

Our results show that "high XET" apples disintegrate with a smaller amount of cellulase than "low XET" apples (see Fig. 2). This is primarily due to XET, because no other endo-acting enzyme activities were found in apple tissue in such large amounts. Relatively large amounts of ß-galactosidase are known to be present in apple [24]. This enzyme was not further investigated here, because it is unable to cleave potential crosslinks like galactan, and no major effect during liquefaction is anticipated. The

findings above provide further evidence for our hypothesis that XET and fungal xyloglucanases act in concert during the degradation of the cellulose-xyloglucan network in apple fruit [8]. These results suggest that XET is a major factor in the processibility of apple fruit during liquefaction.

It might be argued that the accelerated disintegration of tissue is due to structural changes in the fruit rather than to synergism between xyloglucanases. It has been shown for tomatoes [25,26] and persimmon fruit [27] that the mol wt distribution of hemicellulose shifts to lower values as ripening proceeds. Similar observations were made for cellulose in tomatoes [26] and avocado [28]. For apple no such investigations have been done. However, based on the observation that more cellulase is needed to disintegrate apples from February, April and May compared to apples from October, it is expected that these irreversible structural changes play a minor role in our experiments.

There is of course a possibility that an unknown enzyme activity has been overlooked, just like XET has not been recognized for a long time. In principle, our work should be verified with XET-sense and XET-antisense apple constructs. However, production of transgenic apple is still difficult because of their long regeneration time. For tomato such constructs have already been made [29,30].

Under certain circumstances and with apples of a certain degree of ripeness, a cloud was formed during liquefaction. It can be calculated that for these cases the amount of XET activity and that of fungal xyloglucanase activity are in the same order of magnitude (1.7 and *c.* 10 mU, respectively). The ratio between XET and cellulase seems to be rather critical for cloud formation. A minimum amount of cellulase is required to trigger PS→OS endotransglycosylation by XET, while a too large dose degrades the cloud completely. It is important to note that large amounts of XET will not degrade the cloud material. The enzyme shows no activity towards cellulose and very little activity towards, for instance, $[xg]_8$ [10]. These fragments are sufficiently long to bind to cellulose [7]. This suggests that XET is very suitable for breaking xyloglucan crosslinks between cellulose microfibrils, but not to remove the xyloglucan coating from cellulose.

Extraction of xyloglucan from cell wall material is usually much more efficient when strong alkali is used to swell cellulose [31]. From this it was concluded that part of the xyloglucan is entrapped by cellulose fibres. This entrapment explains why native cellulose-xyloglucan complexes contain much more xyloglucan than reconstituted ones [7]. Native apple cellulose-xyloglucan complexes are composed *c.* 40% of xyloglucan [5]. For the cloud material this percentage is a little smaller (*c.* 35%), which suggests that the xyloglucan moiety, in particular, is partially degraded during liquefaction. A schematical representation of the cloud material is shown in Fig. 5: a dispersion of cellulose particles which is prevented from flocculating by the presence of xyloglucan molecules. It is thought that the stabilization is primarily due to steric repulsion, although the presence of GalA (Table I) suggests that electrostatic forces may also play a role. The substrate specificity of XET suggests that the "tails" sticking out in solution comprise several oligosaccharide units. It is unknown if a minimum length of these tails is required for stabilization. The observations described here may be of a similar kind as those referred

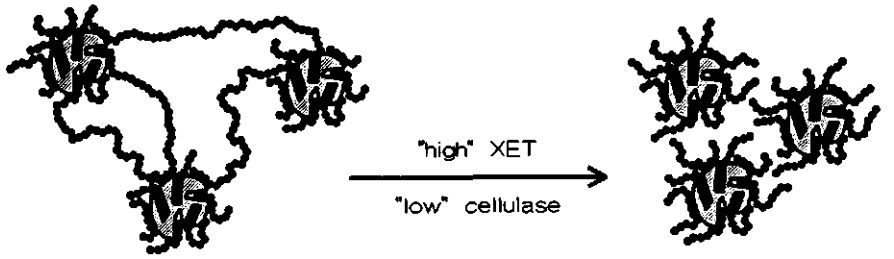

FIGURE 5. Schematic representation of cloud material in apple juice obtained by liquefaction. In the primary cell wall of apples, cellulose microfibrils are interconnected by xyloglucans. Depending on the amount of cellulase, it is possible to cleave predominantly xyloglucan crosslinks during liquefaction of "high XET" apples. Symbols: shaded figures represent a cross section of a cellulose particle; •, individual xyloglucan oligosaccharides.

to as "cell wall swelling" by Redgwell and Fry [23].

The ability of cellulases to disperse cellulose particles has been observed before. Most cellulases are built from two functional domains, the catalytic core and a nonhydrolytic CBD, which are connected by a linker peptide [32]. The two domains can be separated by proteolytic cleavage. The isolated CBDs of (at least) two glucanases from *Cellulomonas fimi* can disperse bacterial cellulose [33], but little is known about the stability of such dispersions. In contrast to this, the primary cell wall of apple contains a "precursor" for a rather stable dispersion of cellulose which can be effected by degradation of pectin and cleavage of xyloglucan crosslinks. Our results show that it is, in principle, possible to obtain a cloudy (apple) juice by the liquefaction process. Success of such an operation depends on the ability to formulate an enzyme preparation with a pectinase and an appropriate xyloglucanase; truly cellulolytic enzymes are probably not important. With respect to cloudy juices, XET might be an enzyme of technological importance.

REFERENCES

[1] McCann, M.C., and Roberts, K. (1991) Architecture of the primary cell wall. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed., Academic Press, New York), 109-129.

[2] Carpita, N.C., and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**, 1-30.

[3] Pilnik, W., and Voragen, A.G.J. (1991) The significance of endogenous and exogenous pectic enzymes in fruit and vegetable processing. In *Food Enzymology* (Fox, P.F., ed., Elsevier Applied Science, London), 1, 303-336.

[4] Pilnik, W., and Voragen, A.G.J. (1991) Effect of enzyme treatment on the quality of processed fruit and vegetables. *Fruit Processing* 1, 125-129.

[5] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and endoglucanases from *Trichoderma viride*. *Plant Physiol.* **104**, 99-107 (this thesis, Chapter 2).

[6] Vincken, J.-P., Beldman, G., and Voragen, A.G.J. Substrate specificity of endoglucanases: what determines xyloglucanase activity? Submitted for publication to *Carbohydr. Res.* (this thesis, Chapter 6).

[7] Vincken, J.-P., de Keizer, A., Beldman, G., and Voragen, A.G.J. (1995) Fractionation of xyloglucan fragments and their interaction with cellulose. *Plant Physiol.* **108**, 1579-1585 (this thesis, Chapter 7).
[8] Vincken, J.-P., van den Broek, L.A.M., van der Lei, D.D., Beldman, G., and Voragen, A.G.J. Fungal and plant xyloglucanases may act in concert during liquefaction of apples. Submitted for publication to *J. Sci. Food Agric.* (this thesis, Chapter 8).
[9] Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828.
[10] Nishitani, K., and Tominaga, R. (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J. Biol. Chem.* **267**, 21058-21064.
[11] Hase, S., Hara, S., and Matsushima, Y. (1979) Tagging of sugars with a fluorescent compound, 2-aminopyridine. *J. Biochem.* **85**, 217-220.
[12] Hase, S., Ibuki, T., and Ikenaka, T. (1984) Reexamination of the pyridylamination used for fluorescence labeling of oligosaccharides and its application to glycoproteins. *J. Biochem.* **95**, 197-203.
[13] Maness, N.O., Miranda, E.T., and Mort, A.J. (1991) Recovery of sugar derivatives from 2-aminopyridine labeling mixtures for high-performance liquid chromatography using UV or fluorescence detection. *J. Chromatogr.* **587**, 177-183.
[14] Sedmak, J.J., and Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Anal. Biochem.* **79**, 544-552.
[15] Vincken, J.-P., Beldman, G., Niessen, W.M.A., and Voragen, A.G.J. (1996) Degradation of apple fruit xyloglucan by endoglucanase. *Carbohydr. Polym.*, in press (this thesis, Chapter 3).
[16] Nishitani, K. (1992) A novel method for detection of endo-xyloglucan transferase. *Plant Cell Physiol.* **33**, 1159-1164.
[17] Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J., and White, A.R. (1993) An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* **89**, 1-3.
[18] Spronk, B.A., Rademaker, G.J., Haverkamp, J., Thomas-Oates, J.E., Vincken, J.-P., Voragen, A.G.J., Kamerling, J.P., and Vliegenthart, J.F.G. Dimers of a GFG hexasaccharide occur in apple fruit xyloglucan. To be submitted (this thesis, Chapter 4).
[19] Voragen, A.G.J., Schols, H.A., and Gruppen, H. (1993) Structural studies of plant cell-wall polysaccharides using enzymes. In *Plant Polymeric Carbohydrates* (Meuser, F., Manners, D.J., and Seibel, W., eds., Royal Society of Chemistry, Cambridge, UK), 3-15.
[20] Biale, J.B. (1964) Growth, maturation, and senescence in fruits. *Sci.* **146**, 880-888.
[21] Biale, J.B., and Young, R.E. (1981) Respiration and ripening in fruits—Retrospect and prospect. In Recent Advances in *The Biochemistry of Fruits and Vegetables* (Friend, J., and Rhodes, M.J.C., eds., Academic Press, New York), 1-39.
[22] Wills, R.B.H., McGlasson, W.B., Graham, D., Lee, T.H., and Hall, E.G. (1989) *Postharvest. An Introduction to the Physiology and Handling of Fruit and Vegetables* (Blackwell Scientific, Oxford).
[23] Redgwell, R.J., and Fry, S.C. (1993) Xyloglucan endotransglycosylase activity increases during kiwifruit (*Actinidia deliciosa*) ripening: implications for fruit softening. *Plant Physiol.* **103**, 1399-1406.
[24] Dick, A.J., Opoku-Gyamfua, A., and DeMarco, A.C. (1990) Glycosidases of apple fruit: a multi-functional ß-galactosidase. *Physiol. Plant.* **80**, 250-256.
[25] Sakurai, N., and Nevins, D.J. (1993) Changes in physical properties and cell wall polysaccharides of tomato (*Lycopersicon esculentum*) pericarp tissues. *Physiol. Plant.* **89**, 681-686.
[26] Maclachlan, G., and Brady, C. (1994) Endo-1,4-ß-glucanase, xyloglucanase and xyloglucan endo-transglycosylase activities versus potential substrates in ripening tomatoes. *Plant Physiol.* **105**, 965-974.
[27] Cutillas-Iturralde, A., Zarra, I., Fry, S.C., and Lorences, E.P. (1994) Implication of persimmon fruit hemicellulose metabolism in the softening process. Importance of xyloglucan endotransglycosylase. *Physiol. Plant.* **91**, 169-176.
[28] O'Donoghue, E.M., Huber, D.J., Timpa, J.D., Erdos, G.W., and Brecht, J.K. (1994) Influence of avocado (*Persea americana*) Cx-cellulase on the structural features of avocado cellulose. *Planta* **194**, 573-584.
[29] Nishitani, K., Okazawa, K., Asada, K., and Kato, I. (1993) *European Patent*, EP 0.562.836.A1.
[30] De Silva, J., Jarman, C.D., Arrowsmith, D.A., Reid, J.S.G., and Edwards M.E. (1993) Endo-1,4-ß-D-glucanase. *World Patent*, WO 93/17101.
[31] Edelmann, H.G., and Fry, S.C. (1992) Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells. *Carbohydr. Res.* **228**, 423-431.
[32] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., and Warren, R.A.J. (1991) Domains of microbial ß-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**, 303-315.
[33] Gilkes, N.R., Kilburn, D.G., Miller, R.C., Warren, R.A.J., Sugiyama, J., Chanzy, H., and Henrissat, B. (1993) Visualization of the adsorption of a bacterial endo-ß-1,4-glucanase and its isolated cellulose-binding domain to crystalline cellulose. *Int. J. Biol. Macromol.* **15**, 347-351.

CHAPTER 10

General Discussion

During the past six years cellulase research has greatly benefitted from the possibilities to produce mutant proteins. These mutants have been successfully used to investigate the structure and function of the different domains of these cellulases. This, together with information on the three-dimensional structures of some of these enzymes has provided a much better (but still incomplete) picture of the mechanism of cellulose hydrolysis [1-5]. In contrast to the degradation of cellulose, relatively little is known about the action of individual glucanases on other, structurally related, substrates such as xyloglucan. In the same period, plant cell wall chemists have elucidated the primary structures of many xyloglucan building units [6-11], while plant physiologists have gathered a body of evidence to show that xyloglucans play a key role in processes that involve rearrangements in cell wall architecture [12,13]. Enzymes like XET[1] [14-17] as well as so-called expansins (nonhydrolytic proteins which are thought to reversibly disrupt noncovalent bonds) [18-20] might be involved in such processes, but their exact function remains to be established. By studying the enzymic liquefaction of apples, we had the unique opportunity of being in the interface of these two fields.

The present work was built on the observations of Renard and coworkers, who showed that one particular endoglucanase worked synergistically with PL in the degradation of apple cell walls [21,22]. The cooperative action of the two enzymes was attended with the solubilization of large amounts of pectic material and xyloglucan. In our study, we have focussed on the degradation of the cellulose-xyloglucan network. The substrate specificity of endoglucanases of *Trichoderma viride* with respect to xyloglucans has been determined in great detail. In addition, we have tried to extrapolate our results obtained with isolated cell wall material to the more complex degradation of apple tissue. This has provided compelling evidence that xyloglucan is the "Achilles heel" of the cell wall during liquefaction of apples, as suggested in Chapter 1. In this last chapter some important implications of our work will be discussed.

CLASSIFICATION OF XYLOGLUCANS

During the biosynthesis of xyloglucan, the basic skeleton of these molecules is first laid down by alternate transfer of Glc and Xyl to a nascent polysaccharide [23]. In a later stage, these structures are "decorated" with Gal, Fuc and/or Ara. The presence of Fuc residues in particular, is often regarded as being characteristic for xyloglucans from

[1] *Abbreviations:* see page viii.

dicotelydons. The xyloglucan from monocotyledons generally lacks Fuc residues. There are a number of exceptions to this rule [24,25], and fucosylation does therefore not appear to be an appropriate criterium for classification. More characteristic, however, are the differences observed in the degree of backbone branching. In Chapter 5 we have shown that potato xyloglucan has an unique building unit structure. This observation has prompted us to compare all known xyloglucan structures with respect to the degree of backbone-branching with Xyl (Fig. 1), and to add some notes to a number of reports in the literature. It is believed that all xyloglucans are composed of either XXXG-type or XXGG-type of building units, as will be discussed below.

XXXG. Many xyloglucans derived from often entirely unrelated species belong to this class [6-11,24-30]. In this group, three out of four Glc residues are substituted with α-D-Xyl*p*-(1→6)-, and some of these can be substituted with ß-D-Gal*p*-(1→2)-. Tamarind xyloglucan does not bear other substituents (Q is a hydrogen; see Fig. 1) but many other xyloglucans, including that of apple as was shown in Chapter 3 and 4, are fucosylated (Q is α-L-Fuc*p*-(1→2)-). Oligosaccharides XXFG, XLFG and XFFG have been characterized in detail [6,9], but the existence of XFLG or XFXG has not (yet) been demonstrated unambiguously. The xyloglucan from sycamore seems to be an exceptional case in that it contains oligosaccharides with additional sidechains at position Y (ß-D-Xyl*p*-(1→2)-, α-L-Ara*f*-(1→2)-, or α-L-Ara*f*-(1→3)-ß-D-Xyl*p*-(1→2)-) and Z (ß-D-Xyl*p*-(1→2)-) [8,10,26]. Substitution at these positions provides resistancy against endoglucanases. Sofar, there has been no clear evidence for the presence of α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-sidechains in this group, as commonly found in *Solanaceae* xyloglucan [31] (see also Chapter 5).

XXGG. In Chapter 5 we have shown that xyloglucan from potato has a different branching pattern than that of the xyloglucans belonging to the previous group. These xyloglucans have clusters of two instead of three branched Glc residues which alternate with a sequence of two "bare" Glc residues. Until now, potato [31] and tomato (Vincken, unpublished results), both belonging to the *Solanaceae*, are the only representatives. There is some evidence suggesting that the xyloglucan from tobacco also is a member of this group (see further). Xyl residues can be substituted with α-L-Ara*f*-(1→2)- and ß-D-Gal*p*-(1→2)-residues. The presence of Fuc as well as the peculiar substitution at position Y and Z has not (yet) been demonstrated for this group.

The literature suggests that a XXG branching pattern also exists; the building units of this type of xyloglucan are composed of two contiguous branched Glc residues, followed by one unbranched Glc residue. Degradation of xyloglucan from *Nicotiana tabacum* by a partially purified *Trichoderma* cellulase yielded predominantly XXG, [SX]G and SSG but also large amounts of Glc [32]. The presence of Glc in this xyloglucan digest might be explained by the action of an endoV-type of endoglucanase (see Chapter 5) in combination with some residual ß-glucosidase in the enzyme preparation used. This, and the fact that tobacco belongs to the *Solanaceae*, suggests that this xyloglucan is more likely to be a member of the XXGG group. In fact, it may be questioned if a "poly-XXG" exists at all. *In vitro* experiments have misleadingly suggested that it is possible to synthesize

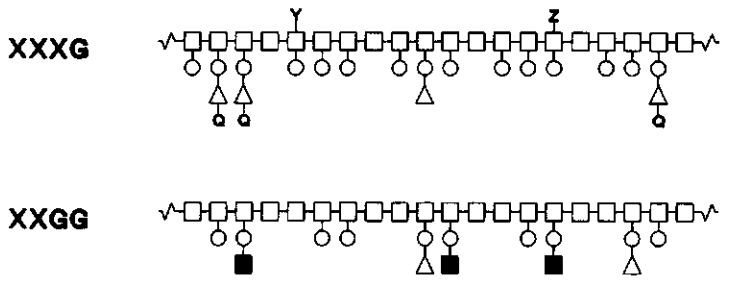

FIGURE 1. An overview of different branching patterns of xyloglucan. □, ß-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; ▵, ß-D-Gal*p*-(1→2)-. Q represents either a hydrogen or an α-L-Fuc*p*-(1→2)-residues. Y can represent a ß-D-Xyl*p*-(1→2)-, an α-L-Ara*f*-(1→2)-, an α-L-Ara*f*-(1→3)-ß-D-Xyl*p*-(1→2)-sidechain, or a hydroxyl. Z can represent a ß-D-Xyl*p*-(1→2)-residue or a hydroxyl.

xyloglucan molecules that are composed of XXG building units [33,34]. Degradation of such an "*in vitro*" polysaccharide by a cellulase preparation did not only yield XXG, but also a large amount of Glc. This suggests that the actual building unit structure of these polymers corresponds to XXGG, and not to XXG. Further, it should be noted that, for instance, sycamore [6] and apple xyloglucan (see Chapter 3) contain small amounts of XXG-type of oligosaccharides. These are probably a result of post-depositional modifications by an α-xylosidase, followed by a ß-glucosidase [17]. XET may be involved in introducing such building units at a mid-chain position, as discussed in detail in Chapter 4.

Xyloglucan derived from immature barley plants [35] and rice seedlings [36] were hypothesized to be composed of XXGGG-type of building units. However, both papers leave room to speculate that the number of unbranched Glc residues is overestimated, because the actual presence of XXGGG was not demonstrated. In principle, the branching pattern of these two xyloglucans may be of a XXGG-type, similar to that of *Solanaceae* xyloglucans. Xyl residues may be substituted with Ara or Gal residues but this extra substitution is less common than in xyloglucan from potato. The xyloglucan from immature barley plants and rice seedlings is insoluble in water, which may be related to the relatively small amount of Ara and Gal residues.

Others. Sofar, α-D-Xyl*p*-(1→6)- was diagnostic for xyloglucans. Recently, a ß-D-Xyl*p*-(1→4)-ß-D-Glc*p*-(1→4)-ß-D-Glc*p* trisaccharide was identified after alkaline extraction of the green alga *Ulva lactuca*, and subsequent enzymic degradation of this extract [37]. In this case, however, Xyl residues seem to be an integral part of the backbone. Although no detailed structure for the polysaccharide is available, this example shows that xyloglucans of a completely different kind also exist. In a later study, similar xyloglucans (or glucoxylans?) were found in a different green alga [38], having molar Xyl:Glc ratios ranging from 0.2 to 1.3. All xyloglucans discussed sofar contain unbranched Glc residues at regular intervals which makes them susceptible to degradation by endoglucanase. An exception to this is a polysaccharide that coats the seeds of *Helipterum eximium* [39].

Every single ß-D-Glc*p*-(1→4)-residue contains two sidechains: ß-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)- and α-L-Ara*f*-(1→2)-. This polysaccharide was resistant against endoglucanase, even after selective removal of the Ara residues with mild acid.

The comparison above suggests that xyloglucans from different sources show more structural similarity than previously thought. In fact, it seems likely that there are only two forms, poly-XXXG and poly-XXGG. All xyloglucans have a building unit length of 4 Glc residues in common, but they differ in the degree of backbone-branching with Xyl. The data of Hayashi and Matsuda [33] and Gordon and Maclachlan [34] suggest that the different forms of xyloglucan may originate from the ability of certain species to mobilize sufficient UDP-Xyl. Further, it is interesting to note that all xyloglucans known to date are composed of building units that have at least two adjacent branched Glc residues. The importance of this observation is that all xyloglucans (except those belonging to "others") possess building units which meet the minimum acceptor requirements of XET [40] (see also Chapter 1).

SYNERGISM IN ENZYMIC DEGRADATION OF CELLULOSE-XYLOGLUCAN NETWORKS

Cellulolytic fungi and bacteria excrete a variety of cellulases, *i.e.* endoglucanases, CBHs, and glucosidases, for the conversion of cellulose to soluble sugars. Endoglucanases primarily cleave internal glucosidic linkages in the disordered, amorphous regions of cellulose, thereby generating new chain-ends for CBHs to act on [41,42]. Next to this "*endo-exo*" type of synergism, also CBHI and CBHII can act in concert ("*exo-exo*") [43-45]. Electron microscopic studies of the action of two CBHs from *Trichoderma reesei* towards cellulose from *Valonia macrophysa* have revealed that the action of CBHII is confined to only one end of the microcrystals [46], while CBHI acts more laterally [47]. Biely and coworkers [48] have shown that CBHII prefers to attack the nonreducing end of cellodextrins and CBHI the reducing end.

The majority of cellulases are composed of two discrete functional domains, a catalytic core and a cellulose-binding domain (CBD), which are connected by a (glycosylated) linker peptide [1]. The domains can be separated by (limited) proteolysis, after which the activity of the enzyme towards cellulose is greatly reduced; the hydrolytic properties towards soluble substrates are not affected [49-51]. Degradation of cotton cellulose with various mixtures of core protein and CBD derived from *Cellulomonas fimi* endoglucanase A has shown that the two domains act synergistically. The core releases predominantly soluble sugars whereas the CBD disrupts the cellulose surface and releases small particles from this. However, none of the mixtures was as effective as the intact enzyme [3]. Thus, in addition to intermolecular synergism ("*endo-exo*" and "*exo-exo*"), cellulose conversion is further facilitated by intramolecular synergism between the catalytic core and the CBD. Din and coworkers [3] have suggested that the C_1 (a system to increase the accessibility of

cellulose) and C_x (a hydrolytic system) factors, as proposed by Reese *et al.* [52] in 1950, correspond to the CBD and core, respectively, and not to CBH and endoglucanase which has long been believed. It is expected that this is also true for other cellulases.

The mechanism of action of CBHI and CBHII is fairly well understood, and also the role of endoglucanase in cellulose conversion seems to be obvious. However, the question why fungi excrete so many endoglucanases is generally not addressed. This thesis provides a possible answer to this question by demonstrating that some endoglucanases have also target substrates other than cellulose. We have shown that *Trichoderma viride* excretes at least three different endoglucanases (Chapter 6): one with a high xyloglucanase activity (endoIV), one for the degradation of cellulose (endoI), and one combining both activities (endoV). Chapter 2 and 8 illustrate that xyloglucanase activity is required for efficient degradation of cellulose in the primary cell walls of plants by endoI and CBH. In fact, this may be regarded as a fourth type of synergism in cellulase systems ("*endo-endo*").

Substrate-Binding and Mode of Action of Xyloglucanases

In our study, we have adopted a different nomenclature for endoglucanases than the one generally used in literature. In Chapter 6, we have tentatively characterized endoIV and endoV as being similar to EGIII (family H or no. 12 [53]) and EGI (family C or no. 7 [53]), respectively. In Chapter 5 it was shown that these two endoglucanases differ in their mode of action towards potato xyloglucan, and it is expected that this mode of action is consistent for all endoglucanases within one family. In Chapter 6 we have suggested that the ability of an endoglucanase to bind unbranched Glc residues of a xyloglucan molecule in its substrate-binding sites may determine whether an endoglucanase will exhibit xyloglucanase activity. This is illustrated in Fig. 2A for "the best xyloglucanase" from *Trichoderma viride*, endoIV, where subsite "IV" plays an important role in substrate-binding. Keeping the two basic types of xyloglucan, poly-XXXG and poly-XXGG, in mind (see discussion above), endoIV seems very well equipped for xyloglucan degradation, because subsite "IV" always interacts with an unbranched Glc residue.

In Chapter 6, the subsite affinities were also calculated for the other xyloglucanase from *Trichoderma viride*, endoV. This yielded a picture as is shown in Fig. 2B. Because our results were not decisive on the subsite affinity of "-IV", this substrate-binding site is indicated with a question mark. However, it is clear that the high xyloglucanase activity of endoV can not be explained by substrate-binding in subsite "IV", because subsite "IV" did not show affinity for Glc residues. Possibly endoV has a subsite "-V", which could serve a similar purpose in xyloglucan degradation as subsite "IV" of endoIV. It is noteworthy that the observed release of a GXXG-type of oligosaccharide from POsxg is compatible with the hypothesis of binding unbranched Glc residues in subsite "-V". Next to the binding of unbranched Glc residues, the endoglucanases may have some structural arrangements for xyloglucanase activity, such as a wide substrate-binding groove to

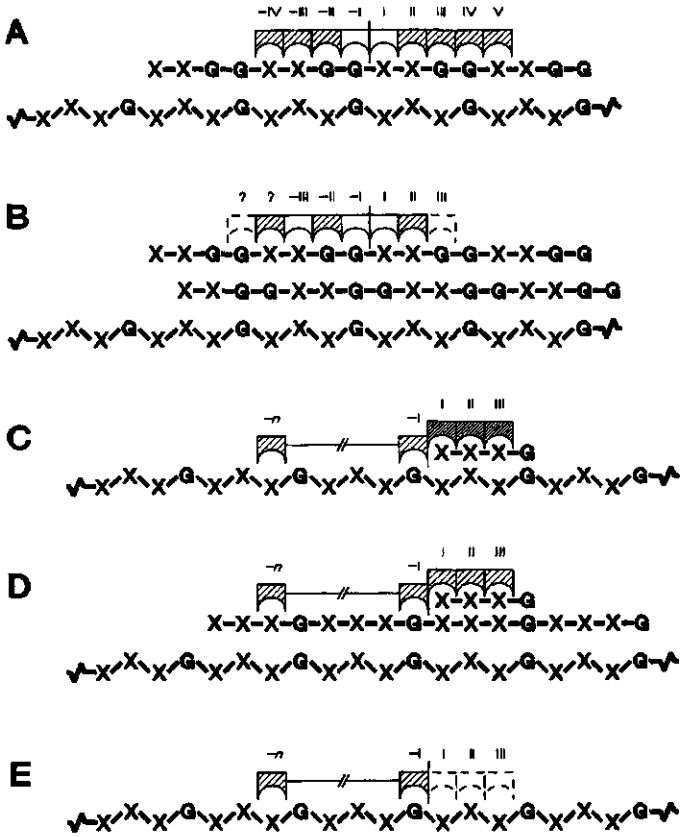

FIGURE 2. Schematic representation of substrate-binding for five different types of "xyloglucanases". A, endoIV from *Trichoderma viride*; B, endoV from *Trichoderma viride*; C, XET from *Vigna angularis*; D, XET from *Tropaeolum majus* (nasturtium) seeds; E, Xyloglucan hydrolase from azuki bean epicotyls. The sum of subsite affinity "-I" and "I" of endoIV and endoV is negative (indicated with open subsites); open subsites are repulsive; shaded subsites are attractive; darker shading indicates stronger substrate-binding. Roman numbers indicate the individual substrate-binding sites. Catalytic residues are located between "-I" and "I". For simplification, xyloglucan molecules are shown as sequences of G and X code letters. Small xyloglucan molecules are drawn straight; larger molecules are thought to have a different conformation as the small ones, and are represented as twisted structures. Note that certain xyloglucanases can only cleave large molecules.

accomodate the heavily branched xyloglucan. Unfortunately, no three-dimensional structures of any of the endoglucanases belonging to the families C and H are available.

Plants have a number of enzymes that can cleave the backbone of xyloglucan. Some of these may act similar as the two enzymes discussed above [24]. Next to these endoglucanases, plants can possess enzymes that are highly specific for xyloglucan, and that can not cleave CMC. These enzymes can catalyze the endotransglycosylation of xyloglucan molecules, and are often referred to as XET (see also Chapter 1). In Chapter 8

and 9, we have shown that such transglycosylases are also present in apple fruit. Until now, only the XETs from *Vigna angularis* [15] and from nasturtium seeds [16] have been purified to homogeneity, and their N-terminal amino acid sequences were used to isolate several cDNA clones from various plant tissues [54-56]. The various cDNA sequences were compared and from these results a phylogenetic tree was constructed [55,57]. It appeared that the nasturtium XET was only distantly related to all other XETs which are currently known. Recently, a novel xyloglucan-specific enzyme was purified from extracts of azuki bean epicotyls, and this enzyme was claimed to lack transglycosylase activity [58]. It is unknown if this enzyme bears any sequence identity with the XETs mentioned above.

Although the various XETs are characterized reasonably well with respect to their cDNA sequences, a rather incomplete picture of their biochemical features exists. However, when combining the data from the literature, we believe that, with respect to (donor) substrate specificity and catalytic mechanism, the xyloglucan-specific glucanases from plants can be subdivided into three groups: (i) cleaves only large molecules and is a strict transglycosylase; (ii) cleaves also smaller molecules and favours transglycosylation over hydrolysis; (iii) cleaves only large molecules and is a strict hydrolase. It seems likely that the differences between these three groups of enzymes find their origin in substrate-binding, as is the case with the endoglucanases of *Trichoderma viride*. However, the (donor) substrate specificity and/or transglycosylase activity suggests that substrate-binding in plant xyloglucanases differs from that of fungal xyloglucanases. Some thoughts on the mechanism(s) of substrate-binding of plant xyloglucanases will be elaborated below. With respect to the following, it should be kept in mind that the different (groups of) enzymes have never been compared directly using similar assay conditions and substrates. This is certainly of great importance, and the (near) future will show if our classification is correct.

Before speculating on substrate-binding by XET, XET is first compared with a different type of enzyme, *i.e.* bacterial ß-(1→3)(1→4)-glucanases. XET and the bacterial ß-(1→3)(1→4)-glucanases can be assigned to the same protein family (no. 16) [53], which implies that these proteins are folded in a similar way. From two of the ß-(1→3)(1→4)-glucanases (*Bacillus*) the three-dimensional structure has been solved [59-61], and it may be expected that some of this structural information also applies to XET. The ß-(1→3)(1→4)-glucanases are compact proteins with a sandwich-like jellyroll architecture. A groove, where the actual catalysis takes place, spans the concave side of the molecule. The other side of the enzyme contains a Ca^{2+} binding site which probably plays a role in stabilizing the protein, together with one disulfide bridge. This seems to be in agreement with Fry's observations [14] that addition of $CaCl_2$ or mercaptoethanol influences XET activity. Another important observation is that the catalytic site residues of these ß-(1→3)(1→4)-glucanases, Glu105 and Glu109, are not located in the center of the substrate-binding groove, but towards the "reducing end" of the groove [61]. If we extrapolate this to XET, less substrate-binding sites towards the reducing end of the catalytic site are expected (see Fig. 2C). Finally, the amino acid sequence Asp-Glu-Ile-Asp-*Ile*-Glu-Phe-

Leu-Gly, which was postulated to play an important role in the actual cleavage of ß-glucan by the ß-(1→3)(1→4)-glucanases [62], is conserved in all XETs, with the exception of the italicized Ile residue (replaced by Phe in XETs) [56,57]

Based on the observation that the average mol wt of xyloglucan remains the same during treatment with XET from *Vigna angularis*, it was concluded that this XET has exclusively transglycosylase activity (xyloglucan as acceptor substrate), and no hydrolase activity (water as acceptor substrate) [15,57]. Similar results have been reported recently for a tomato XET [63]. In order to explain this, Nishitani [57] has suggested that first the acceptor substrate should bind to the enzyme (see Fig. 2C), before the actual binding and subsequent cleavage of the donor molecule takes place. In this way water may be excluded from the catalytic site. It has been shown that the transglycosylation reaction occurs only when the acceptor substrate meets certain requirements. No transglycosylation was observed with cellobiose, cellotetraose to cellohexaose, laminarihexaose, XGG and GXG as acceptor substrate [14,15,40]. XXG appeared to be the smallest acceptor molecule, and from this it was concluded that XET requires acceptor substrates with two adjacent X elements. These results suggest that the binding of acceptor molecules is mediated by the Xyl sidechains, rather than by the glucan backbone. Further, it has been shown that XXXG is a better acceptor than XXG [40], whereas XXXGXXXG-based structures (and presumably larger molecules as well) seem equally effective as XXXG [15]. Therefore, three Xyl-binding sites ("I", "II", and "III") are indicated in Fig. 2C.

Another interesting feature of the *Vigna angularis* XET is that a minimum length of the donor substrate (10 kDa or more) is required for activity [15]. Given the dimensions of the XET protein (*c.* 32 kDa), it seems very unlikely that this specificity is determined by an impressive number of substrate-binding sites [61]. Another possibility is that XET only binds xyloglucans with a certain conformation in its putative substrate-binding sites "-*n*" to "-I" (exact number unknown), and that this conformation depends on the mol wt of the xyloglucan. The observation that xyloglucan molecules with a mol wt smaller than 10 kDa do not stain with iodine, whereas larger ones do, supports this view [64]. With respect to iodine staining, similar observations have been done for amylose. Investigations of the conformation of amylose in solution [65,66] have suggested that amylose is built from several helical segments (responsible for iodine staining), which are interrupted by more random parts.

Summarizing, the mechanism of action of *Vigna* XET may be pictured as follows. First, the Xyl residues of the acceptor substrate (either an oligosaccharide or the nonreducing building unit of a polysaccharide) strongly bind to substrate-binding sites "I" to "III". Second, the substrate-binding sites to the left of the catalytic residue bind segments of a xyloglucan donor molecule with the appropriate conformation. Both the donor and the acceptor molecule are now precisely positioned, and the actual transglycosylation can proceed. In order to explain the release of the hybrid xyloglucan molecule from the enzyme, we postulate that the conformation of the donor molecule has changed as a result of the cleavage. The hybrid molecule is not recognized anymore by substrate-binding sites "-*n*" to "-I", and is ejected. The repulsive forces in these substrate-

binding sites should exceed the attractive binding of Xyl residues of the hybrid molecule in substrate-binding sites "I" to "III". In principle, it can not be ruled out that a donor molecule interacts with substrate-binding sites "-*n*" to "III", *i.e.* a mid-chain xyloglucan oligosaccharide binds in subsites "I" to "III". The product of this reaction would be the donor molecule, which is very inefficient in catalytic terms. Therefore, it would not be surprising when subsites "I" to "III" specifically bind oligosaccharides or nonreducing building units of larger xyloglucan molecules, having a different conformation than the donor molecules.

The nasturtium XET is a member of the second group of plant xyloglucanases (Fig. 2D). Contrary to the XET from *Vigna* [15], the nasturtium XET seems also capable of xyloglucan hydrolysis (water as an acceptor substrate) [16,55,67,68], although the transglycosylation reaction is favoured [68]. An explanation for this might be that subsites "I" to "III" do not have such a high affinity for xyloglucan acceptor molecules as those in the *Vigna* XET. As a result of this water may get the opportunity to compete as an acceptor substrate with xyloglucan molecules. Therefore, substrate-binding sites "I" to "III" are indicated in Fig. 2D with a lighter shading pattern compared to Fig. 2C. The presence of both hydrolases and transglycosylases within one enzyme family is not unique for XETs, but has also been observed with α-amylases and cyclodextrin glycosyltransferases [53,69,70]. Although a number of these enzymes have been studied in much greater detail than XETs, the basis for the actual transglycosylation activity is still not understood [71]. A second important difference between the *Vigna* and the nasturtium XET is that the latter enzyme can cleave small (for instance $[xg]_2$) [16] as well as large xyloglucan donor molecules [67,68]. Presumably, the nasturtium XET does not discriminate between xyloglucans with a different conformation. In this respect the nasturtium XET behaves like endoIV or endoV. We speculate that there is a relationship between the lower subsite affinity of subsite "I" to "III" of nasturtium XET and its less stringent donor substrate specificity. Because the acceptor substrate is not as tightly bound as is the case with the *Vigna* XET, the hybrid xyloglucan molecules are much easier released from the enzyme, and an extra impulse by conformational changes of the donor molecule is not required.

The xyloglucanase from azuki beans described by Tabuchi *et al.* [58] may be the first member of a third group of plant xyloglucanases. This enzyme probably shows a similar donor substrate specificity as the *Vigna* XET, because only large xyloglucan molecules are cleaved (indicated by a twisted molecule in Fig. 2E). Further, this enzyme does not have any transglycosylase activity under the conditions tested. It seems likely that subsites "I" to "III" do not bind Xyl sidechains of xyloglucan at all (subsites "I" to "III" not shaded in Fig. 2E).

The speculations above suggest that the endoglucanases from *Trichoderma viride* and plant xyloglucanases differ considerably in substrate-binding. The *Trichoderma* endoglucanases recognize (part of) any ß-(1→4)-glucan backbone, whereas the *Vigna* XET is very specific for xyloglucan, probably recognizes certain conformations in the donor

substrate and Xyl sidechains in the acceptor substrate, and only catalyzes transglycosylation reactions. The nasturtium XET and the azuki xyloglucanase are also highly specific for xyloglucan, but have an intermediary position with respect to their mechanism of catalysis (transglycosylation or hydrolysis), and presumably differ in their ability to cleave smaller xyloglucan fragments (nasturtium). The special requirements for plant xyloglucanases make it very difficult to perform subsite mapping as described for endoglucanases from *Trichoderma* in Chapter 6.

In Chapter 5 we have shown that POsxg is a very useful substrate to detect differences in the mode of action of endoglucanases. It will be worthwhile to investigate the action of XET towards POsxg. It is expected that the plant xyloglucanases will cleave in a similar manner as endoIV (XXGG↓XXGG), because the other alternative (XXG↓GXXGG) will generate molecules which are not suitable as acceptor substrates in transglycosylation reactions. Hereby, we assume that multiple forms of XET have similar acceptor requirements [40], but this has not (yet) been substantiated. Further, potato xyloglucan is a good source for new acceptor substrates such as LSGG. It would be interesting to investigate whether XET can utilize oligosaccharides with a disaccharide sidechain at the nonreducing end as acceptor substrate.

IMPLICATIONS FOR FRUIT JUICE PROCESSING

In Chapter 1 of this thesis a short overview was given on the introduction of enzyme preparations in fruit juice processing. The liquefaction process was discussed as one of the more recent developments in this field. For this process, technologists have relied on rather crude cocktails, containing multiple enzyme activities. However, emerging genetic techniques will enable them in the near future to switch from these ill-defined enzyme mixtures to much more specific, recombinant single enzyme components [72]. By combining these components, tailor-made preparations can be formulated which are easy to adapt to any kind of application or (maturity of) fruit. The main objective of our study was to investigate which glucanases should be used in these formulations to achieve an optimal liquefaction of apples.

Previous research in our laboratory had resulted in the purification of nine glucanases from *Trichoderma viride*: endoI to endoVI and exoI to exoIII [73]. In our study, we have focussed on the substrate specificity of the endoglucanases. The six endoglucanases were subjected to subsite analysis, and it appeared that endoII and endoVI were very similar to endoI and endoV, respectively (data not shown). In fact, endoI and endoII differed only in their binding to and activity towards crystalline cellulose [74]; the hydrolytic properties towards soluble substrates were similar. This, and a comparison of the mol wt of the two enzymes, suggest that endoII represents the endoI catalytic core (thus, endoI without CBD). For similar reasons, endoVI probably represents the endoV core protein. It has been reported before that endoglucanases can be truncated in the culture filtrates of

Trichoderma viride [75]. EndoIII has four substrate-binding sites (data not shown). This, in combination with other characteristics of the enzyme [73,74], suggests that endoIII is similar to CBHII from the literature [76-78]. From the exoglucanases, only exoIII has been used; exoI and exoII were available in small amounts. ExoIII is the most abundant protein in the *Trichoderma* preparation [73]. Further, the properties of exoIII correspond very well to those of CBHI [79], which suggests that these enzymes are similar. In order to verify these comparisons, N-terminal sequencing of our glucanase-proteins was performed. However, it appeared that the N-termini of all glucanases were blocked. The endoIV protein was fragmented by CNBr treatment, and an internal sequence of twenty amino acids was obtained (PTTASWSGSGRNIRANVAYD) which closely matched (90%) part of the sequence reported in the literature (PTTASWSYSGSNIRANVAYD) [80].

Based on the above reasonings, we have restricted our investigations to endoI, endoIV and endoV. With respect to tailor-made enzyme preparations for liquefaction of apple fruit, we have shown that not just any endoglucanase can be used. EndoIV is important, not only for loosening the cell wall in cooperation with PL, but it can also play a role in fine-tuning the actual cellulose degradation in the primary cell wall (Chapter 2). EndoV is probably more suitable for this purpose because it is the most versatile endoglucanase. It can be used for stripping the xyloglucan coating from cellulose as well as for cellulose degradation itself. Further, it seems reasonable to expect that endoV will act synergistically with pectinases. Therefore, the combination of endoI plus endoIV (Chapter 2) was replaced by endoV in liquefaction experiments (Chapter 8). In Chapter 8, it was demonstrated that xyloglucanase activity plays a role in fine-tuning the degradation of cellulose in apple fruit tissue, which was in accordance with earlier results using apple WUS. Next to this, another function of xyloglucanase activity in the degradation of apple fruit tissue was proposed, *e.g.* generating oligosaccharides which can serve as acceptor substrate for XET in apple. This synergism between fungal and apple xyloglucanases might explain why raw apple material disintegrates much faster than blanched apple material upon treatment with a combination of PL, endoV and CBH. In Chapter 9, additional evidence for this hypothesis was obtained by showing that the amount of cellulase needed for complete disintegration of apple tissue is inversely proportional to the level of XET activity in apple. Although our data support an important role for XET in apple liquefaction, it should be kept in mind that other (unknown) enzymes might be induced at the same stage of maturity as XET, and that structural changes in the apple can not be ruled out. A number of questions remains. It is, for instance, not known to what extent the size of an enzyme influences its efficiency in liquefaction. The relatively small size of endoIV (23.5 kDa) compared to endoV (57 kDa) may proove an important factor in penetrating apple tissue. The difference in size of these enzymes is such that a different diffusion may be expected [81,82]. Further, the role of endoIII remains to be clarified. If this enzyme is similar to CBHII, then a faster conversion of cellulose is to be expected ("*exo-exo*" synergism).

An interesting observation in Chapter 9 was that a controlled degradation of the cellulose-xyloglucan network can result in a cloudy apple juice. Until now, such juices

were produced by a conventional pressing operation, but not by an operation involving cellulases. The clouds from conventional processes consisted of a positively charged protein nucleus with a negatively charged coating of pectin (Fig. 3A) [83]. Electrostatic repulsion of negatively charged particles prevented the cloud from flocculating. The cloud is destroyed by treatment with PG (plus PME) or PL which results in a partial degradation of the coating, and a subsequent aggregation of cloud particles [84,85]. The action of PME in combination with the presence of Ca^{2+} also has a negative effect on the cloud stability [86]. As the demethylation of pectin progresses, cloud particles will eventually precipitate with Ca^{2+}. PME from plants is more efficient in this respect than fungal PME. Plant PME deesterifies in a blockwise-fashion and consequently the resulting pectins are very sensitive to Ca^{2+} [87]. Fungal PME acts more at random but, in this case more methyl groups have to be removed before "egg-box" structures can be formed.

Our results show that a strictly controlled liquefaction can result in a cloud of a completely different nature. These clouds are built predominantly from cellulose microfibrils and xyloglucan (Chapter 9). The stability of this type of cloud probably finds its origin in steric repulsion; xyloglucans prevent spontaneous contact formation of cellulose particles (see Fig. 3B). The observation that XETs are not active towards cellulose [14], and that *Vigna* XET degrades xyloglucan only partially [15], suggest that XET may be a very suitable enzyme for making cloudy apple juice by liquefaction. It is speculated that cloud stability can be affected by many enzymes. EndoI, CBH (both enzymes are less efficient on cellulose that is coated with xyloglucan) and especially endoV are undesirable, because these glucanases can degrade the cellulose moiety of the particles (especially when allowed to act together). EndoIV and endoV core protein will trim the xyloglucan "tails" sticking out in solution. However, it is unknown whether a minimum length of these tails is required for a stable cloud. Therefore, the effect of endoIV and endoV core protein, or xyloglucanases in general, on cloud stability needs to be further investigated. Cloud stability may also be manipulated by nonhydrolytic proteins such as CBDs. Depending on their origin, CBDs can differ considerably in their affinity for substrates such as amorphous cellulose, crystalline cellulose and chitin [88-90]. In principle, CBDs have the ability to disrupt cellulose microfibrils [91], and to disperse cellulose particles [92]. With respect to plant cell wall cellulose, it is speculated that they can play a role in exposing xyloglucans that were previously entrapped inside cellulose fibres. An advantage of this is that hydration of the cloud particles may be improved, which presumably has a positive effect on cloud stability. It is important to realize that xyloglucan molecules are much more accessible for xyloglucanases in this situation.

The discussion above points out that the stability of cellulose-xyloglucan clouds may be influenced by several components from cellulase preparations. This implies that the new concept for a cloudy juice can not be realized by using the classical approach of formulating enzyme preparations for liquefaction, *i.e.* combining the excretion products of different fungi, or the same fungus grown under different conditions. Most certainly, this will introduce undesirable enzyme activities, which emphasizes the need for tailor-made enzyme preparations. At this moment, the new "cloudy juice concept" relies heavily on a

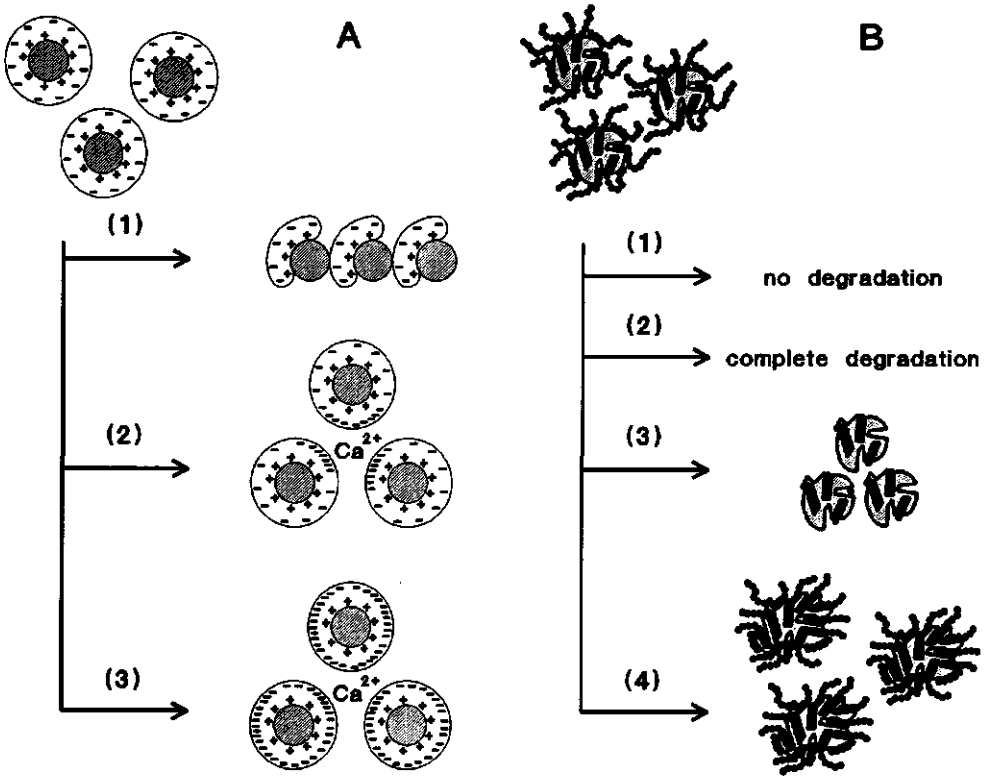

FIGURE 3. Schematic representation of two different clouds in apple juice and factors influencing their stability. A, "conventional" cloud in apple juice, obtained after pressing, is composed of a protein nucleus (shaded circle) with a coating of pectin (open circle) [83]. Cloud stability is lost upon action of PG or PL (1) [84], plant PME (2) or fungal PME (3). B, a cloud in apple juice obtained by controlled liquefaction. Shaded figures represent a cross section of cellulose particles; •, individual xyloglucan oligosaccharides. The influence of various enzymes and enzyme domains is hypothesized. XET (1) will not degrade the xyloglucan "tails" further because its activity declines when xyloglucan chains become shorter. In principle, cloud loss will be achieved with endoI, endoV or CBH (2). A combination of these enzymes will enhance this process. Action of endoIV and endoV core protein (3) will trim the xyloglucan tails. The implications of this are still unclear; steric repulsion is diminished which probably leads to destabilization of the cloud. CBDs can disrupt the cellulose surface (4). CBDs will increase the hydration of cloud particles, and in the absence of xyloglucanases a more stable cloud is expected.

ripening-related endogenous enzyme, XET. There are two options to make the process less dependent on the degree of ripening of the raw material. First, the underlying mechanism of the stability of cellulose-xyloglucan clouds should be further studied. If the length of the xyloglucan tails appears to be unimportant for stability, then controlled liquefaction to a cloudy juice may be achieved relatively easy by using pectinases in combination with a pure xyloglucanase. If a minimum length of the xyloglucan tails is required, an option might be to include XET in tailor-made enzyme preparations. With

amino acid sequences of XET available, this will probably be possible in near future [54-57]. It may be anticipated that, with respect to cell wall degradation, exogenous XET does not cut crosslinks as fast as endogenous XET because it has to penetrate the cell wall by diffusion. A disadvantage of most XETs is that its depolymerizing mode has to be triggered by oligosaccharides. With respect to our application, the xyloglucanase from azuki bean [58] seems to be most suitable: this enzyme does not need oligosaccharides for depolymerization of xyloglucan (hydrolase), and looses activity with decreasing length of xyloglucan molecules.

Juice manufacturers often observe that the amount of enzyme required for complete liquefaction depends on the maturity of the apple fruit. Our results indicate that the presence of XET in apple tissue, and its synergism with fungal endoglucanases, provides a plausible explanation for these observations (Chapter 8 and 9). In order to substantiate this further, activity spectra of various enzyme preparations used in industrial liquefaction are needed, as well as an inventory on their performance as a function of time. An interesting thought is that the throughput of apples in the liquefaction process could be increased if all apples would contain a high level of XET. In such a case, tissue will disintegrate much faster and, consequently, cell wall degradation will not suffer from a limited diffusion of enzymes. Construction of apples with an elevated level of XET by genetic engineering would be necessary to achieve this, but this is still a long way to go. Further, it is not known whether this has an effect on the physiological processes in the apple fruit.

Finally, it is important to note that our results have a much broader field of application than just apples. Cellulose and xyloglucan are major cell wall polysaccharides in many fruits and vegetables, and also XET occurs ubiquitously [14]. This thesis shows that modification of cellulose-xyloglucan networks deserves more attention with respect to (fruit juice) processing than has been the case.

REFERENCES

[1] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., and Warren, R.A.J. (1991) Domains of microbial ß-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55**, 303-315.

[2] Béguin, P., and Aubert, J.-P. (1994) The biological degradation of cellulose. *FEMS Microbiol. Rev.* **13**, 25-58.

[3] Din, N., Damude, H.G., Gilkes, N.R., Miller, R.C., Warren, R.A.J., and Kilburn, D.G. (1994) C_1-C_x revisited: intramolecular synergism in a cellulase. *Proc. Natl. Acad. Sci. USA* **91**, 11383-11387.

[4] Singh, A., and Hayashi, K. (1995) Microbial cellulases: protein architecture, molecular properties, and biosynthesis. *Adv. Appl. Microbiol.* **40**, 1-44.

[5] Teeri, T.T., and Koivula, A. (1995) Cellulose degradation by native and engineered fungal cellulases. *Carbohydrates in Europe* **12**, 28-33.

[6] York, W.S., van Halbeek, H., Darvill, A.G., and Albersheim, P. (1990) Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and Fast-Atom-Bombardment Mass Spectrometry. *Carbohydr. Res.* **200**, 9-31.

[7] York, W.S., Harvey, L.K., Guillén, R., Albersheim, P., and Darvill, A.G. (1993) Structural analysis of tamarind seed xyloglucan oligosaccharides using ß-galactosidase digestion and spectroscopic methods. *Carbohydr. Res.* **248**, 285-301.

[8] York, W.S., Impallomeni, G., Hisamatsu, M., Albersheim, P., and Darvill, A.G. (1995) Eleven newly characterized xyloglucan oligoglycosyl alditols: the specific effects of sidechain structure and location on ^{1}H NMR chemical shifts. *Carbohydr. Res.* **267**, 79-104.

[9] Hisamatsu, M., Impallomeni, G., York, W.S., Albersheim, P., and Darvill, A.G. (1991) A new undecasaccharide subunit of xyloglucans with two α-L-fucosyl residues. *Carbohydr. Res.* **211**, 117-129.

[10] Hisamatsu, M., York, W.S., Darvill, A.G., and Albersheim, P. (1992) Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues. *Carbohydr. Res.* **227**, 45-71.

[11] Guillén, R., York, W.S., Pauly, M., An, J., Impallomeni, G., Albersheim, P., and Darvill, A.G. (1995) Metabolism of xyloglucan generates xylose-deficient oligosaccharide subunits of this polysaccharide in etiolated peas. *Carbohydr. Res.* **277**, 291-311.

[12] McCann, M.C., and Roberts, K. (1994) Changes in cell wall architecture during cell elongation. *J. Exp. Bot.* **45**, 1683-1691.

[13] Roberts, K. (1994) The plant extracellular matrix: in new expansive mood. *Curr. Opin. Cell Biol.* **6**, 688-694.

[14] Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828.

[15] Nishitani, K., and Tominaga, R. (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J. Biol. Chem.* **267**, 21058-21064.

[16] Fanutti, C., Gidley, M.J., and Reid, J.S.G. (1993) Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1→4)-ß-D-glucanase) from the cotelydons of germinated nasturtium seeds. *Plant J.* **3**, 691-700.

[17] Fry, S.C. (1995) Polysaccharide-modifying enzymes in the plant cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 497-520.

[18] McQueen-Mason, S.J., Durachko, D.M., and Cosgrove, D.J. (1992) Two endogenous proteins that induce cell wall extension in plants. *Plant Cell* **4**, 1425-1433.

[19] McQueen-Mason, S.J., and Cosgrove, D.J. (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc. Natl. Acad. Sci. USA* **91**, 6574-6578.

[20] Shcherban, T.Y., Shi, J., Durachko, D.M., Guiltinan, M.J., McQueen-Mason, S.J., Shieh, M., and Cosgrove, D.J. (1995) Molecular cloning and sequence analysis of expansins—a highly conserved, multigene family of proteins that mediate cell wall extension in plants. *Proc. Natl. Acad. Sci. USA* **92**, 9245-9249.

[21] Renard, C.M.G.C., Searle-van Leeuwen, M.J.F., Voragen, A.G.J., Thibault, J.-F., and Pilnik, W. (1991) Studies on apple protopectin. II. Apple cell wall degradation by pure polysaccharidases and their combinations. *Carbohydr. Polym.* **14**, 295-314.

[22] Renard, C.M.G.C., Schols, H.A., Voragen, A.G.J., Thibault, J.-F., and Pilnik, W. (1991) Studies on apple protopectin. III. Characterization of material extracted by pure polysaccharidases from apple cell walls. *Carbohydr. Polym.* **15**, 13-32.

[23] Gibeaut, D.M., and Carpita, N.C. (1994) Biosynthesis of plant cell wall polysaccharides. *FASEB J.* **8**, 904-915.

[24] Hayashi, T. (1989) Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 139-168.

[25] McDougall, G.J., and Fry, S.C. (1994) Fucosylated xyloglucan in suspension-cultured cells of the graminaceous monocotyledon, *Festuca arundinacea*. *J. Plant Physiol.* **143**, 591-595.

[26] Kiefer, L.L., York, W.S., Albersheim, P., and Darvill, A.G. (1990) Structural characterization of an arabinose-containing heptadecasaccharide enzymically isolated from sycamore extracellular xyloglucan. *Carbohydr. Res.* **197**, 139-158.

[27] Renard, C.M.G.C., Lomax, J.A., and Boon, J.J. (1992) Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and alkali-extracted xyloglucans. *Carbohydr. Res.* **232**, 303-320.

[28] Ohsumi, C., and Hayashi, T. (1994) The oligosaccharide units of the xyloglucans in the cell walls of bulbs of onion, garlic and their hybrid. *Plant Cell Physiol.* **35**, 963-967.

[29] Hayashi, T., and Takeda, T. (1994) Compositional analysis of the oligosaccharide units of xyloglucans from suspension-cultured poplar cells. *Biosci. Biotech. Biochem.* **58**, 1707-1708.

[30] Braccini, I., Hervé du Penhoat, C., Michon, V., Goldberg, R., Clochard, M., Jarvis, M.C., Huang, Z.-H., and Gage, D.A. (1995) Structural analysis of cyclamen seed xyloglucan oligosaccharides using cellulase digestion and spectroscopic methods. *Carbohydr. Res.* **276**, 167-181.

[31] Ring, S.G., and Selvendran, R.R. (1981) An arabinogalactoxyloglucan from the cell wall of *Solanum tuberosum*. *Phytochem.* **20**, 2511-2519.

[32] Mori, M., Eda, S., and Kato, K. (1980) Structural investigation of the arabinoxyloglucan from *Nicotiana tabacum*. *Carbohydr. Res.* **84**, 125-135.

[33] Hayashi, T., and Matsuda, K. (1981) Biosynthesis of xyloglucan in suspension-cultured soybean cells. Occurrence and some properties of xyloglucan 4-ß-D-glucosyltransferase and 6-α-D-xylosyltransferase. *J. Biol. Chem.* **256**, 11117-11122.

[34] Gordon, R., and Maclachlan, G. (1989) Incorporation of UDP-[^{14}C]glucose into xyloglucan by pea membranes. *Plant Physiol.* **91**, 373-378.
[35] Kato, Y., Iki, K., and Matsuda, K. (1981) Cell-wall polysaccharides of immature barley plants. II. Characterization of a xyloglucan. *Agric. Biol. Chem.* **45**, 2745-2753.
[36] Kato,Y., Ito, S., Iki, K., and Matsuda, K. (1982) Xyloglucan and ß-D-glucan in cell walls of rice seedlings. *Plant Cell Physiol.* **23**, 351-364.
[37] Lahaye, M., Jegou, D., and Buleon, A. (1994) Chemical characteristics of insoluble glucans from the cell wall of the marine green alga *Ulva lactuca* (L.) Thuret. *Carbohydr. Res.* **262**, 115-125.
[38] Ray, B., and Lahaye, M. (1995) Cell-wall polysaccharides from the marine green alga *Ulva "rigida"* (Ulvales, Chlorophyta). Extraction and chemical composition. *Carbohydr. Res.* **274**, 251-261.
[39] Mabusela, W.T., Stephen, A.M., Rodgers, A.L., and Gerneke, D.A. (1990) A highly substituted glucan that coats the seeds of *Helipterum eximium*. *Carbohydr. Res.* **203**, 336-340.
[40] Lorences, E.P., and Fry, S.C. (1993) Xyloglucan oligosaccharides with at least two α-D-xylose residues act as acceptor substrates for xyloglucan endotransglycosylase and promote the depolymerisation of xyloglucan. *Physiol. Plant.* **88**, 105-112.
[41] Beldman, G., Voragen, A.G.J., Rombouts, F.M., and Pilnik, W. (1988) Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from *Trichoderma viride*. *Biotechnol. Bioeng.* **31**, 173-178.
[42] Wood, T.M. (1989) Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi. In *Enzyme Systems for Lignocellulose Degradation.* (Coughlan, M.P., ed., Elsevier, London), 19-35.
[43] Fägerstam, L.G., and Pettersson, L.G. (1980) The 1,4-ß-glucan cellobiohydrolases of *Trichoderma reesei* QM 9414. *FEBS Lett.* **119**, 97-101.
[44] Nidetzky, B., Hayn, M., Macarron, R., and Steiner, W. (1993) Synergism of *Trichoderma reesei* cellulases while degrading different celluloses. *Biotechnol. Lett.* **15**, 71-76.
[45] Nidetzky, B., Steiner, W., Hayn, M., and Claeyssens, M. (1994) Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. *Biochem. J.* **298**, 705-710.
[46] Chanzy, H., Henrissat, B., Vuong, R., and Schülein, M. (1983) The action of 1,4-ß-D-glucan cellobiohydrolase on *Valonia* cellulose microcrystals. An electron microscopic study. *FEBS Lett.* **153**, 113-118.
[47] Chanzy, H., and Henrissat, B. (1985) Undirectional degradation of *Valonia* cellulose microcrystals subjected to cellulase action. *FEBS Lett.* **184**, 285-288.
[48] Biely, P., Vrsanska, M., and Claeyssens, M. (1993) Mode of action of *Trichoderma reesei* ß-1,4-glucanases on cellooligosaccharides. In Proceedings of the Second TRICEL Symposium on Trichoderma reesei *Cellulases and other Hydrolases* (Suominen, P., and Reinikainen, T., eds., Foundation for Biotechnical and Industrial Fermentation Research, Helsinki, Finland), 99-108.
[49] Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. (1988) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* **170**, 575-581.
[50] Gilkes, N.R., Warren, R.A.J., Miller, R.C., and Kilburn, D.G. (1988) Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *J. Biol. Chem.* **263**, 10401-10407.
[51] Srisodsuk, M., Reinikainen, T., Penttilä, M., and Teeri, T.T. (1993) Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. *J. Biol. Chem.* **268**, 20756-20761.
[52] Reese, E.T., Sui, R.G.H., and Levinson, H.S. (1950) Biological degradation of soluble cellulose derivatives. *J. Bacteriol.* **59**, 485-497.
[53] Henrissat, B. (1994) Classification of glycosyl hydrolases based on amino acid sequence similarities. *Cellulase Nomenclature Meeting*, Lake Tahoe, USA.
[54] De Silva, J., Jarman, J.D., Arrowsmith, D.A., Stronach, M.S., Chengappa, S., Sidebottom, C., and Reid, J.S.G. (1993) Molecular characterization of a xyloglucan-specific endo-(1→4)-ß-D-glucanase (xyloglucan endo-transglycosylase) from nasturtium seeds. *Plant J.* **3**, 701-711.
[55] De Silva, J., Arrowsmith, D., Hellyer, A., Whiteman, S., and Robinson, S. (1994) Xyloglucan endotransglycosylase and plant growth. *J. Exp. Bot.* **45**, 1693-1701.
[56] Okazawa, K., Sato, Y., Kakagawa, T., Asada, K., Kato, I., Tomita, E., and Nishitani, K. (1993) Molecular cloning and cDNA sequencing of endo-xyloglucan transferase, a novel class of glycosyl transferase that mediates molecular grafting between matrix polysaccharides in plant cell walls. *J. Biol. Chem.* **268**, 25364-25368.
[57] Nishitani, K. (1995) Endo-xyloglucan transferase, a new class of transferase involved in cell wall construction. *J. Plant Res.* **108**, 137-148.
[58] Tabuchi, A., Kamisaka, S., and Hoson, T. (1995) Novel xyloglucan-hydrolyzing enzymes in the cell wall of azuki bean epicotyls. *Plant Cell Physiol.* **36**, s47.
[59] Keitel, T., Simon, O., Borriss, R., and Heinemann, U. (1993) Molecular and active-site structure of a *Bacillus* 1,3-1,4-ß-glucanase. *Proc. Natl. Acad. Sci. USA* **90**, 5287-5291.

[60] Hahn, M., Olsen, O., Politz, O., Borriss, R., and Heinemann, U. (1995) Crystal structure and site-directed mutagenesis of *Bacillus macerans* endo-1,3-1,4-ß-glucanase. *J. Biol. Chem.* **270**, 3081-3088.
[61] Hahn, M., Keitel, T., and Heinemann, U. (1995) Crystal and molecular structure at 0.16-nm resolution of the hybrid *Bacillus* endo-1,3-1,4-ß-D-glucan 4-glucanohydrolase H(A16-M). *Eur. J. Biochem.* **232**, 849-858.
[62] Borriss, R., Büttner, K., and Mäntsälä, P. (1990) Structure of the ß-(1,3)(1,4)-glucanase gene of *Bacillus macerans*: homologies to other ß-glucanases. *Mol. Gen. Genet.* **222**, 278-283.
[63] Arrowsmith, D.A., and de Silva, J. (1995) Characterisation of two tomato fruit-expressed cDNAs encoding xyloglucan *endo*-transglycosylase. *Plant Mol. Biol.* **28**, 391-403.
[64] Sulova, Z., Lednická, M., and Farkaš, V. (1995) A colorimetric assay for xyloglucan-endotransglycosylase from germinating seeds. *Anal. Biochem.* **229**, 80-85.
[65] Szejtli, J., and Augustat, S. (1966) Über die Konfiguration der Amylosemoleküle in wässriger Lösung. *Stärke* **18**, 38-52.
[66] Kodama, M., Noda, H., and Kamata, T. (1978) Conformation of amylose in water. I. Light-scattering and sedimentation-equilibrium measurements. *Biopolym.* **17**, 985-1002.
[67] Edwards, M., Dea, I.C.M., Bulpin, P.V., and Reid, J.S.G. (1986) Purification and properties of a novel xyloglucan-specific endo-(1→4)-ß-D-glucanase from germinated nasturtium seeds (*Tropaeolum majus* L.). *J. Biol. Chem.* **261**, 9489-9494.
[68] Farkas, V., Sulova, Z., Stratilova, E., Hanna, R., and Maclachlan, G. (1992) Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. *Arch. Biochem. Biophys.* **298**, 365-370.
[69] Jespersen, H.M., MacGregor, E.A., Henrissat, B., Sierks, M.R., and Svensson, B. (1993) Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic $(ß/\alpha)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes. *J. Prot. Chem.* **12**, 791-805.
[70] Wind, R.D., Liebl, W., Buitelaar, R.M., Penninga, D., Spreinat, A., Dijkhuizen, L., and Bahl, H. (1995) Cyclodextrin formation by the thermostable α-amylase of *Thermoanaerobacterium thermosulfurigenes* EM1 and reclassification of the enzyme as a cyclodextrin glycosyltransferase. *Appl. Environ. Microbiol.* **61**, 1257-1265.
[71] Penninga, D., Strokopytov, B., Rozeboom, H.J., Lawson, C.L., Dijkstra, B.W., Bergsma, J., and Dijkhuizen, L. (1995) Site-directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 affect activity and product specificity. *Biochem.* **34**, 3368-3378.
[72] Dalbøge, H., and Heldt-Hansen, H.P. (1994) A novel method for efficient expression cloning of fungal enzyme genes. *Mol. Gen. Genet.* **243**, 253-260.
[73] Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., and Voragen, A.G.J. (1985) The cellulase of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases. *Eur. J. Biochem.* **146**, 301-308.
[74] Beldman, G., Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F., and Pilnik, W. (1987) Adsorption and kinetic behaviours of purified endoglucanases and exoglucanases from *Trichoderma viride*. *Biotechnol. Bioeng.* **30**, 251-257.
[75] Ståhlberg, J., Johansson, G., and Pettersson, G. (1988) A binding-site-deficient, catalytically active, core protein of endoglucanase III from the culture filtrate of *Trichoderma reesei*. *Eur. J. Biochem.* **173**, 179-183.
[76] Van Tilbeurgh, H., Pettersson, G., Bhikabhai, R., de Boeck, H., and Claeyssens, M. (1985) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-ß-glucan cellobiohydrolase II. *Eur. J. Biochem.* **148**, 329-334.
[77] Teeri, T.T., Lehtovaara, P., Kauppinen, S., Salovuori, I., and Knowles, J. (1987) Homologous domains in *Trichoderma reesei* cellulolytic enzymes: gene sequence and expression of cellobiohydrolase II. *Gene* **51**, 43-52.
[78] Penttilä, M., André, L., Lehtovaara, P., Bailey, M., Teeri, T.T., and Knowles, J.K.C. (1988) Efficient secretion of two fungal cellobiohydrolases by *Saccharomyces cerevisiae*. *Gene* **63**, 103-112.
[79] Suominen, P.L., Mäntylä, A.L., Karhunen, T., Hakola, S., and Nevalainen, H. (1993) High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. *Mol. Gen. Genet.* **241**, 523-530.
[80] Ward, M., Wu, S., Dauberman, J., Weiss, G., Larenas, E., Bower, B., Rey, M., Clarkson, K., and Bott, R. (1993) Cloning, sequence and preliminary structural analysis of a small, high pI endoglucanase (EGIII) from *Trichoderma reesei*. In Proceedings of the Second TRICEL Symposium on Trichoderma reesei *Cellulases and other Hydrolases* (Suominen, P., and Reinikainen T., eds., Foundation for Biotechnical and Industrial Fermentation Research 8, Helsinki, Finland), 153-158.
[81] Carpita, N., Sabularse, D., Montezinos, D., and Delmer, D.P. (1979) Determination of the pore size of cell walls of living plant cells. *Sci.* **205**, 1144-1147.
[82] Baron-Epel, O., Gharyal, P.K., and Schindler, M. (1988) Pectins as mediators of wall porosity in soybean cells. *Planta* **175**, 389-395.
[83] Yamasaki, M., Yasui, T., and Arima, K. (1964) Pectic enzymes in the clarification of apple juice. Part I. Study on the clarification reaction in a simplified model. *Agric. Biol. Chem.* **28**, 779-787.

[84] Yamasaki, M., Kato, A., Chu, S.Y., and Arima, K. (1967) Pectic enzymes in the clarification of apple juice. Part II. The mechanism of clarification. *Agric. Biol. Chem.* **31**, 552-560.
[85] Pilnik, W., and Voragen, A.G.J. (1991) The significance of endogenous and exogenous pectic enzymes in fruit and vegetable processing. In *Food Enzymology* (Fox, P.F., ed., Elsevier Applied Science, London) **1**, 303-336.
[86] Pilnik, W., and Voragen, A.G.J. (1991) Effect of enzyme treatment on the quality of processed fruit and vegetables. *Fruit Processing* **1**, 125-129.
[87] Kohn, R., Dongowski, G., and Bock, W. (1985) Die Verteilung der freien und veresterten Carboxylgruppen im Pektinmolekül nach Einwirkung von Pektinesterase aus *Aspergillus niger* und Orangen. *Nahrung* **29**, 75-85.
[88] Coutinho, J.B., Gilkes, N.R., Kilburn, D.G., Warren, R.A.J., and Miller, R.C. (1993) The nature of the cellulose-binding domain affects the activities of a bacterial endoglucanase on different forms of cellulose. *FEMS Microbiol. Lett.* **113**, 211-218.
[89] Linder, M., Lindeberg, G., Reinikainen, T., Teeri, T.T., and Pettersson, G. (1995) The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. *FEBS Lett.* **372**, 96-98.
[90] Tomme, P., Driver, D.P., Amandoron, E.A., Miller, R.C., Warren, R.A.J., and Kilburn D.G. (1995) Comparison of a fungal (family I) and bacterial (family II) cellulose-binding domain. *J. Bacteriol.* **177**, 4356-4363.
[91] Din, N., Gilkes, N.R., Tekant, B., Miller, R.C., Warren, R.A.J., and Kilburn, D.G. (1991) Non-hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulase. *Bio/Technol.* **9**, 1096-1099.
[92] Gilkes, N.R., Kilburn, D.G., Miller, R.C., Warren, R.A.J., Sugiyama, J., Chanzy, H., and Henrissat, B. (1993) Visualization of the adsorption of a bacterial endo-ß-1,4-glucanase and its isolated cellulose-binding domain to crystalline cellulose. *Int. J. Biol. Macromol.* **15**, 347-351.

SUMMARY

Commercial enzyme preparations are frequently applied in the large-scale production of apple juice. The liquefaction process, for instance, aims at a complete degradation of the cell wall polysaccharides which determine the strength of apple tissue to a large extent. Enzymic liquefaction is very attractive from an industrial point of view, because it offers a high juice yield with a relatively low energy input. The commercial preparations used for this purpose are generally undefined mixtures of enzymes obtained by fermentation. In many cases the required activities are present in only limiting amounts. Nowadays, it is possible to produce relatively pure enzymes individually using genetic techniques. This provides the opportunity to formulate tailor-made enzyme preparations which contain all important enzymes for liquefaction in an optimal ratio.

In order to formulate tailor-made preparations it is necessary to know which enzymes are required for liquefaction. The degradation of pectic polysaccharides has been studied extensively in this respect. Relatively little is known about the breakdown of the cellulose-xyloglucan network, which comprises approximately 50% of the cell wall polysaccharides and determines the strength of the cell wall. This thesis describes (i) the primary structure of apple xyloglucan, (ii) the interaction between cellulose and xyloglucan, and (iii) the enzymes that play a role in the degradation of the cellulose-xyloglucan network of apple cell walls.

For model studies, cell wall material was prepared from ground apple fruit tissue by extensive extraction with water. Various polysaccharides were subsequently extracted from these cell walls with alkaline solutions of different strength (0.05 M, 1 M, and 4 M KOH). Pectin was removed from the 4 M KOH extract by anion-exchange chromatography, resulting in a fraction which consisted of predominantly xyloglucan (Chapter 2). Methylation analysis of this polysaccharide was consistent with a ß-(1→4)-glucan backbone containing the following elements: ß-Glc*p*, α-Xyl*p*-(1→6)-ß-Glc*p*, ß-Gal*p*-(1→2)-α-Xyl*p*-(1→6)-ß-Glc*p* and α-Fuc*p*-(1→2)-ß-Gal*p*-(1→2)-α-Xyl*p*-(1→6)-ß-Glc*p*. According to the current nomenclature these elements are abbreviated as G, X, L en F, respectively.

The apple xyloglucan was degraded to oligosaccharides with a purified endoglucanase (endoIV) derived from *Trichoderma viride*. Most fragments were isolated from this mixture using chromatography (separation based on molecular weight and charge). The purified oligosaccharides were characterized by the analysis of monosaccharide composition and molecular weight (in some cases with fragmentation of the molecule), ^{1}H-NMR spectroscopy, and enzymic degradation studies (Chapter 3 and 4). A hepta- (XXXG), octa- (XLXG), nona- (XXFG), and decasaccharide (XLFG) were the most important building units of apple xyloglucan. From this it was concluded that apple xyloglucan has a X○▲G-type of branching pattern, where "○" represents a X or L element, and "▲" a X, L or F element (Chapter 10). Small building units, such as a tri- (XG), two penta- (XXG and FG), and a hexasaccharide (GFG), were also found. These

small building units might be formed from the larger ones as a result of the action of endogenous enzymes during apple maturation (Chapter 4).

The endoglucanases of *Trichoderma viride* differ greatly in their substrate specificity. The activity of endoIV towards xyloglucan is 50 times that of endoI, whereas both enzymes show similar activity towards carboxymethyl cellulose (Chapter 2). Apparently, cleavage of the ß-(1→4)-glucan backbone by endoI is hindered by the presence of many sidechains. It was known from the literature that xyloglucans with a lower degree of backbone-branching than in apple can also occur in plant cell walls. An example of this is potato xyloglucan, and it was expected that this would be an interesting substrate to investigate the action of the different endoglucanases further. The exact structure of potato xyloglucan was, however, unknown. The branching pattern of potato xyloglucan was elucidated (Chapter 5), using a similar procedure as described for apple xyloglucan. Potato xyloglucan differs from that of apple in (i) the presence of α-Ara*f*-(1→2)-α-Xyl*p*-(1→6)-ß-Glc*p* elements (S), (ii) the absence of F elements, and (iii) the branching pattern which corresponds to ●●GG ("●" represents a X, L or S element). Especially the last remark is very interesting, because this suggests that all xyloglucans, of different origin, have building units with a backbone length of four Glc residues in common (Chapter 10).

In Chapter 6 the substrate specificity of three endoglucanases from *Trichoderma viride* was further investigated. Although the three enzymes have a similar activity towards carboxymethyl cellulose, it appeared that their activity towards natural substrates greatly differed. EndoI has a relatively low activity towards xyloglucan, but a relatively high activity towards cellulose; the opposite applies for endoIV. EndoV is the most versatile endoglucanase, because of its relatively high activity towards both cellulose and xyloglucan. The action of endoI and endoV towards apple xyloglucan, and to a lesser extent that of endoIV, is hindered by the presence of Fuc residues in this substrate (Chapter 3 and 6). EndoIV and endoV degrade potato xyloglucan in a different fashion (Chapter 5). EndoIV releases predominantly ●●GG oligosaccharides, whereas endoV predominantly produces G●●G, ●●G (●, see above) and Glc. However, determination of the exact position of the X, L, and S elements in the different oligosaccharides is necessary to understand the mode of action of these two enzymes completely. Potato xyloglucan is, despite its relatively low degree of backbone branching, not a good substrate for endoI. The types of oligosaccharides released by endoI from potato xyloglucan are similar to those released by endoV (Chapter 6).

The question why endoIV and endoV have a high xyloglucanase activity and endoI a low is also addressed in Chapter 6. Based on the bond cleavage frequencies and the conversion rate of different cellodextrins, the number of substrate-binding sites was determined for the three enzymes. EndoIV appears to have more substrate-binding sites (at least 9) than endoI (5). Our results were not decisive for endoV. These results, combined with those of the release of oligosaccharides from apple xyloglucan, suggest that binding of G elements of xyloglucan into the substrate-binding sites of an endoglucanase is important for xyloglucanase activity. Because endoIV has many substrate-binding sites,

this enzyme seems very well equipped to bind molecules which are composed of X○▲G or ●●GG building units (●, see above). This is in accordance with the high xyloglucanase activity of endoIV. Chapter 10 discusses whether substrate-binding by fungal and plant xyloglucanases is based on a similar principle.

Sofar, only the activity of endoglucanases towards xyloglucans, which were isolated from cell walls, has been discussed. In order to investigate whether xyloglucanase activity is important in the liquefaction process, apple cell walls as well as apple fruit tissue were treated with various (mixtures of) glucanases. The cellulose in isolated cell wall material was more efficiently degraded when xyloglucanase activity was present (Chapter 2). Apparently, the accessibility of cellulose for true cellulolytic enzymes (such as endoI and CBH) is improved by removal of its xyloglucan coating by endoIV. Similar observations were done during liquefaction of apple fruit tissue. Chapter 7 shows that extensive degradation of xyloglucan is required for removal of the coating from cellulose, because fragments composed of five oligosaccharides still bind to cellulose.

Comparison of the results of various liquefaction trials (apples from different batches, similar amounts of glucanases) showed that cellulose degradation did not correlate to the extent of disintegration of apple tissue (Chapter 8). It was also shown that an endogenous enzyme, xyloglucan endotransglycosylase (XET), is active during liquefaction of apples. Based on these two observations it was postulated that XET and fungal enzymes act in concert during the liquefaction of apples. In this hypothesis, the accelerated disintegration of certain apples is explained by XET activity. In Chapter 9, apples of different stages of maturity were screened for the presence of various enzymes. XET appeared to be the most abundant, endo-acting enzyme in apple, and its activity was related to the stage of ripening of the apple. The apples from various stages of maturity were treated with pectin lyase and different amounts of a glucanase preparation derived from *Trichoderma viride*. The amount of glucanase needed for a complete disintegration of apple fruit tissue was inversely proportional with the amount of XET that was present in the tissue. These observations provided additional evidence for the hypothesis mentioned above. Further, it was observed that a dispersion of cellulose-xyloglucan particles was formed upon treatment of ripe apples with PL and a relatively small amount of the glucanase preparation. The underlying mechanism for the stability of this dispersion remains unknown.

Summarizing, this thesis shows that (i) not any endoglucanase can be used in tailor-made enzyme preparations for liquefaction of apples, (ii) xyloglucanase activity plays an important role in fine-tuning the degradation of cellulose in apple cell walls, and (iii) xyloglucan is the "Achilles-heel" of the apple cell wall, the degradation of which is of technological importance. An example of the latter is the release of cellulose particles from the plant cell wall by xyloglucanase. These particles might be used as cloudifyer in fruit juices.

SAMENVATTING

Bij de industriële bereiding van appelsap wordt op grote schaal gebruik gemaakt van commerciële enzympreparaten. Een voorbeeld hiervan is het vervloeiingsproces. Gedurende dit proces worden de diverse celwand polysachariden, hoofdverantwoordelijk voor de stevigheid van het appelweefsel, zo volledig mogelijk afgebroken. Het vervloeiingsproces combineert een hoge sap opbrengst met een relatief lage energiebehoefte, hetgeen dit proces vanuit industrieel oogpunt bijzonder aantrekkelijk maakt. De gebruikte enzympreparaten zijn vaak ongedefinieerde, door fermentatie verkregen, mengsels van vele enzymen, waarin de gewenste enzymen niet zelden in limiterende hoeveelheden voorkomen. Sinds kort is het mogelijk om afzonderlijke enzymen in relatief zuivere vorm te produceren *m.b.v.* moleculair biologische technieken. Daardoor wordt de mogelijkheid geschapen om de belangrijkste enzymen voor het vervloeiingsproces in de juiste verhouding te mengen, zodat de bestaande preparaten op termijn vervangen kunnen worden door meer uitgekiende mengsels van enzymen.

De centrale vraag bij het samenstellen van uitgekiende enzymmengsels is: "Welke enzymen zijn belangrijk?" Voor pectine, een belangrijk polysacharide in de appelcelwand, is deze vraag grotendeels beantwoord. Voor cellulose en xyloglucaan, twee polysachariden die samen een even groot aandeel in de appelcelwand hebben als pectine, was dit tot nu toe minder duidelijk. Dit proefschrift beschrijft de fijnstructuur van appelxyloglucaan, de interactie tussen xyloglucaan en cellulose, en de enzymen die betrokken zijn bij de afbraak van het cellulose-xyloglucaan netwerk in appelcelwanden.

Voor modelstudies was het noodzakelijk te beschikken over zowel appelcelwanden als een gezuiverd appelxyloglucaan. Door appelweefsel veelvuldig met water te extraheren werd een materiaal verkregen dat *vnl.* appelcelwanden bevatte. Dit onoplosbare materiaal werd vervolgens geëxtraheerd met alkalische oplossingen (0.05 M, 1 M en 4 M KOH) om het xyloglucaan uit de celwand los te maken (Hoofdstuk 2). Het 4 M KOH extract bleek naast veel xyloglucaan, ook pectine te bevatten. *M.b.v.* anionenuitwisselings-chromatografie werd het aanwezige pectine uit het 4 M KOH extract verwijderd. Het resterende materiaal bestond uit vrijwel zuiver xyloglucaan. Methyleringsanalyse ondersteunde de aanwezigheid van de volgende elementen in een ß-(1→4)-glucaan hoofdketen: ß-Glc*p*, α-Xyl*p*-(1→6)-ß-Glc*p*, ß-Gal*p*-(1→2)-α-Xyl*p*-(1→6)-ß-Glc*p* en α-Fuc*p*-(1→2)-ß-Gal*p*-(1→2)-α-Xyl*p*-(1→6)-ß-Glc*p*. Deze elementen worden volgens de gangbare nomenclatuur afgekort tot respectievelijk G, X, L en F.

Het polymere appel xyloglucaan werd *m.b.v.* een gezuiverd endoglucanase (endoIV), afkomstig van de schimmel *Trichoderma viride*, afgebroken tot oligosachariden. *M.b.v.* chromatografische technieken (scheiding op molecuulgrootte en op lading in alkalisch milieu) werden de meeste fragmenten uit dit mengsel geïsoleerd. Deze oligosachariden werden gekarakteriseerd op basis van bepaling van de suikersamenstelling en het molecuulgewicht (in enkele gevallen met fragmentatie van het molecuul), ^{1}H-NMR spectroscopie en enzymatische afbraakstudies (Hoofdstuk 3 en 4). Hieruit werd

geconcludeerd dat een hepta- (XXXG), octa- (XLXG), nona- (XXFG) en decasacharide (XLFG) de belangrijkste bouwstenen van appelxyloglucaan zijn. In zijn algemeenheid kan gesteld worden dat appelxyloglucaan een X○▲G-type vertakkingspatroon heeft, waarin "○" een X of L, en "▲" een X, L of F element kan vertegenwoordigen (Hoofdstuk 10). Kleinere bouwstenen als een tri- (XG), twee penta- (XXG en FG) en een hexasacharide (GFG) werden ook gevonden. Het lijkt aannemelijk dat de kleine bouwstenen tijdens de ontwikkeling van de appel uit de grotere gevormd worden door de werking van endogene enzymen (Hoofdstuk 4).

De endoglucanases van *Trichoderma viride* kunnen sterk verschillen in hun substraatspecificiteit. EndoIV heeft een ongeveer vijftig maal hogere activiteit *t.a.v.* xyloglucaan dan endoI, terwijl de activiteit van beide enzymen *t.a.v.* carboxymethylcellulose ongeveer gelijk is (Hoofdstuk 2). Blijkbaar werkt endoI minder goed op sterk vertakte ß-(1→4)-glucanen dan endoIV. Uit de literatuur was het bestaan van xyloglucanen met een minder sterk vertakte hoofdketen dan die van bv. appelxyloglucaan bekend. Een voorbeeld hiervan is aardappelxyloglucaan en verwacht werd dat dit een interessant substraat zou zijn om de werking van de verschillende endoglucanases verder te bestuderen. Echter, de fijnstructuur van aardappelxyloglucaan was onbekend. Deze werd (gedeeltelijk) ontrafeld (Hoofdstuk 5), gebruikmakend van een soortgelijke procedure als beschreven voor appel xyloglucaan. Aardappelxyloglucaan verschilt van dat van appel in (i) de aanwezigheid van α-Ara*f*-(1→2)-α-Xyl*p*-(1→6)-ß-Glc*p* elementen (S), (ii) de afwezigheid van F elementen, en (iii) het vertakkingspatroon dat voor aardappel voldoet aan ●●GG (waarin "●" een X, L of S element kan vertegenwoordigen). Vooral het laatste punt is erg belangrijk, omdat dit suggereert dat alle xyloglucanen, van verschillende origine, bouwstenen met een ruggegraat van vier Glc residuen gemeen hebben (Hoofdstuk 10).

In Hoofdstuk 6 wordt de substraatspecificiteit van een drietal endoglucanases van *Trichoderma viride* bestudeerd. Alhoewel de drie enzymen een nagenoeg gelijke activiteit *t.a.v.* carboxymethylcellulose hebben, blijkt hun activiteit *t.a.v.* meer natuurlijke substraten sterk verschillend. EndoI heeft een relatief lage xyloglucanase activiteit en een relatief hoge activiteit *t.a.v.* cellulose; voor endoIV geldt het omgekeerde. EndoV is het meest veelzijdige endoglucanase vanwege zijn relatief hoge activiteit *t.a.v.* zowel cellulose als xyloglucaan. De activiteit van endoI en endoV *t.a.v.* appelxyloglucaan, en in mindere mate die van endoIV, wordt negatief beïnvloed door de aanwezigheid van Fuc residuen in dit substraat (Hoofdstuk 3 en 6). Hoofdstuk 5 laat zien dat endoIV en endoV aardappelxyloglucaan op verschillende wijze kunnen afbreken. EndoIV maakt *vnl.* ●●GG oligosachariden vrij, terwijl endoV vooral G●●G, ●●G (●, zie boven) en Glc vrijmaakt. Echter, exacte toekenning van de plaats van de X, L en S elementen in de verschillende oligosachariden is noodzakelijk om het werkingsmechanisme van deze twee enzymen volledig te kunnen begrijpen. Aardappelxyloglucaan blijkt, ondanks de lagere vertakkingsgraad van de hoofdketen, geen goed substraat voor endoI te zijn. Wat betreft het vrijmaken van oligosachariden uit aardappel xyloglucaan lijkt endoI op endoV (Hoofdstuk 6).

Verder probeert Hoofdstuk 6 een antwoord te geven op de vraag waarom endoIV en endoV xyloglucanase activiteit hebben en endoI nauwelijks. Op basis van splitsingspatronen van cellodextrines, alsmede de snelheid waarmee dit gebeurt, wordt voor de drie afzonderlijke enzymen het aantal substraatbindingsplaatsen bepaald. EndoIV blijkt meer substraatbindingsplaatsen (minstens 9) te hebben dan endoI (5). Op basis van onze resultaten kunnen geen sluitende conclusies *t.a.v.* endoV getrokken worden. Deze resultaten, in combinatie met die van het vrijmaken van oligosachariden uit appel xyloglucaan, suggereren dat binding van G elementen van xyloglucaan in de substraatbindingsplaatsen van het endoglucanase belangrijk is voor xyloglucanase activiteit. EndoIV lijkt met zijn vele substraatbindingsplaatsen bij uitstek geschikt om ketens opgebouwd uit X○▲G of ●●GG (zie bovenstaande) te binden, hetgeen bevestigd wordt door zijn hoge xyloglucanase activiteit. In hoofdstuk 10 wordt gespeculeerd of de substraatspecificiteit van sommige xyloglucanases uit planten op een zelfde principe berust.

Tot dusverre is alleen de activiteit van endoglucanases *t.a.v.* uit de plantecelwand geïsoleerde xyloglucanen aan de orde geweest. Om te bestuderen of xyloglucanase activiteit belangrijk is in vervloeiing van appelcelwanden, werden zowel geïsoleerd celwandmateriaal als appelweefsel blootgesteld aan behandelingen met verschillende glucanases. De afbraak van cellulose in geïsoleerd celwandmateriaal verloopt efficiënter in aanwezigheid van xyloglucanase activiteit (Hoofdstuk 2). Blijkbaar wordt de toegankelijkheid van cellulose voor strikt cellulolytische enzymen als endoI en cellobiohydrolase bevorderd door verwijdering van de xyloglucaan laag door endoIV. Soortgelijke waarnemingen worden gedaan tijdens vervloeiing van appelweefsel. In Hoofdstuk 7 wordt gesuggereerd dat een vergaande afbraak van xyloglucaan noodzakelijk is ter verwijdering van deze laag omdat fragmenten bestaande uit vijf oligosachariden nog steeds aan cellulose binden.

Vergelijking van de resultaten van verscheidene vervloeiingsproeven (appels uit verschillende partijen, identieke enzymdosering) laat zien dat cellulose afbraak en de mate waarin appelweefsel uiteenvalt niet gecorreleerd zijn (Hoofdstuk 8). Tevens werd aangetoond dat tijdens vervloeiing van appelweefsel een endogeen enzym, xyloglucaan endotransglycosylase (XET), actief is. Op basis van deze twee waarnemingen werd een hypothese opgesteld waarin XET en exogene xyloglucanases samenwerken tijdens het vervloeiingsproces. Hierbij zou een hoge XET activiteit een versnelde desintegratie van appelweefsel verklaren. In Hoofdstuk 9 worden appels uit verschillende ontwikkelingsstadia geanalyseerd op het voorkomen van diverse enzymen waaronder XET. XET blijkt het meest voorkomende endo-splitsende enzym in appel te zijn, en de activiteit van dit enzym is sterk gerelateerd aan de rijpheid van de appel. De appels afkomstig uit de verschillende ontwikkelingsstadia werden vervloeid met pectine lyase en verschillende hoeveelheden van een glucanase preparaat afkomstig van *Trichoderma viride*. De hoeveelheid glucanase nodig voor volledige desintegratie van het appelweefsel was omgekeerd evenredig met de XET activiteit in het appelweefsel. Dit is een sterke

aanwijzing dat bovengenoemde hypothese correct is. Verder werd waargenomen dat onder bepaalde omstandigheden tijdens vervloeiing een dispersie van cellulose-xyloglucaan deeltjes onstaat. Het mechanisme achter de stabiliteit van deze dispersie is nog onbekend (Hoofdstuk 9 en 10).

Samenvattend kan gezegd worden dat (i) niet ieder willekeurig endoglucanase gebruikt kan worden in de formulering van uitgekiende enzymmengsels voor de vervloeiing van appels, (ii) xyloglucanase activiteit een belangrijke rol speelt in optimalisering van de cellulose afbraak in appelcelwanden, (iii) xyloglucaan de "Achilles-hiel" van de appelcelwand is en dat diens afbraak van technologisch belang kan zijn. Bij dit laatste punt moet vooral gedacht worden aan het "losknippen" van cellulose deeltjes uit de plantecelwand *m.b.v.* een xyloglucanase. Deze cellulose deeltjes zouden een toepassing als "cloudifyer" in vruchtesappen kunnen hebben.

CURRICULUM VITAE

Jean-Paul Vincken werd geboren op 29 juni 1962 te Weert. In 1980 behaalde hij het Gymnasium-B diploma aan het Bisschoppelijk College te Weert. In datzelfde jaar begon hij de studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. In september 1988 slaagde hij voor het doctoraal examen met als hoofdvakken levensmiddelenchemie en proceskunde en als bijvak organische chemie.

In de periode van juni tot october, 1988, was Jean-Paul werkzaam als "backcountry ranger" in het North Cascades National Park (State of Washington, Verenigde Staten). Aansluitend vervulde hij zijn dienstplicht als sergeant bij het 12e pantserinfanterie bataljon "Garde Jagers" te Schaarsbergen. In december 1989 zwaaide hij vervroegd af om als toegevoegd onderzoeker bij de sectie Levensmiddelenchemie en -microbiologie van de vakgroep Levensmiddelentechnologie van de Landbouwuniversiteit Wageningen te gaan werken. Sedert september 1993 is hij als post-doc aan bovengenoemde sectie verbonden. Het in dit proefschrift beschreven onderzoek werd in 1994 afgerond.