Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans

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Proefschrift

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STELLINGEN

1. De conclusie van Lahaye et al. dat de katalytische werking van Aspergillus aculeatus endo-1,4-ß-D-galactanase geremd wordt bij hoge substraatconcentraties is twijfelachtig.

   Lahaye, M., Vigouroux, J. and Thibault, J.-F.: Carbohydr Polym., 15, 1991, 431-444.

2. De bewering van Siddiqui dat selderij arabinogalactaan samengesteld is uit zowel 1,3- als 1,4-gebonden galactose-eenheden is discutabel.

   Siddiqui, I.R.: J. Agric. Food Chem., 38, 1990, 28-41.

3. Een voorwaarde voor invoering van kwaliteitszorg in de industrie is een positieve relatie tussen kwaliteit en prijs van een produkt.

4. Aan de houdbaarheidstermijn op de verpakking van koudgerookte vis moet men niet te veel waarde hechten.

5. Mobiliteit en vrijheid worden vaak ten onrechte gezien als synoniemen.

6. Uit esthetisch oogpunt verdient het aanbeveling de trottoirs in winkelcentra reeds bij aanleg te voorzien van een deklaag bestaande uit kauwgom.

7. Het is onjuist het gebruik van een platenspeler als ouderwets te bestempelen.

8. Aangezien een goede 1 aprilgrap voldoende waarschijnlijk is om tijdelijk geloofwaardig te zijn, is een inventarisatie van 1 aprilgrappen gelanceerd binnen onderzoeksinstellingen een goede indicatie van wat binnen afzienbare tijd haalbaar zal worden.

9. De tendens in de media om de resultaten van wetenschappelijk onderzoek in percentages weer te geven kleineert wetenschap tot simpel telwerk.

10. Bij dissertaties kan men twee soorten stellingen als vijandelijk betitelen: de originele omdat ze zo moeilijk te veroveren zijn, en de voor de hand liggende stellingen waarmee men anderen op hun gepubliceerde fouten wijst, vanwege hun toon.

Stellingen behorend bij het proefschrift "Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans" door J.W. van de Vis. Wageningen, 21 juni 1994.

4095I

VOORWOORD

Het schrijven van het voorwoord betekent dat je een fase in je werk afsluit. Dit is dan ook het moment om terug te blikken op vier jaar onderzoek. De resultaten van dit onderzoek zijn totstand gekomen dankzij de steun en ervaring van anderen. Zonder iemand te kort te willen doen, wil ik hiervoor een aantal mensen bedanken.

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LIST OF ABBREVIATIONS

A_1, A_2,..	arabinose, arabinobiose,..
AG	arabinogalactan
AIS	alcohol insoluble solids
Araf	arabinofuranoside
Arap	arabinopyranoside
arafur B	arabinofuranosidase B
AUA	anhydro uronic acid
CM	carboxymethyl
CMC	carboxymethylcellulose
CDTA	1,2-cyclohexane diaminetetra-acetate
DEAE	diethylaminoethyl
DP	degree of polymerization
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
endo-ara	endo-arabinanase
endo-gal	endo-galactanase
F*nn*	fractions obtained from a type I arabinogalactan preparation by precipitation with ethanol, *nn* indicates ethanol concentration (% v/v) at which the precipitate was collected
FID	flame ionization detection
FPLC	fast protein liquid chromatography
Fuc	fucose
G_1, G_2, G_3..	galactose, galactobiose, galactotriose..
Gal*p*	galactopyranose
ß-D-Gal	ß-D-galactosidase
GC-MS	gas chromatography-mass spectrometry
GLC	gas liquid chromatography
Glc	glucose
HPAEC	high performance anion-exchange chromatography
HPLC	high performance liquid chromatography
HPSEC	high performance size exclusion chromatography
HTP	hydroxyl apatite

K_m	Michaelis-Menten constant
M_1; M_2,..	mixture of arabinose and galactose; of arabinobiose, arabinotriose and galactobiose,..
Man	mannose
MHR	modified hairy regions
M_w	molecular weight
M_r	relative molar mass
NMR	nuclear magnetic resonance
PAD	pulsed amperometric detection
pI	isoelectric point
PNP	*p*-nitrophenyl
Rha	rhamnose
RI	refractive index
SDS-PAGE	sodium dodecyl sulphate-polyacryl amide gel electrophoresis
TFA	trifluoroacetic acid
U	Unit (µmol/min)
V_{max}	maximum velocity
v:v	volume to volume
w:v	weight to volume
Xyl	xylose

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Biomass, which is defined as all organic matter except fossil fuels, represents an enormous potential reserve in material and energy for man. Since traditional energy resources, which are expected to become increasingly costly, are finite, research and development programs have been undertaken for exploitation of agricultural biomass as an alternative resource of energy and chemicals. Moreover, because of accumulation of agricultural surpluses and byproducts of processing agricultural produce research on conversion of biomass into useful products is considered to be important (Smith et al., 1986).

Most of the agricultural biomass is lignocellulosic material, which represents 50% of all biomass on earth with an estimated annual production of 50 x 10^9 tons (Goldstein, 1981). Lignocellulosics consist of cellulose, hemicelluloses, lignin and a.o. proteins, oil/fat and minerals, Typical lignocellulosics, which are essentially byproducts of agriculture, are corn stover, wheat-, rice-, barley-, timothy- and sorghum straw, cotton gin trash, sunflower stalks, bagasse and aspen chips (Dale, 1987).

Biomass rich in cellulose, hemicellulose and lignin is in several cases also grown as agricultural or forestry crops, such as sugar beet, sweet sorghum, jerusalem artichoke, chicory, conifers, poplar, eucalyptus and willow (Dale, 1987; Linko et al., 1989; Teissier du Gros, 1988).

Constituents of agricultural biomass can be converted into various chemicals and thus serve as renewable resource for industry. A major drawback is the fact that these chemicals have to compete with chemicals produced at a lower price from

mineral oil.

AGRICULTURAL BIOMASS AS RESOURCE OF ENERGY AND FUELS

The major constituents of agricultural biomass (carbohydrates, proteins, lignin, fats and oils) can be converted to energy or fuels by various processes (figure 1).

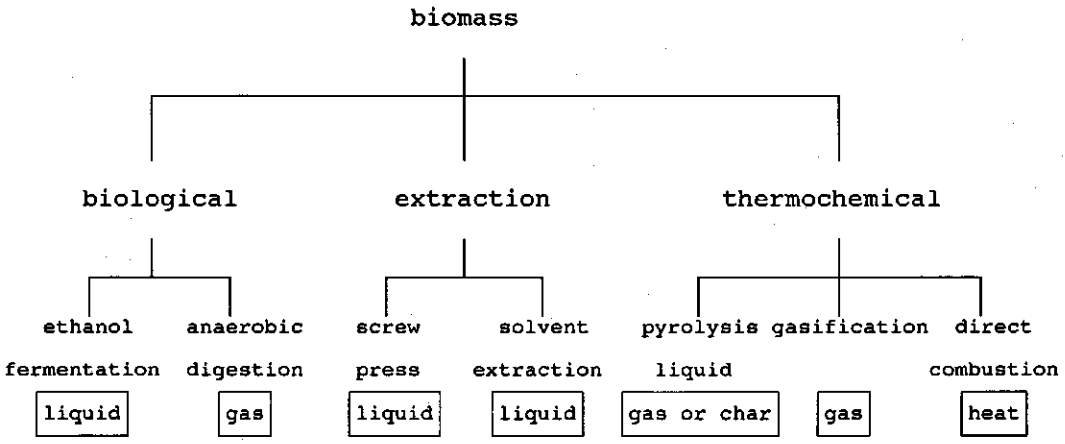

Figure 1: Some options for converting biomass to heat or liquid or gaseous fuels (Stout, 1984).

Via the biological "route" carbohydrates can be converted by hydrolysis into fermentable sugars, which are subsequently fermented to ethanol. Ethanol is generally suitable for fuel in spark-ignition engines (Sweeten and Redell, 1985). Another biological option, anaerobic digestion, is a process in which substrates (carbohydrates, oils/fats and protein) of agricultural waste are degraded by bacteria to predominantly methane and a digester effluent. The latter can be utilized as fertilizer or feedstuff (Sixt and Sahm, 1987; Sweeten and Redell, 1985).

Oils, which may also be present in plant biomass, are obtained from seeds by pressing or solvent extraction. They can be used for diesel application, but have to be processed prior to use

(Lipinsky et al., 1982).
Direct combustion, gasification and pyrolysis are thermochemical processes for conversion of biomass. The primary purpose of direct combustion is to produce heat for space heating or mechanical work (Lepori and Soltes, 1985). Gasification is a partial oxidation process that results primarily in combustible gases. Pyrolysis is a nonoxidative process that results in gases, liquids and char. The objective of pyrolysis is, generally, to maximize either the liquid or the solid fraction (Lepori and Soltes, 1985). Gasification and pyrolysis are suitable to convert a wide variety of biomass residues (Lepori and Soltes, 1985).

AGRICULTURAL BIOMASS AS RESOURCE OF CHEMICALS FOR INDUSTRY

In addition to the conversion of lignocellulosic material to energy or fuels its constituents can also be converted to chemicals used in industrial processes.
Pentoses and hexoses obtained by hydrolysis of carbohydrates can be converted to furfural and hydroxymethylfurfural, respectively. Both compounds are important raw materials from which a great variety of chemicals and polymers (polyethers, polyamides, polyesters, polyurethanes, polybutadiene) can be produced (Nimz, 1986). Various chemicals are also obtained by fermentation. Using glucose as raw material, e.g. polyhydroxybutyrate, organic acids, amino acids, organic solvents, microbial polysaccharides can be produced (Gaset and Delmas, 1986; Stringer, 1986).
Next to these carbohydrate-derived applications, chemicals have been derived from oils and fats (e.g. oleochemicals, soaps [Stowell, 1987]), lignin (e.g. vanillin and DMSO [Nimz, 1986]) and protein (e.g. cosmetics and emulsions for photography [Van Gelder, 1992]).

SACCHARIFICATION OF AGRICULTURAL BIOMASS

Plant biomass consists mainly of cellulose which is interlaced

with xyloglucans or glucuronoarabinoxylans in cell walls. The cellulose-xyloglucan framework is embedded in a pectin matrix (Carpita and Gibeaut, 1993). Development of cost-effective processes is important in order to exploit this enormous renewable resource of chemicals and fuels. Hemicelluloses are second to cellulose the most abundant renewable polysaccharides present in agricultural biomass.

Conversion of cellulose and hemicelluloses into monomeric sugars (saccharification) makes these polysaccharides suitable as a resource for fuels and chemicals. Saccharification can be achieved by the use of two competing methods, viz. acid hydrolysis and enzymic hydrolysis (Dale, 1987; El-Gammal and Sadek, 1988).

Enzymic hydrolysis has advantages over acid hydrolysis as this, in combination with appropriate pretreatments, can offer higher yields of sugars (Dale, 1987). Enzymic conversion is highly specific and is much less likely to involve degradation products of fermentable sugars, lignin and other plant components (Dale, 1987; El-Gammal and Sadek, 1988). These degradation products are found to inhibit fermentation (Wilson et al., 1989). However, enzymic hydrolysis tends to be slower and more expensive than acid hydrolysis (Dale, 1987; Perez et al., 1980). Pretreatment of biomass is required as polysaccharides in lignocellulosic materials are relatively resistant to enzymic degradation (Detroy et al., 1980; 1981). The main obstacles are the degree of crystallinity of cellulose, the lignin seal surrounding the cellulose fibres and the sites available for enzymic attack (Lee and Fan, 1983; Gharpuray et al., 1983). In addition, because of the economics of bioconversion and its associated technology, and the estimated price of the product in relation to existing products, lignocellulosic materials have remained relatively unexploited (Smith et al., 1986).

In order to solve these problems research has to be conducted in five fields (Bauer et al., 1988; Dale, 1987; Kormelink, 1992; Takagi, 1987 and Voragen et al., 1986):

1. Studies of mechanisms of enzyme action

2. Production of enzymes
3. Chemical (composition and structural features of polysaccharides, architecture of the cell wall, content of lignin etc.) and physical characterization of raw materials
4. Chemical, physical, biochemical pretreatment, or combinations thereof
5. Technological processes
6. Economical studies

In order to understand the enzymic hydrolysis of specific plant cell wall polymers knowledge of their occurrence, structure and interpolymer cross-links (covalent, non-colalent or both) in the plant cell wall is of prominent importance.

Two distinct structural models of primary cell walls in flowering plants are known. Type I cell wall is representative of all Dicotyledonae (e.g. potato, apple, coffee and soy) and some Monocotylodonae (e.g. onion); type II cell wall is representative of the Poaceae, (i.e. the Gramineae [e.g. wheat]) and of closely related monocot families.

Type I cell wall. In the type I primary cell wall three structurally independent, but interacting, domains are distinguished: the cellulose-xyloglucan framework, the matrix of pectic polysaccharides and the third domain which consists of structural proteins (extensins).

In the cellulose-xylan framework a network of cellulose microfibrils is present. These microfibrils, which are comprised of several dozen chains of 1,4-ß-D-linked glucans, are aggregrated by H-bridges. These microfibrils are interlaced by xyloglucans. In xyloglucans, repeating units consisting of a three consecutive 1,4-ß-linked D-glucose residues substituted at C_6 with xylose units are linked by a single unbranched glucose residue. In some of these xylose side-chains galactopyranose and arabinofuranose units are attached at C_2. The galactose residues may be substituted at C_2 with fucopyranose. Because of the presence of xylose units and α-L-Fuc(1->2)-ß-D-Gal(1->2)-ß-D-Xyl trisaccharide side-chains only one side of the chain can H-bond to another xyloglucan or a cellulose microfibril and, therefore, stacking of

xyloglucans is prevented. As xyloglucan and cellulose are present in equal amounts in primary cell walls, Carpita and Gibeaut (1993) propose that woven xyloglucans (as in a chain-link fence) interlace celllose microfibrils. Microfibrils can also be interlocked by other polysaccharides present in much lower amounts such as galactomannans.

This cellulose-xyloglucan framework is embedded in a pectin matrix. In pectin three regions can be distinguished: 1. rhamnogalacturonan I (RG I), a ramified and discrete domain consisting of a backbone of 1,4-α-linked galacturonic acid residues interrupted by 1,2-ß-linked L-rhamnose residues. Many of the rhamnose residues may be subsituted at C_4 side-chains such as arabinans and arabinogalactans (McNeil et al., 1984; Talmadge et al., 1973). The non-reducing ends of these side-chains may be feruloylated. Oxidative coupling of ferulate residues to diferulate may cross-link pectin molecules (Jarvis et al, 1981). Arabinogalactans are also found to be associated with non-extensin proteins. Arabinogalactan-proteins are able to bind specifically to ß-linked hexose units (Jermyn and Yeow, 1975), and therefore, they may be associated with polysaccharides found in the cell wall.

The second region in pectin, rhamnogalacturonan II (RG II), contains at least 12 different monosaccharides. Amounts of the latter molecule found generally in cell walls are too scarce to be major structural polysaccharide in plant cell walls (Carpita and Gibeaut, 1993).

The third region is a smooth region (homogalacturonan) of unbranched galacturonic residues. The helical chains of galacturonans are interconnected by calcium bridges (junction zones [Rees, 1977]).

Extensins which comprise the third independent domain, are highly basic glycoproteins bearing mono- to tetrasaccharide side-chains. In extensin two regions are distinguished; a flexous region warped around the cellulose fibrils and rod-like regions which serve as spacers (Lamport, 1986). In the rod-like region isodityrosine units cross-link at least two different polypeptide extensin backbones (Fry 1982).

Extensins are proposed to be incorporated like pins stuck into thick cloth in the plant cell wall (Lamport, 1986).

Type II cell wall. The type II cell wall differs significantly from the type I cell wall. In type II cell wall the interlocking of cellulose microfibrils is performed by glucuronoarabinoxylans, pectin is present in small amounts and the role of extensin is replaced by cross-linking phenolic compounds.

Glucuronoarabinoxylans, which functionally replace pectin in the cell wall, are linear chains of 1,4-linked ß-D-xylose units which are substituted at C_3 with single-unit arabinofuranose and, less frequently, at C_2 with single-unit glucuronic acid. The xylose residues of the xylan chain may also be doubly substituted at C_2 and C_3 with arabinofuranose (Wilkie, 1979). The degree of branching affects significantly formation of H-bridges between glucuronoarabinoxylans, which may result, contrary to xyloglucans in type I cell walls, to stacking of these polysaccharides. H-bridges can also be formed between glucuronoarabinoxylan and cellulose (i.e. the cellulose-glucuronoarabinoxylan framework) (Carpita and Gibeaut, 1993).

Formation of H-bridges does not play a major role in fixation of the architecture of the cell wall, contrary to esterified and etherified phenolic acids (Scalbert, 1985). Carboxyl groups of phenolic acids such as ferulic and coumaric acid are attached at C_5 of arabinose units (Kato and Nevins, 1985). Esterified and etherified phenolic acids cross-link glucuronoarabinoxylans wrapped around cellulose microfibrils (Scalbert et al., 1985).

Plant cell walls are considered to be a network of cellulose microfibrils as cellulose represents approximately half the amount of plant biomass (Bauer et al., 1988). Therefore, most of the research on bioconversion of biomass has been focused on the degradation of cellulose. It was found that synergistic action between cellulases and pectinases resulted in higher degree of degradation of sugar beet pulp and potato fibre

(Beldman et al., 1984). Physical pretreatment of beer brewers' spent grain resulted in increased release of sugars by fungal enzyme preparations (Beldman et al., 1987). Hemicelluloses, which are also a source of fermentable sugars, hamper saccharification of cellulose. Therefore, bioconversion of hemicelluloses must be optimised in order to develop a competitive industry (Dale, 1987; Wilson et al., 1989).
From the research on the biodegradation of hemicelluloses, until now, most of the research has been concentrated on xylans as these are the most abundant hemicellulose polysaccharides. They constitute up to 35% of the total dry weight of plants (Dekker, 1979; Timell, 1967). A detailed study on the saccharification of xylans using xylanases and accessory enzymes has been performed by Kormelink (1992).
As well as xylans, arabinans and arabinogalactans are hemicellulosic components of plant cell walls. For improvement of the overall economics of the bioconversion of biomass it is important not only to utilize xylans but also other hemicelluloses such as arabinans and arabinogalactans. The enzymic saccharification of arabinans can be achieved by a mixture of endo-arabinanase and arabinofuranosidase B (Voragen et al., 1987). Ways to accomplish enzymic saccharification of arabinogalactans are subject of the present study.

AIM AND APPROACH OF THIS RESEARCH

The aim of this study is application of galactan degrading enzymes in saccharification of arabinogalactans in the scope of bioconversion of biomass. Arabinogalactans will be isolated from various sources and their structural features will be investigated. A study of galactan degrading enzymes purified from galactanolytic fungi will be carried out. Galactan degrading enzymes will be used in combination with well characterized arabinanases in order to degrade different types of arabinogalactans present in biomass.
In chapter 2 an overview of the structural features of arabinogalactans from various plants and of galactan degrading

enzymes including their mode of actions are presented. For the degradation studies a variety of arabinogalactans found in various plants were isolated. Structural features of arabinogalactans were studied (chapter 3). Galactanases, which were used in combination with arabinanases to investigate degradation of an arabinogalactan, belonging to the so-called type I (chapter 2), are purified from Aspergillus sp. and characterized with respect to their physico-chemical and kinetic properties and mode of actions (chapter 4). In order to be able to achieve further saccharification of type I arabinogalactans, a ß-D-galactosidase is also purified from an Aspergillus sp.. Its physico-chemical and kinetic properties and mode of action are given in chapter 5. In chapter 6 various type I arabinogalactans, which differed in structural features, were used in enzymic degradation studies. Interactions between galactan degrading enzymes and arabinanases in degradation of these substrates were investigated.

For degradation of type II arabinogalactans a galactanase was purified from an Aspergillus sp. derived enzyme preparation. This purified and characterized galactanase was used in combination with arabinanases to study their interaction in degradation a type II arabinogalactan (chapter 7). Finally, concluding remarks are given in chapter 8.

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CHAPTER 2

ARABINOGALACTANS AND THEIR DEGRADATION BY GALACTANASES AND ß-GALACTOSIDASES

INTRODUCTION

Arabinogalactans belong to the group of the hemicelluloses (Dekker, 1985; Wilkey, 1985). The term hemicellulose was coined in the 19th century (Schulze, 1891) for non-cellulosic polysaccharides other than starches and fructosans which can be extracted with dilute aqueous alkali. Arabinogalactans are heteropolysaccharides. A common structural feature of these molecules is a backbone of ß-D-galactopyranose residues, which is in most cases substituted with appendages of arabinose and galactose. Arabinogalactans found in various plants vary considerably with respect to their structural characteristics. In general, arabinogalactans represent only a minor proportion of plant biomass, with the exception of larch arabinogalactans and Acacia gums (Clarke et al., 1979). Despite their presence in minor proportions, bioconversion of arabinogalactans is important as they take part in the rigid structure of several plant cell walls.

It is obvious that for enzymic conversion of arabinogalactans in biomass detailed knowledge is required of their structural characteristics and of the degradation mechanism of their galactan backbones by galactanases and ß-galactosidases. For the understanding of the mechanism of breakdown of arabinogalactan it is only usefull to describe the degradation mechanisms of enzymes which are devoid of contaminating activities. Therefore, a state of the art concerning these topics is presented in this chapter.

ARABINOGALACTANS

Arabinogalactans in which 0-85 mole% of the galactose residues are substituted with arabinose, have been found in numerous higher plants, as reviewed by several authors (Clarke et al., 1979; Dey and Brinson, 1984; Fincher et al., 1983; Stephen, 1983; Timell, 1964; 1965; 1967; 1982). Arabinogalactans found in plants are classified by Aspinall (1973) as type I and type II arabinogalactans.

In plant cell walls type I and II arabinogalactans may be either present as side-chains of rhamnogalacturonan I which is a constituent of the pectin matrix (Fry, 1986) or as a single polymer. In the latter case arabinogalactans are also found to be associated with other constituents in plant cell walls by non-covalent interactions. Type II arabinogalactans may also be found as side-chains of protein (arabinogalactan-protein, AGP) and of a glucuronomannan backbone found in e.g. gum ghatti (Clarke et al., 1979; Fincher et al., 1983). Type II arabinogalactans which lack any attachement to other constituents of plant cell walls are found in exudate gums from e.g. Acacias and in the heartwood of the genus Larix (Clarke et al., 1979; Dey and Brinson, 1984; Fincher et al., 1983; Stephen, 1983; Timell; 1964; 1965; 1967; 1982).

TYPE I ARABINOGALACTANS

Arabinogalactans of type I are found in several tissues of higher plants, except in grasses and cereals (Clarke et al., 1979, Stephen, 1983). They have been isolated from fruit, beans, seeds, bulbs, leaves, tubers and bark as reviewed by Clarke et al. (1979), Dey and Brinson (1984) and Stephen (1983). An overview of the type I arabinogalactans which are described in this chapter is presented in table 1.

Type I arabinogalactans are polysaccharides with a 1,4-ß-D-linked backbone of galactopyranose residues substituted with α-L-arabinofuranose side-chains of varying degrees of polymerization (DP). The arabinose content of type I

arabinogalactans varies between 0% w/w for garlic (Allium sativum) (Das et al., 1977) to 43% w/w for soybean (Glycine max) flour (Labavitch et al., 1976). In several preparations substitution with ß-D-galactopyranose is also found. In some cases also other sugars (rhamnose, xylose, glucose and uronic acid) have been found in preparations of type I arabinogalactans (Clarke et al., 1979; Stephen, 1983).

As type I arabinogalactans from various sources vary with respect to branching of the 1,4-linked galactan backbone, type I arabinogalactans can be subdivided into four groups: 1. linear homogalactans; 2. branched homogalactans; 3. galactans substituted with arabinose side-chains; 4. galactans substituted with uronic acid.

1. Linear homogalactans. Linear 1,4-ß-D-galactans were found in preparations extracted from lupin (Hirst et al., 1947), potato (Jarvis et al., 1981) and tobacco (Eda and Kato, 1978).

2. Branched homogalactans. Galactans branched at C_6 with galactose were found in garlic (Das et al., 1977), onion (Redgwell and Selvendran, 1986) and white willow (Toman et al., 1972). Approximately 33, 10, and 4% of the galactopyranose residues in the backbone are substituted, respectively. In onion and garlic galactans the backbones are substituted with galactopyranose residues as single-unit side-chains (Redgwell and Selvendran, 1986; Das et al., 1977), whereas in the white willow galactan the presence of longer side-chains was indicated (Toman et al., 1972).

3. Galactans substituted with arabinose side-chains. Type I arabinogalactans may carry arabinose appendages at C_3 or C_6 of the 1,4-linked galactose residues of the galactan backbone. The galactan backbone of soy arabinogalactan is substituted at C_3 with arabinose side-chains. Structural features of soy arabinogalactan reported in two articles differ with respect to the length of the side-chains. Morita (1965) proposed double unit 1,5-linked arabinofuranose side-chains (see figure 1), whereas Labavitch et al. (1976) suggested the presence of heavily branched arabinan side-chains.

In type I arabinogalactans extracted from e.g. Becium

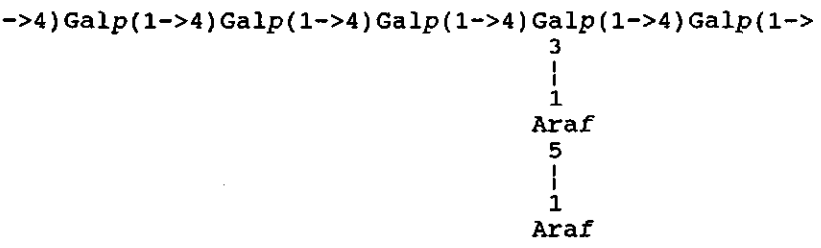


Figure 1: Proposed structure of soybean arabino-1,4-galactan

filamentosum seed (Rahman Khan et al., 1987) and potato tuber (Wood and Siddiqui, 1972) 1,5-linked arabinose side-chains were found to be linked to C_6 of the 1,4-linked galactose residues. In the former arabinogalactan the arabinofuranose side-chains are terminated with L-rhamnopyranose residues (Rahman Khan et al., 1987). The arabinose side-chains attached to the main galactan chain of potato tuber have been indicated to be linear 1,5-arabinans (Voragen et al., 1987). A water-soluble arabinogalactan, not covalently linked to pectin, was extracted from potato (Wood and Siddiqui, 1972). In this water-soluble arabinogalactan, in contrary to the type I arabinogalactan from potato studied by Jarvis et al. (1981), also single-unit galactose side-chains occur at C_6 of 1,4-linked galactose residues in the backbone.

4. Galactans substituted with uronic acid. A type I arabinogalactan consisting of 1,4-linked backbone which is substituted at C_6 with single-unit galacturonic acid has been isolated from delignified compression wood of tamarack (Larix laricina) (Jiang and Timell, 1972). A similar galactan is present in red spruce (Schreuder et al., 1966).

Data presented in table 1 show that not all type I arabinogalactans found in plants are part of the pectic matrix. Type I arabinogalactans from Becium filamentosum, garlic, tamarck, and white willow do not appear to be associated with pectin. In the case of type I arabinogalactans extracted from potato it appears that these polysaccharides

Table 1: Overview of data of type I arabinogalactans found in higher plants

Type of side-chain	Backbone substituted at	Gal*p* residues in backbone substituted (%)	Covalently linked to pectin	Source	References
-	-	-	yes	lupin seed	a
-	-	-	yes	potato tuber	b
-	-	-	yes	tobacco leaf	c
Gal*p*(1->	O-6	33	no	garlic bulb	d
Gal*p*(1->	O-6	10	yes	onion bulb	e
[Gal*p*(1->6)]$_n$Gal*p*(1->	O-6	4	no	white willow bark	f
Ara*f*(1->5)Ara*f*(1->	O-3	20-25	yes	soy flour	g
Rha*p*(1->5)Ara*f*(1->, Rha*p*(1->5)[Ara*f*(1->5)]$_n$Ara*f*(1->	O-6	33	no	Becium filamentosum seed	h
[Ara*f*(1->5)]$_n$Ara*f*(1->, Gal*p*(1->	O-6	3-5	no	potato tuber	i
GalpA(1->	O-6	5	no	tamarack compression wood	j

a: Hirst et al., 1947
b: Jarvis et al., 1981
c: Eda and Kato, 1978
d: Das et al., 1977
e: Redgwell and Selvendran, 1986
f: Toman et al., 1972
g: Morita, 1965
h: Rhaman Khan et al., 1987
i: Wood and Siddiqui, 1972
j: Jiang and Timell, 1972

were water-soluble (Wood and Siddiqui, 1972) and covalently linked to pectin (Jarvis et al., 1981).

TYPE II ARABINOGALACTANS

Arabinogalactans of type II are more widely spread in plant kingdom than type I. They are found in flowering plants from every taxonomic group tested. These polysaccharides occur in leaves, stems, roots, floral parts, seeds and media of suspension cultured cells.

The basic structural similarity of type II arabinogalactans is that they consist of a branched chain of ß-D-galactopyranose residues, which are predominantly 1,3-linked in the backbone and to a varying degree 1,6-linked. The galactose branches, which are 1,6-linked in many cases, may be substituted with α-L-arabinofuranose and to a lesser extent with ß-L-arabinopyranose residues. L-Arabinose appendages may also be attached to 1,3-linked galactose residues. An usual structural presentation for the type II arabinogalactan is a comb-like organization (Clarke et al, 1979; Stephen, 1983).

The ratio galactose/arabinose may vary between 10:90 and 85:15, however, most preparations contain more galactose than arabinose. In arabinogalactans of type II several other sugar residues can be present: L-rhamnopyranose (up to 11%), D-mannopyranose (up to 16%), D-xylopyranose (up to 7%), D-glucopyranose (up to 4%), D-glucopyranosyl uronic acid and its 4-O-methyl derivative (up to 28%), and D-galactopyranosyl uronic acid and its 4-O-methyl derivative (up to 26%) (Fincher et al., 1983).

In many cases type II arabinogalactans are found to be associated with protein (arabinogalactan-protein, AGP). Larch arabinogalactans are an exception (Clarke et al., 1979; Fincher et al., 1983) to this. When no information is available regarding associated protein these polysaccharides are often referred to as arabinogalactans (Fincher et al., 1983). In a model of an arabinogalactan-protein the ß-D-galactopyranose residue of the reducing terminus of

arabinogalactan is covalently linked to hydroxyproline of a polypeptide backbone (figure 2). The polypeptide backbone, which may consist of a single chain or several cross-linked chains, is substituted with several arabinogalactan chains. In the arabinogalactan substituents sequences of 1,3-linked ß-D-galactopyranose residues exist as open helices. In these substituents blocks of 1,3-linked galactan backbone are interrupted by periodate-susceptible residues. These blocks may consist of 1,6-linked galactopyranose residues or arabinofuranose residues. The presence of bulky side-chains linked to the galactan backbone, the flexibility of the helices, and the flexibility of the protein core will lead to an overall "wattle blossom" appearance.

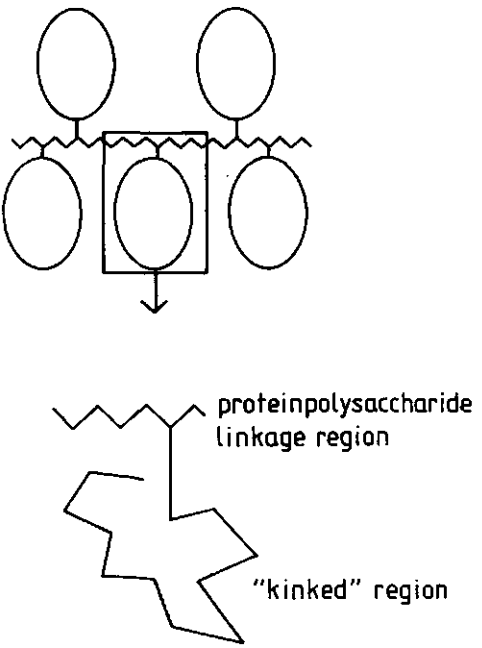


Figure 2: Hypothetical structure of an arabinogalactan-protein (Fincher et al., 1983).

Larch arabinogalactans, which are not associated with protein, and exudate gum arabinogalactans appear to have in common a general lack of attachment to other components present in the cell wall. Larch arabinogalactans are abundant in the

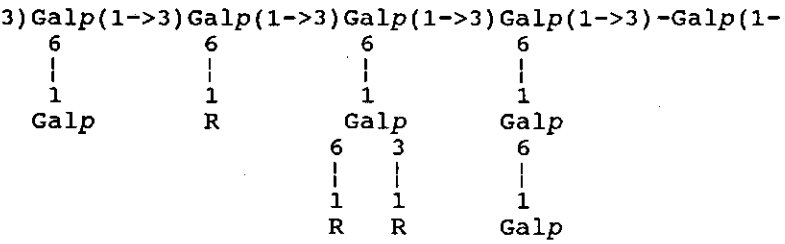
heartwood of all species of larch and can comprise up to 35% of the total heartwood (Clarke et al., 1979; Timell, 1967). Large amounts of arabinogalactan as constituents of gums are also found in the Acacia species (Stephen, 1983). Arabinogalactan-based gums comprise the single largest group of gums (Aspinall, 1969).

Arabinogalactans of type II can be subdivided into two groups with respect to the presence of arabinopyranose: 1. arabinogalactans containing arabinopyranose residues; 2. arabinogalactans without arabinopyranose residues. An overview on data of arabinogalactans of type II described is given in table 2.

Arabinogalactans containing arabinopyranose residues

Arabinogalactans containing arabinopyranose residues have been isolated from Larix and Acacia species and rape seed (Aspinall, 1969; Larm et al., 1976; Timell, 1965).

A model of arabinogalactan from western larch (Larix occidentalis) is described, because its essential structural



R= Galp(1-> or α-L-Araf-(1-> or ß-L-Arap-(1->3)-α-L-Araf(1->

Figure 3: General structural features of western larch (Larix occidentalis) arabino-1,3/6-galactan.

features are typical for many of the type II arabinogalactans (figure 3) (Clarke et al, 1979; Fincher et al., 1983). The backbone of this arabinogalactan consists of 1,3-linked ß-D-galactopyranose residues. These residues may be substituted at C_6 with single galactopyranose or arabinofuranose residues, or

with disaccharides such as ß-Ara*p*-1,3-α-Ara*f*, ß-Ara*p*-1,3-ß-Gal*p*, ß-Gal*p*-1,6-ß-Gal*p* and larger side-chains (Bouveng, 1961; Bouveng and Lindberg, 1956).

The main chain of larch arabinogalactan is sometimes interrupted by 1,6-linked galactopyranose residues, as reported for tamarack (Larix laricina) arabinogalactan (Haq and Adams, 1961). Glucuronic acid residues were found to be substituted at C_6 of the 1,3-linked galactan backbone of e.g. mountain larch (Larix lyallii) arabinogalactan (Timell, 1965).

In arabinogalactans preparations from some larch species two populations are present, one with a high M_w (37-100 kD) and a second with a lower M_w value (7.5-18 kD). These populations account for 70-95% and 5-30% of the total arabinogalactan preparations, respectively. As intrinsic viscosities for both populations are similar, a less extensive branching for the low M_w value than for the high M_w value population is suggested (Clarke et al., 1979; Timell, 1967).

A highly branched arabinogalactan is present as constituent in gum arabic from Acacia senegal. In this arabinogalactan next to outer chains of arabinopyranose and arabinofuranose, peripheral residues of D-glucuronic acid or its 4-methyl ether in terminal or near terminal positions and in some cases L-rhamnopyranose residues in outer chains are found (figure 4) (Akiyama et al., 1984; Anderson et al., 1966; Anderson and Stoddart, 1966; Aspinall, 1969). In general, these gums are more highly ramified than arabinogalactans from larch.

In gum arabic varying numbers of arabinogalactan side-chains, which are homogeneous with respect to M_w values, are attached to a protein core (Connolly et al., 1987). Gums from other Acacia species are in essence similar, but vary considerably in their overall composition and structural features (Aspinall, 1969).

In arabinogalactan-protein from mesquite gum (Prosopis juliflora) highly complex arrangements of L-rhamnose, 4-Me-D-glucuronic acid are substituted at C_6 of every 1,3-linked residue in the galactan backbone.

Table 2: Overview of data of type II arabinogalactans found in higher plants

Type of side-chain	Galactose residues substituted at	*Galp* residues in backbone substituted (%)	Covalently linked to pectin	Source	References
Ara*p*(1->3)Ara*f*(1->, Gal*p*(1->6)Gal*p*(1->, Ara*p*(1->3)Gal*p*(1->, Ara*f*(1->	O-6	>90	no	western larch	a,b
similar to western larch	O-3, O-6	>80	no	tamarack	c
Ara*p*(1->3)Ara*f*(1->, Galp(1->6)Gal*p*(1->, Ara*f*(1->, Glc*p*A(1->	O-6	>90	no	mountain larch	c
Ara*p*(1->3)Ara*f*(1->3)[Gal*p*(1->6)]$_n$Gal*p*(1->	O-3, O-6 O-6	>90	no	Acacia *senegal*	d
Rhap(1->4)Glc*p*A(1->6)[Gal*p*(1->6)]$_n$Gal*p*(1->, 4-OMe-Glc*p*A(1->6)[Gal*p*(1->6)]$_n$Gal*p*(1->, Ara*f*(1->, Ara*f*(1->3)Ara*f*(1->, Gal*p*(1->3)Ara*f*(1->, Ara*p*(1->3)Ara*f*(1->, Ara*f*(1->3)Ara*f*(1->3)Ara*f*(1->, Ara*p*(1->3)Ara*f*(1->3)Ara*f*(1->	O-3				

a: Bouveng and Lindberg, 1956 d: Anderson et al., 1966
b: Bouveng, 1961
c: Timell, 1965

Table 2-Continued

Type of side-chain	Galactose residues substituted at	Gal*p* residues in backbone substituted (%)	Covalently linked to pectin	Source	References
Araf 1 | 5 Araf(1->2)Araf 1 | 5 Araf(1->2)Araf(1-2)Araf(1->, 5 | 1 Araf(1->2)Araf(2<-1)Araf 5 5 | | 1 1 Araf Araf	0-6	>90	no	rape seed	e
Araf(1->?)Araf(1->, Araf(1->	0-6	55-70	no	wheat endosperm	f
[Araf(1->5)]$_n$Araf(1->, [Gal*p*(1->6)]$_n$Gal*p*(1->	0-6	66	no	sugar cane	g
Araf(1->3)Gal*p*(1->, Araf(1->, Gal*p*(1->	0-6	66	no	Coffea arabica	h,i

e: Larm et al., 1976
f: Fincher et al., 1974
g: Blake et al., 1983
h: Wolfrom and Patin, 1965
i: Hashimoto, 1971

Table 2-Continued

Type of side-chain	Galactose residues substituted at	Galp residues in backbone substituted (%)	Covalently linked to pectin	Source	References
	O-3 O-6	12-13 40-60	yes	<u>Angelica acutiloba</u> root	j
Ara*f*(1->5)Ara*f* 1 | 3 Gal*p*(1->6)Gal*p*(1->6)Gal*p*(1->6)Gal*p*(1->, 3 | 1 Ara*f*	O-6				
Gal*p* 1 | 3 Gal*p*(1->6)Gal*p*(1->6)Gal*p*(1->6(Gal*p*(1->6)Gal*p*(1->, 3 | 1 Gal*p*	O-6				
Ara*f* 1 | 3 Gal*p*(1->6)Gal*p*(1->6)Gal*p*(1->6)Gal*p*(1->6)Gal*p*(1-> 3 | 1 Ara*f*	O-6				

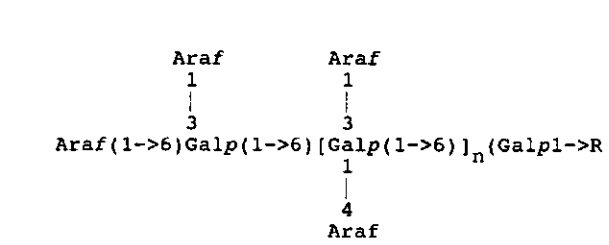
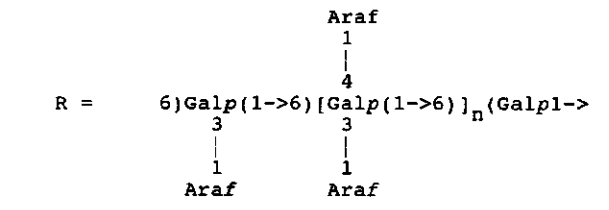
j: Kiyohara and Yamada, 1989

Table 2-Continued

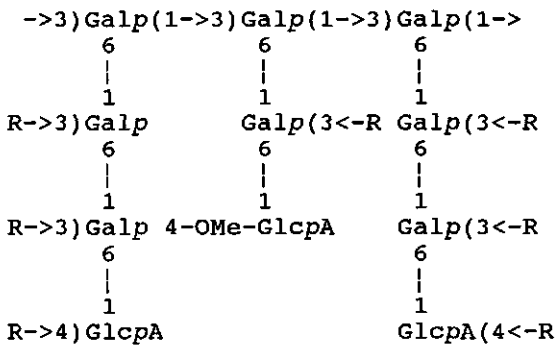
Type of side-chain	Galactose residues substituted at	Gal*p* residues in backbone substituted (%)	Covalently linked to pectin	Source	References
Ara*f*(1->3)Gal*p*(1->, [Gal*p*(1->6)]$_n$Gal*p*(1->, Ara*f*(1->, Gal*p*(1->	O-6	33	no	Coffea robusta	k
similar to Angelica acutiloba, Fuc*p*(1->2)Ara*f*(1->, Ara*f*(1->,	O-3, O-6 O-3 O-3	41	no	radish leaf	l
[Ara*f*(1->5)]$_n$Ara*f*(1->, [Gal*p*(1->6)]$_n$Gal*p*(1->, Rha*p*(1->, Glc*p*A(1->	O-6	66	no	tobacco leaf	m
[Ara*f*(1->5)]$_n$Ara*f*(1->3)[Gal*p*(1->6)]$_n$Gal*p*(1->, [Ara*f*(1->5)]$_n$Ara*f*(1->	O-6 O-3	56	yes	apple juice	n

Value of n=2
k: Bradbury and Halliday, 1990
l: Tsumuraya et al., 1984
m: Akiyama et al., 1982
n: Will and Dietrich, 1992

Table 2-Continued

Type of side-chain	Galactose residues substituted at	Galp residues in backbone substituted (%)	Covalently linked to pectin	Source	References
	O-3 O-6	25 87	yes	wine	o
	O-6				

Value of n=2
o: Brillouet et al., 1990



R= Rhap(1->, Araf(1->, Galp(1->3)Araf(1, Arap(1->3)Araf(1, Arap(1->3)Arap(1->3)Araf(1->, Arap(1->3)Araf(1->, Arap(1->3)Araf(1->3)Araf(1->

Figure 4: Partial core structure of gum arabic

A striking difference in structural features of mesquite gum compared to gum arabic is the presence of isolated D-galactopyranose and L-arabinopyranose residues within chains of L-arabinofuranose residues (Aspinall, 1969).
As described earlier, the content of 1,6-linked galactose residues of arabinogalactan and arabinogalactan-proteins may vary. An arabinogalactan devoid of 1,3-linkages has been isolated from rape seed (Brassica napus). Larm et al (1976) presented a tentative structure of this water-soluble arabinogalactan. The galactan backbone was indicated to consist of a 1,6-linked backbone of galactose residues, substituted with highly branched 1,2- and 1,5-linked arabinan side-chains. The arabinopyranose residues are found in terminal positions in the molecule (Larm et al., 1976).

Arabinogalactans without arabinopyranose residues

Arabinogalactans which are indicated not to be composed of arabinopyranose residues have been isolated from wheat, sugar cane, Coffea arabica and robusta, Angelica acutiloba, radish, tobacco, apple and grape.
The arabinogalactan constituent of the wheat endosperm, which

was proposed to contain arabinose in the furanose form, consists of a 1,3-linked galactan backbone substituted at C_6 with arabinofuranose as single-unit or as short side-chains. In this arabinogalactan-protein galactopyranose residues of arabinogalactan chains are covalently linked to hydroxyproline at frequent intervals in a peptide chain (Fincher et al., 1974).

An arabinogalactan devoid of protein was extracted from sugar cane. The arabinogalactan has a backbone of 1,3-linked galactose residues in which two out of every three carry a galactose or arabinofuranose side-chain attached at C_6. It differs from the arabinogalactans found in larch with respect to the relative high amount of arabinofuranose residues (43 mole % vs 14 mole %). The preparation contains two populations with M_w values of the orders 20-25 kD and 1000-2000 kD (Blake et al., 1983).

An arabinogalactan which carries short side-chains has been isolated from delignified Coffea arabica (Wolfrom and Patin, 1965; Hashimoto, 1971). In the 1,3-ß-galactan backbone about two-thirds of the galactose residues are substituted at C_6 either with Araf(1->3)Gal(1-> side-chains or single-unit arabinofuranose or galactose residues.

An arabinogalactan, which differs from the latter with respect to the degree of substitution at C_6 (one-third vs. two-third) has been isolated from delignified Coffea robusta. This arabinogalactan contains also non-terminal arabinose and small amounts of 1,6-linked galactose (Bradbury and Halliday, 1990).

A neutral and two acidic arabinogalactans were reported to be linked at C_4 of rhamnopyranose units in the rhamnogalacturonan backbone of pectin extracted from the roots of Angelica acutiloba. The neutral arabinogalactan is composed of a 1,3-linked galactan backbone substituted at C_3 with 1,6-linked galactose side-chains which possess unbranched arabinofuranose side-chains at position 3. Single-unit galactose side-chains can also be linked at position 3 of the 1,6-linked galactose side-chains. This neutral arabinogalactan is attached to galacto-oligosaccharides which are in turn subsituted at the

rhamnogalacturonan core. Structural features of the acidic type II arabinogalactans were not reported in detail (Kiyohara et al., 1987; Kiyohara and Yamada, 1989).

Two arabinogalactan-proteins extracted from radish leaves (Raphanus sativus) differ from the neutral pectic arabinogalactan from Angelica acutiloba with respect to the presence of L-fucose. This characteristic feature of radish leaf AGPs has been rarely found in type II arabinogalactans (Clarke et al., 1979, Fincher et al., 1983). The 1,3-linked main chain is substituted with side-chains composed of one to three consecutive 1,6-linked galactose residues subsituted at C_3 with α-L-Fuc*p*(1->2)α-L-Ara*f*(1-> and Ara*f*(1->. Also small amounts of uronic acids were found in the preparations (Tsumuraya et al., 1984).

Arabinogalactan and arabinogalactan-protein from radish seeds differ from the latter polymers with respect to the absence of fucose and the presence of small amounts of xylose and glucose (Tsumuraya et al., 1987).

An arabinogalactan-protein which is substituted at C_6 of the 1,3-linked main chain of galactose with arabinofuranose, 1,5-linked arabinofuranose, traces of arabinopyranose, ß-D-glucuronic acid and L-rhamnose side-chains, has been isolated from tobacco (Nicotiana tabacum) leaves (Akiyama et al., 1982).

An arabinogalactan covalently linked to apple pectic substances appears to consists of of a 1,3/1,6-linked galactan backbone (De Vries et al., 1983). Pectic polysaccharides in apple juice have also been reported to carry type II arabinogalactan side-chains. Structural features of a neutral arabinogalactan were studied by Will and Dietrich (1992). This arabinogalactan was found to consist of a 1,3-linked galactan backbone subsituted by 1,6-with terminal arabinofuranose as 1,5-linked short side-chains located predominantly in position 3 of the 1,6-linked galactan side-chains. More than 70% of the arabinofuranose residues was found in terminal positions (Will and Dietrich, 1992).

An arabinogalactan in wine derived from Carignan noir grapes

was reported to carry 1,6-galacto-oligomeric side-chains which are heavily substituted at C_3 and C_4 with single-unit arabinofuranose. Some 6-linked galactose units are subsituted at C_3 only, while others are unsubstituted (Brillouet et al., 1990). Brillouet et al. (1990) suggested that the wine arabinogalactan was associated with pectin. In an arabinogalactan-protein from Carignan noir grapes, however, most of the arabinofuranose residues were 1,3-linked to 1,6-linked galactan chains (Saulnier et al., 1992).

GALACTANASES AND ß-GALACTOSIDASES

In contrast to the numerous reports on type I and II arabinogalactans, little information is available on enzymes degrading the galactan structures of these arabinogalactans. Galactanases are hydrolytic enzymes, which degrade galactan backbones of arabinogalactans. Therefore, these enzymes belong to the group of the hemicellulases (Dekker and Richards, 1976). Three types of galactanases have been reported that are specific for 1,4- (EC 3.1.2.89), 1,3- (EC 3.2.1.90) and 1,6-ß-D-galactopyranosyl linkages (not classified yet).
In general, galactanases can attack their substrates in either an exo or an endo fashion. In an exo-mechanism the polysaccharide is degraded in a stepwise manner by successive removal of terminal monosaccharide or oligosaccharide units, usually from the non reducing end. An endo-galactanase degrades a substrate in a random fashion.
ß-galactosidases which are able to attack polymeric substrates will also be described. An overview of data on galactanases and ß-galactosidases described is given in table 3.

MODE OF ACTION OF GALACTANASES AND A ß-GALACTOSIDASE ON TYPE I ARABINOGALACTANS

Type I arabinogalactans degrading galactanases have been reported to be produced by several organisms (Dekker and Richards, 1976). These galactanases are specific for 1,4-ß-D-

Table 3: Overview of data on enzymes degrading galactan structures in type I arabinogalactans

Source	Type of enzyme	Major end-products released	Mode of action	References
	galactanase			
Bacteria				
Bacillus subtilis WT 168		Gal_4	exo- and endo-mechanism	Labavitch et al., 1976
Bacillus subtilis K 50		Gal_2	exo- and endo-mechanism	Emi et al., 1971
Bacillus subtilis var. amylosacchariticus		Gal_3, (Gal, Gal_2)	endo-mechanism	Emi and Yamamoto, 1972 Yamamoto and Emi, 1988
Bacillus subtilis		Gal_2, (Gal_4)	exo-mechanism	Nakano et al., 1990
Bacillus sp. S-2		Gal_3, Gal_4	endo-mechanism	Tsumura et al., 1991
Bacillus sp. S-39		Gal_2, Gal_3	endo-mechanism	Tsumura et al., 1991
Fungi				
Penicillium citrinum		Gal, Gal_2	endo-mechanism	Nakano et al., 1985
Aspergillus aculeatus		Gal, Gal_2	endo-mechanism	Layahe et al., 1991
	galactosidase			
Plant				
Carrot (Daucus carota L.)		Gal	-	Konno et al., 1986

Table 4: Overview of data on enzymes degrading galactan structures in type II arabinogalactans

Source	Type of enzyme	Major end-products released	Mode of action	References
Fungi	**galactanase**			
Rhizopeus niveus		$A_x G_y$, Ara	endo-mechanism	Hashimoto et al., 1969 Hashimoto, 1971
Irpex Lacteus		Gal	exo-mechanism	Tsumuraya et al., 1990
Aspergillus niger		Gal_2	endo-mechanism	Brillouet et al., 1991
	galactosidase			
Plant				
Radish (Rhapanus Sativus L.)		Gal	-	Sekimata et al., 1989

A_xG_y= arabinogalacto-oligosaccharides

galactopyranosyl linkages and, therefore, carry the systematic name 1,4-ß-D-galactan galactanohydrolase (Dekker and Richards, 1976).

Several bacterial and fungal 1,4-galactanases have been purified to a homogeneous state. A 1,4-galactanase purified from Bacillus subtilis WT 168 (Labavitch et al., 1976) and from Bacillus strain K 50 (Emi et al., 1971) degrades the polymeric substrate by both exo- and endo-mechanisms (i.e. a single chain multiple attack mechanism (Robyt and French, 1967). These galactanases released galactotetraose and galactobiose as major degradation products, respectively.

Three endo-1,4-galactanases isolated from B. subtilis var. amylosacchariticus release mainly galactotriose and to a lesser extent galactobiose and galactose as final reaction products (Emi and Yamamoto, 1972; Yamamoto and Emi, 1988).

An exo-galactanase purified from a Bacillus subtilis strain accumulated galactobiose almost exclusively with a small amount of galactotetraose as byproduct. The formation of galactotetraose indicated the ability of the enzyme to transfer galactobiose to other galactobiose residues (Nakano et al., 1990). Temperature (60 °C) and pH optima (6.5-7) of this exo-galactanase are similar to values found for the other Bacillus galactanases (Emi and Yamamoto, 1972; Yamamoto and Emi, 1988).

In contrast to these Bacillus galactanases, with pH optima in the range 6-7, two endo-galactanases purified from alkalophilic Bacillus sp. S-2 and S-39 show activities at higher pHs. The former (S-2) possesses an optimum at pH 10 and the latter enzyme shows optimal activity at both pH 4.0 and 9.0. Endo-galactanase S-2 preferentially produces galactotriose and galactotetraose and the S-39 releases galactobiose and galactotriose as major products (Tsumura et al., 1991).

Two fungal endo-galactanases from Penicillium citrinum, which are similar in their properties (Nakano et al., 1985) and one from Aspergillus aculeatus (Lahaye et al., 1991) released galactose and galactobiose as the end products of galactan

hydrolysis. The Penicillium galactanases I and II also show glycosyl transferase activity (Nakano et al., 1985, 1986, 1988).
Up to now only one ß-galactosidase active towards type I arabinogalactan has been purified to a homogeneous state. This ß-galactosidase, purified from cell suspension cultures of carrot, is able to degrade a 1,4-galactan in an exo-fashion (Konno et al., 1986).

MODE OF ACTION OF GALACTANASES AND A ß-GALACTOSIDASE ON TYPE II ARABINOGALACTANS

Type II arabinogalactans degrading galactanases have only been purified from fungi. Hashimoto et al. (1969) found four 1,3-galactanases produced by Rhizopus niveus. The mode of action of only one of these galactanases has been investigated (Hashimoto, 1971). This enzyme releases arabino-1,3-galactooligosaccharides and arabinose. However, the latter may be released by a contaminating arabinofuranosidase.
An exo-galactanase from Irpex lacteus specifically releases galactose from 1,3-ß-galactan chains. This enzyme is also able to bypass branching points, thus liberating intact side-chains (Tsumuraya et al., 1990).
A galactanase from Aspergillus niger that releases 1,6-galactobiose and to a lesser extent galactose from de-arabinosylated grape arabinogalactan-protein is a novel enzyme. The enzyme acts in an endo-fashion and therefore this enzyme was named endo-1,6-ß-D-galactanase (Brillouet et al., 1991).
A ß-galactosidase purified from radish seeds releases galactose from de-arabinosylated radish arabinogalactan-proteins. The enzyme is able to cleave both 1,3- and 1,6 linkages in arabinose-reduced radish arabinogalactan-proteins. However, the enzyme was found to degrade preferentially 1,6-linkages in these arabinogalactan-proteins. Because of its mode of action the enzyme was suggested to be an exo-galactanase (Sekimata et al., 1989).

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CHAPTER 3

PREPARATION AND CHARACTERIZATION OF TYPE I AND II ARABINOGALACTANS FROM VARIOUS SOURCES

INTRODUCTION

Arabinogalactans, which can be grouped in arabino-4-galactans (type I) and arabino-3,6-galactans (type II) (Aspinall, 1973) are ubiquitous in higher plants. Structural features of arabinogalactans belonging to the same group may differ considerably (see chapter 2).
In order to investigate optimal enzymic saccharification of type I and II arabinogalactans found in biomass, a variety of arabinogalactans were isolated and characterized from a number of sources.

MATERIALS AND METHODS

MATERIALS

Type I arabinogalactans were prepared from citrus pomace (a gift from Méro Rousselot Satia, Paris, France), onion powder (kindly provided by ATO-DLO, Wageningen, The Netherlands) and potato fibre (kindly provided by AVEBE, Veendam, The Netherlands). A type I arabinogalactan from soy meal was kindly provided by NOVO Nordisk, Dettingen, Switzerland, and denoted as soy AG.
Green coffee beans (Coffea arabica, kindly provided by Douwe Egberts, Utrecht, The Netherlands) were used for extraction of type II arabinogalactan. Type II arabinogalactan from larch wood ("stractan") was purchased from St. Regis Paper company, Tacoma, WA, USA).

EXTRACTION OF TYPE I ARABINOGALACTANS

Prior to extraction of arabinogalactan the raw materials were subjected to various purification steps: From potato fibre the residual starch was removed enzymically (Rombouts et al., 1988), from onion powder the lipid material was removed by extraction in a soxhlett apparatus using methanol-chloroform (1:1 v/v) (Mankarios et al., 1980) and from citrus pomace the oligomeric sugars were removed by refluxing for 1 h with 80% v/v aqueous ethanol.

Arabinogalactan extracts from citrus pomace, potato fibre and onion powder were obtained by refluxing for 20 h at 90 °C with an aqueous solution of 4 N NaOH containing 0.1 M $NaBH_4$ (Labavitch et al., 1976). After dialysis against distilled water, 0.5 M potassium phosphate buffer, pH 7.0 was added until an 0.01 M extract was obtained. This buffered extract was passed through a column (5 x 25 cm) of DEAE-cellulose using a 0.01 M potassium phosphate buffer pH 7.0 as eluent (Labavitch et al., 1976). The material which passed directly through the column was collected, dialysed against distilled water and lyophilized.

The resulting crude arabinogalactan extracts from potato fibre, onion powder and citrus pomace are denoted as potato AG, onion AG and citrus AG, respectively.

GRADED ETHANOL PRECIPITATION OF ALKALI EXTRACTED TYPE I ARABINOGALACTANS

Citrus AG, onion AG and potato AG, as 2% w/v aqueous solutions in water, were fractionated by precipitation with ethanol of increasing concentration (increments of 20%). After each increase in ethanol concentration, the mixture was stored at 4 °C for 16 h and the precipitate was collected by centrifugation (30 min at 43 000 g). Fractions precipitated in 20%, 40%, 60% and 80% v/v ethanol were designated as F20, F40, F60 and F80, (e.g. the precipitate obtained by increasing the 20% v/v ethanol concentration in the potato AG solution to 40%

v/v ethanol is designated as potato F40). The precipitates were disolved in distilled water and freeze-dried. The final supernatant (80% v/v ethanol), designated as S, was freeze-dried after dialysis against distilled water.
In order to remove polymeric material consisting of glucose or of xylose and glucose from citrus F40 this fraction was treated with endo-glucanase IV (Beldman et al., 1985). A reaction mixture of 3.8 µg endo-glucanase IV and 1 mg citrus F40 per ml of 0.05 M sodium acetate buffer pH 5.0 was incubated for 40 h at 30 °C. Reaction products were removed by dialysis and the AG was precipitated by addition of ethanol to a final concentration of 80% v/v. This treated citrus F40 fraction is designated as citrus TF40.

EXTRACTION OF TYPE II ARABINOGALACTANS FROM GREEN COFFEE BEANS

Extraction of type II arabinogalactan from 1 kg green coffee beans was performed according to Wolfrom and Patin (1965). Prior to removal of lignin the procedure was slightly modified: A step in which ground coffee beans were defatted by extraction with 3.5 l chloroform in a soxhlett apparatus for 6 h (Folstar, 1976) was added to the procedure. Removal of Ca-bound pectins from the defatted coffee beans was performed by extraction with CDTA (Jarvis, 1982).
By treatment with sodium chlorite and acetic acid, to remove lignin, holocellulose of green coffee beans was obtained. The holocellulose was thoroughly wetted by stirring overnight with water of 37 °C. The material was extracted with water by refluxing for 5 h. The solution was filtered, concentrated under reduced pressure at 40 °C, made to 0.2 M NaOH, 0.01 M $NaBH_4$ and stirred at 60 °C for 4 h under a stream of nitrogen. After dialysis against distilled water and concentration until the volume was reduced to 5 l cold ethanol was added to a final concentration of 70 % v/v. The precipitate was dialysed and freeze dried. The obtained coffee arabinogalactan is denoted as coffee AG.
A partly de-arabinosylated coffee arabinogalactan (denoted as

coffee galactan) was obtained by treatment of coffee AG with a pure arabinofuranosidase B (Rombouts et al., 1988).

CARBOHYDRATE ANALYSIS

Polysaccharides were hydrolysed by pretreatment 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 constituent neutral sugars were converted to their alditol acetates and analysed by gas chromatography (Englyst and Cummings, 1984). Conditions of GC-analysis are described by Gruppen et al. (1989). Total neutral sugars and uronic acid were determined colorimetrically by an automated orcinol sulfuric acid (Tollier and Robin, 1979) and m-hydroxydiphenyl (Thibault, 1979) assay, respectively. In the latter procedure 96% w/w H_2SO_4 containing 0.0125 M sodiumtetraborate was used. Correction was made for the interference of neutral sugars in the determination of uronic acid.

METHYLATION ANALYSIS

Glycosidic linkage composition was investigated by methylation analysis according to Hakomori (1964) as modified by Sandford and Conrad (1966). Care was taken to ensure total methylation. If, after methylation, any material remained insoluble in $CHCl_3$/MeOH (50/50 v/v), the procedure was repeated. Methylated polysaccharides were purified by dialysis, freeze dried and subsequently subjected to two different methods of hydrolysis. Arabinogalactans were hydrolysed in 90% (v/v) formic acid for 5 h at 100 °C followed by hydrolysis in 0.125 M sulfuric acid for 16 h at 100 °C. Arabinan was hydrolysed in 2 M TFA for 1 h at 121 °C. After reduction to partially methylated alditols using $NaBD_4$, these alditols were acetylated according to Englyst and Cummings (1984). The methylated alditol acetates were separated and quantified on a fused silica capillary column (0.32 mm x 30 m; wall coated with DB1701; 0.25 mm, J & W Scientific, Folsom, California, USA) in a Carlo ERBA

Fractovap 4160 GC (Carlo ERBA Instrumentazione, Milan, Italy) equipped with an FID. Sample composition was calculated using effective carbon response (E.C.R.) factors (Sweet et al., 1975).
Identification of the compounds was confirmed by GC-MS. A HP 5890 gas chromatograph equipped with a fused silica column (CPSIL 19CB, 0.35 mm x 26 m; 0.18 mm, Chrompack Nederland BV, Middelburg, The Netherlands) coupled to a mass spectrometer (HP MSD 5970-B) (Hewlett-Packard, Rockville, Midland, USA), was used.

ANALYSIS OF ALKALI EXTRACTED AND FRACTIONATED ARABINOGALACTAN PREPARATIONS BY HPLC

Apparent molecular weight distributions of alkali extracted and subfractionated type I arabinogalactan preparations were determined by high performance size exclusion chromatography (HPSEC). HPSEC was performed on a SP 8700 HPLC (Spectra Physics, San Jose, California, USA) equipped with three gel permeation columns (each 7.5 x 300 mm) in series, Bio-gel TSK-40 XL, -30 XL and -20 XL (Biorad Laboratories, Richmond, California, USA) in combination with a TSK XL guard column (6 x 40 mm) at 30 °C using 0.4 M sodium acetate buffer (pH 3.0) as eluent at a flowrate of 0.8 ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector. A mixture of dextrans was used as standard.

RESULTS

TYPE I ARABINOGALACTANS

Sugar composition of crude arabinogalactan preparations

The sugar compositions of potato AG, onion AG, soy AG and citrus AG shown in table 1 confirmed that arabinogalactans were present in these preparations.
The obtained preparations differed in purity (the total sugar

Table 1: Sugar composition[a] of crude AG extracts

Sugar (mol%)	Source potato fibre	onion powder	soy meal	citrus pomace
Rhamnose/ Fucose	0.5	0.2	1.8	0.2
Arabinose	12	7.5	38	72
Xylose	2.2	trace	1.4	5.5
Mannose	0.3	5.2	0	trace
Galactose	77	71	57	14
Glucose	4.9	15	0	7.9
AUA	2.6	1.0	2.4	0.4
Total sugar content[b]	87	71	75	96
Molar Gal/Ara ratio	6.4	9.5	1.5	0.19

[a] The sugar composition is expressed in mole percentages with the total sugars set at 100 mole %.
[b] Total sugar content is expressed as weight percentage (% w/w) of the freeze dried extract.
trace= < 0.05 mole%

content was found to be in the range of 71-96% w/w) and their molar Gal/Ara ratio. Citrus AG, with the lowest Gal/Ara ratio of 0.19, showed more the characteristics of an arabinan. The proportions of glucose and xylose in potato AG, onion AG and citrus AG points to the presence of xyloglucans.

Sugar composition of ethanol precipitated fractions

The sugar composition of fractions collected by graded ethanol fractionation of potato, onion and citrus AG is given in table 2. It can be seen that the collected fractions differed significantly in their molar Gal/Ara ratios. For all raw materials the F40 fractions contained the major part of the (arabino)galactans with highest molar Gal/Ara ratios. From the relatively high arabinose content of F40 fractions collected from potato AG and citrus AG it was inferred that the presence of arabinans is likely.

Table 2: Sugar composition of fractions collected from potato, onion and citrus AG by ethanol fractionation.

Fractions	yield[b]	Molar proportions (%)[a] Rha/ Fuc	Ara	Xyl	Man	Gal	Glc	AUA	TSC[c]	Gal/ Ara
Potato										
F20	12	0.1	16	2.1	0	75	6.5	0.4	94	4.7
F40	64	0.2	6.6	1.8	0	86	5.2	0.7	96	13
F60	4	0.8	7.2	2.0	0	86	4.2	0	80	12
F80	9	1.5	51	2.8	0.6	34	6.9	3.4	62	0.67
Onion										
F20	1	0	3.5	0	7.6	59	29	0.8	16	17
F40	29	0.1	0.3	0	0.1	99	0.3	0.8	100	330
F60	10	0.3	1.0	0	0.1	97	0.6	0.6	103	97
F80	2	0.7	50	0.3	1.4	42	4.0	1.7	78	0.84
S	48	0.9	22	0	19	2.3	53	2.5	33	0.10
Citrus										
F20	6	0.1	43	5.7	0.1	42	8.5	0.5	92	0.98
F40	27	0.8	6.7	23	0	38	32	0.2	98	5.7
F80	4	0.3	37	0.3	0	59	2.2	1.0	100	1.6
S	63	0.1	98	0	0	1.0	0	0.5	100	0.01
TF40	n.d.	0.8	16	3.7	0	74	5.1	0	101	4.6

[a] Abbreviations: Rha = rhamnose; Fuc = fucose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glc = glucose; AUA= anhydro uronic acid.
[b] The yield is expressed as weight percentage of the crude extraxt.
[c] TSC= Total sugar content which is expressed as weight percentage (% w/w) of the freeze dried extract.
n.d.= not determined

Fraction S could only be collected in trace amounts from potato AG and, therefore, could not be analysed.
From onion AG two very pure galactans (F40 and F60) could be obtained. Together these fractions made up 29 and 10% w/w of the onion AG, respectively. The citrus AG appeared to be an arabinan-arabinogalactan mixture. Arabinan was the major component and collected in fraction S. Citrus F40 was the second major fraction to S and only in this fraction an arabinogalactan fraction could be collected in a reasonable amount (fraction F60 could only be collected in trace

amounts). After treatment of citrus F40 with endo-glucanase IV (TF40) glucose and xylose could be removed in a substantial amount. This points to the presence of xyloglucan in citrus F40.

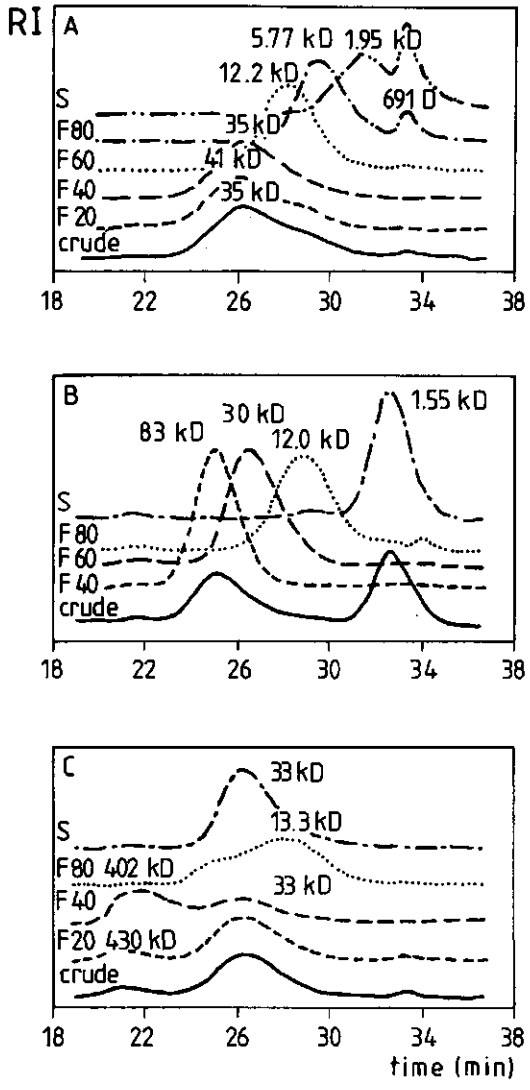

Figure 1: HPSEC analysis of potato AG (**A**), onion AG (**B**) and citrus AG (**C**) and fractions thereof obtained by graded ethanol fractionation.

HPSEC analysis of crude AGs and ethanol precipitated fractions thereof

HPSEC analysis of potato AG and ethanol precipitated fractions thereof shows that the apparent M_w values of the peak fractions of potato AG and F40 were similar (figure 1A). The apparent M_w value found for F20 was somewhat higher. The broad shape of the peak widths in the chromatograms of fraction potato F20 and F40 indicate that more than one population was present. It can be seen (figure 1A and table 2) that the arabinose enriched fractions F80 and S both had low apparent M_w values.
In the onion AG preparation (figure 1B) two populations can be observed differing in their apparent M_w values of the peak fractions (1.55 and 83 kD). It appeared that the apparent M_w value found for onion F40 was similar to the latter value. The apparent M_w value detected for onion F60 was lower (30 kD). The low M_w population present in onion AG was recovered in fraction S. The onion F20 fraction was not analysed because of its low sugar content. The chromatograms in figure 1C show that in the citrus AG preparation also two populations (33 and 430 kD) were present. Two fractions with similar apparent M_w values were also found in the citrus F40 fraction, although with different proportions. Data in table 2 and figure 1 show that the arabinan in fraction S had a M_w value similar to the low molecular weight fraction present in citrus AG.

Methylation analysis of crude AGs and ethanol precipitated F40 fractions

The results of methylation analysis (table 3) of the potato F40 were in accordance with a type I arabinogalactan structure which consists of a backbone of 1,4-linked galactopyranose residues substituted at C_6 by single-unit galactose and 1,5-arabinan side-chains. However, based on the methylation data the presence an 1,5-arabinan population cannot be excluded.
The results in table 3 indicated that the 1,4-linked galactan

present in onion F40 substituted at C_6 by single-unit galactose was ramified to a higher extent compared to the arabinogalactan in potato F40.

The soy AG preparation was suggested to contain a type I arabinogalactan which was slightly branched at C_6 with short arabinose and/or branched arabinans as side-chains. The presence of an arabinan population in this soy AG is very likely (table 3).

The citrus AG preparation consisted of a mixture of a branched arabinan and a type I arabinogalactan (figure 1, tables 2 and 3). The results obtained for TF40 arabinogalactan may be

Table 3: Glycosidic linkage composition of (arabino)galactans present in crude extracts and F40 fractions thereof.

mode of glycosidic linkage (mole%)	potato		onion		soy	citrus	
	AG	F40	AG	F40	AG	AG	TF40
1-Araf	0.8	0.9	0.8		14	24	6.1
1,3-Araf						0.7	1.1
1,5-Araf	20	11	10.0	0.6	19	27	14
1,3,5-Araf					7.2	6.0	2.0
1,2,5-Araf			1.0		3.4	3.5	1.2
1,2,3,5-Araf					5.9	7.6	3.7
1-Galp	2.4	2.0	8.2	18	2.3	1.1	4.0
1,3-Galp							
1,4-Galp	64	74	61.1	67	45	18	50
1,6-Galp							
1,4,6-Galp	1.0	4.1	6.6	11	0.5	0.3	0.7
1-Glcp			0.6				
1,4-Glcp	5.9	4.4	0.9			2.6	5.8
1,6-Glcp			9.2				
1,4,6-Glcp	3.1	2.2				3.7	7.0
1-Manp	1.0					1.3	1.5
1,4-Manp							
1-Rhap							
1,2-Rhap							
1,2,4-Rhap							
1-Xylp	0.6	0.5			0.7	2.5	1.2
1,4-Xylp	0.9	0.7					

interpreted as substitution at C_6 of the 1,4-linked galactan backbone with branched arabinan side-chains. Substitution at C_6 of the galactan backbone with single-unit galactose side-chains cannot be excluded. A branched arabinan may also be present in this fraction. Potato F40 and citrus TF40 also contained 1,4-linked glucopyranose, 1,4,6-linked glucopyranose, terminal xylopyranose, 1,4-linked xylopyranose and terminal mannopyranose containing polysaccharides. The present set of data does, however, not allow to exclude whether the detected xylose and glucose points to the presence of a xyloglucan in these preparations, to covalent linkage of these sugars to the backbone of the arabinogalactans or to both.

TYPE II ARABINOGALACTANS

Sugar composition of coffee arabinogalactan, coffee galactan and stractan

The sugar composition of the arabinogalactans present in coffee AG, coffee galactan and stractan (table 4) differed

Table 4: Sugar composition[a] of arabino-1,3/6-ß-D-galactans

Sugar (mol%)	coffee AG	coffee galactan	stractan
Rhamnose/ Fucose	0.4	0.8	0.4
Arabinose	24	6.0	17
Xylose	0	0	0
Mannose	3.9	2.6	0
Galactose	65	90	83
Glucose	2.1	0.4	0
AUA	4.1	0.4	1.3
Total sugar content[b]	85	74	88
Molar Gal/Ara ratio	2.7	15	4.9

[a] The sugar composition is expressed in mole percentages with the total sugars set at 100 mole %.
[b] Total sugar content is expressed as weight percentage (% w/w) of the freeze dried extract.

with respect to purity (total sugar content varied between 74 and 88% w/w) and their molar Gal/Ara ratio (in the range of 2.7 to 15). Although coffee AG was treated with sufficient arabinofuranosidase B, carbohydrate analysis revealed that the coffee galactan was only partially de-arabinosylated.

Methylation analysis of coffee arabinogalactan and stractan

Analysis of glycosidic linkages (table 5) indicated that coffee AG consisted of a 1,3-linked galactan backbone which

Table 5: Glycosidic composition of coffee AG and stractan

Mode of glycosidic linkage (mole%)	coffee AG	stractan
1-Araf	9.4	4.0
1-Arap	0	4.3
1,2-Araf		
1,3-Araf		5.0
1,5-Araf	8.3	
1,3,5-Araf	1.0	
1,2,5-Araf	0.5	
1,2,3,5-Araf		
1-Galp	5.8	29
1,3-Galp	34	1.2
1,6-Galp		19
1,2-Galp		0.9
1,3,6-Galp	27	19
1,3,4-Galp		2.1
1,2,3,6-Galp		5.6
1,3,4,6-Galp		4.0
1,2,3,4,6-Galp		5.4
1-Glcp		
1,4-Glcp	0.6	
1,6-Glcp		
1,4,6-Glcp	0.6	
1-Manp	6.7	
1,4-Manp		
1-Rhap		
1,2-Rhap		
1,2,4-Rhap		
1-Xylp		
1,4-Xylp	0.5	

carried side-chains in which arabinose is present as single-unit arabinose, branched arabino-oligomeric units or arabinogalacto-oligomeric units. Galactose may also be substituted as single-unit side-chain at C_6. The presence of mannose, glucose and xylose may indicate that these sugars can be bound to C_3 or C_6 of galactose or to C_2, C_3 or C_5 of arabinose residues.

The results obtained for stractan may be interpreted as a 1,3-linked galactan backbone heavily substituted at C_6 with short appendages consisting of 1,6-linked galactose residues or a 1,3-linked backbone interrupted by 1,6-linked galactose residues which are substituted at C_6 and C_3, respectively, by appendages of galactose, arabinopyranose and arabinofuranose. Side-chains of arabinofuranose residues, a disaccharide such as ß-Arap-1,3-Araf or longer chains of 1,3-linked arabinofuranose may be linked at C_6 or C_3 of the galactan backbone. The more heavily substituted galactopyranose residues may indicate undermethylation or the presence of regions in stractan which are more ramified.

DISCUSSION

Type I and II arabinogalactans were isolated and characterized from various sources to be used in screening, assays for enzymes degrading galactan structures and for studying the mode of action of such purified enzymes on these substrates.

The arabinogalactans of both types were characterized by methylation analysis. Although this procedure was repeated for completeness of methylation of the arabinogalactans, it appeared that ratios of terminal/branched residues found were not satisfactory for all preparations. The methylated arabinogalactans could not be effectively hydrolyzed by using TFA and, therefore, an aliquot of these samples was subjected to hydrolysis using formic acid and, subsequently, sulfuric acid.

Type I arabinogalactans. For degradation studies to be performed it was of interest to further purify crude

arabinogalactan extracts by using graded ethanol precipitation. It appeared that a contaminating xyloglucan population present in onion AG powder could be eliminated by graded ethanol precipitation. However, in the case of citrus AG a contaminating xyloglucan population could be only partly eliminated graded ethanol precipitation, followed by treatment with endo-glucananse IV and ethanol precipitation. In contrast to the removal of xyloglucan present in onion AG, graded ethanol precipitation of potato AG and citrus AG did not result in elimination of the contaminating arabinan, as the results presented in table 3 point to the presence of the arabinan populations in the F40 fractions collected at 40% v/v ethanol from potato AG and citrus AG. Type I arabinogalactans precipitating at this ethanol concentration were found to be the major part of the (arabino)galactans with highest galactose/arabinose ratios present in potato AG, onion AG and citrus AG.

Methylation analysis of the arabinogalactan in potato F40 indicated the presence of single-unit galactose side-chains and 1,5-linked arabinose side-chains linked to C_6 the 1,4-linked galactan backbone. These results are similar to data reported by Wood and Siddiqui (1972).

The degree of substitution with single-unit galactose side-chains at C_6 was found to be higher for onion F40 than for potato F40. The very pure ß-D-galactan present in onion F40 fraction had a degree of branching somewhat higher than reported by Redgwell and Selvendran (14 vs 10%) (1986).

Methylation analysis of soy AG pointed to the presence of an arabinan and a type I arabinogalactan, which may be substituted to a small extent at C_6 by short or branched arabinose side-chains, in this extract. The structural features of the arabinogalactan clearly differed from data reported by Morita et al. (1965) and Labavitch et al. (1976). These authors described a more branched arabinogalactan structure in which appendages of arabinose were linked to C_3 of the 1,4-linked galactan backbone.

The 1,4-linked main chain of galactose residues of citrus TF40

arabinogalactan fraction was suggested to be substituted at C_6 by branched arabinans and galactose single-unit side-chains. Similar structural features were reported for a type I arabinogalactan extracted from citrus pectin (Voragen et al., 1987).

Type II arabinogalactans. The glycosidic linkage composition of coffee bean AG points to a more complex structure than reported in literature by Hashimoto (1971) and Wolfrom and Patin (1965). In the model proposed by these authors the 1,3-linked galactan backbone is substituted at C_6 either with Araf(1->3)Gal(1-> side-chains or single-unit arabinofuranose or galactose residues. However, the degree of substitution of 1,3-linked galactose residues in this preparation appeared to be lower than reported by Wolfrom and Patin (1965) (44 vs. 67%).

Treatment of coffee AG with arabinofuranosidase B did not result in complete removal of arabinose residues. A reduction of 17 to 3.7% w/w arabinose was obtained. This may indicate the presence of arabinofuranose residues in the main chain as suggested by Hashimoto (1971) and Bradbury and Halliday (1990).

The types of linkages present in stractan, which is a more heavily branched polymer, are in agreement with the model of western larch or mountain larch arabinogalactans, as reviewed by Timell (1965).

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CHAPTER 4

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[1]

ABSTRACT

Two galactanases, purified from experimental enzyme preparations derived from Aspergillus niger and from Aspergillus aculeatus were found to be similar in a number of properties. They had similar molecular weights (M_r=42-43 kD) and both showed highest activity on potato galactan. Optimal activity was measured at 50-55 °C, pH 4.0-4.3; optimal stability was observed in the pH range of 5 to 7 at 30 °C. A sodium acetate buffer was found to be the best incubation buffer. Digestion of (arabino)-galactan resulted initially in a large shift in the apparent M_w value of the peak fraction of the bulk of (arabino)galactan and formation of low galacto-oligomers, mainly tetra- and trimers. In the final stage of the reaction mono- and dimer accumulated as end products. Therefore a multiple attack mechanism was suggested for the endo-1,4-ß-D-galactanases. The galactanases did not hydrolyse

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arabino-1,3/6-ß-D-galactan.
The effect of the temperature on their stability, specific activities and affinity for potato arabinogalactan differed; in absence of substrate the A. niger and A. aculeatus endo-galactanase were stable up to 60 °C and 35 °C, respectively. The specific activities on potato arabinogalactan in a sodium acetate buffer pH 5.0, 30 °C were found to be 158 and 244 U/mg, respectively. The K_m values estimated for potato arabinogalactan were 0.77 and 0.31 g/l, respectively.
For breakdown of potato arabinogalactan combined action of arabinanases from A. niger and endo-galactanase was required. Arabinofuranosidase B did not stimulate the degradation of potato arabinogalactan by the endo-galactanases under conditions used, whereas endo-1,5-α-L-arabinanase had an immediate synergistic effect with both endo-galactanases, indicating the presence of linear 1,5-α-L-arabinan side-chains.

INTRODUCTION

1,4-ß-D-Galactanases have been shown to occur in several microorganisms (Dekker, 1976). Purification of galactanases to a homogeneous state has only been reported for various strains of Bacillus subtilis (Emi et al., 1971; Labavitch et al., 1976; Nakano et al., 1990; Tsumura et al., 1991; Yamamoto and Emi, 1988) and one fungus Penicillium citrinum (Nakano et al., 1985). Interaction between galactanases and arabinanases during breakdown of arabinogalactans has not been studied until now.
In plant cell walls two types of arabinogalactans are present. Type I L-arabino-1,4-ß-D-galactans and type II L-arabino-1,3/6-ß-D-galactans which have a branched backbone (Clarke et al., 1979 and Stephen, 1983). The (arabino)galactans are considered to be involved in making up the rigid structure of primary cell walls, as indicated in a recently updated tentative model of the primary cell wall (Carpita and Gibeaut, 1993).

Studies have been carried out on the role of galactanases in the processing of fruit and vegetables in order to facilitate juice and colour extraction, tissue maceration and tissue liquefaction. According to Chesson (1980) galactanases do not play a role in maceration of vegetables. Studies on the action of cell wall degrading enzymes on an apple cell wall preparation performed by Renard et al. (1989) and Voragen et al. (1980, 1982) indicate that 1,4-ß-D-galactanase action does not appear to be very important in tissue liquefaction. However, it may be required in order to achieve complete saccharification of solubilized cell wall polysaccharides. Monogastric animals lack enzymes which can split ß-glycosidic bonds (except for ß-galactosidase). Treatment of feed by galactanases may facilitate digestion of animal feed (Beudeker, 1988).
The objective of this study was to isolate and characterize endo-1,4-ß-D-galactanases from fungal enzyme preparations derived from Aspergillus niger and Aspergillus aculeatus and to investigate the enzymes with regard to substrate specificity, mode of action and interaction with arabinanases in the degradation of arabinogalactans.

MATERIALS AND METHODS

SUBSTRATES

Type I arabinogalactans (denoted as AGs) were isolated from high methoxy apple pectin (Obi Pectin Ltd, Bischofzell, Switzerland), citrus pectin NF (Sunkist Growers Inc., California ,USA) and potato fibre (kindly provided by Avebe, Veendam, The Netherlands) as described in chapter 3. Tetragalactose was obtained by partial enzymic degradation of citrus AG by 1,4-ß-D-galactanase of Bacillus subtilis as described by Labavitch et al. (1976). A potato galactan was obtained by removal of arabinose residues from potato AG with arabinofuranosidase B. Other substrates used were 1,5-α-L-arabinan and an arabinose rich ramified pectic material from

apple (designated as MHR, Schols et al., 1990), larch wood arabinogalactan (see chapter 3), arabinoxylan ex oats spelts (Koch and Light, Colnbrook, Bucks, UK), polygalacturonic acid (ICN, Cleveland, Ohio, USA) and CM-cellulose (Akucell AF type 0305, Akzo, Arnhem, The Netherlands). Highly esterified pectin, DE 93% was from the laboratory collection.
In addition, p-nitrophenyl derivatives of α-L-arabinofuranose, (Sigma, Chemical Company, St.Louis, Missouri, USA), α/ß-D-galactopyranose, ß-D-glucopyranose and α/ß-D-xylopyranose (Koch and Light) were also used.

CARBOHYDRATE ANALYSIS OF SUBSTRATES

Neutral sugar composition of AGs, potato galactan and MHR was determined by Seaman hydrolysis and conversion to their alditol acetates as described by Englyst and Cummings (1984) or by the method of Albersheim et al. (1967), as indicated. In the latter method the samples were subjected to hydrolysis with 2 M TFA (1 h, 121 °C). Conditions of GC-analysis of the alditol acetates are given by Gruppen et al. (1989).
Galacturonic acid was determined by spectrophotometry, as described in chapter 3. Methylation analysis was carried out as described in chapter 3.

ENZYME PREPARATIONS

Pectinase 29, an experimental enzyme preparation derived from Aspergillus niger was kindly provided by Gist-Brocades, Seclin, France. Pectinex Ultra-SP-L a technical enzyme preparation derived from Aspergillus aculeatus was a gift from Novo Nordisk, Dettingen, Switzerland.

ENZYME ASSAYS

All enzyme activities were expressed in International Units (U). One unit of enzyme activity is defined as the amount which liberates 1 μmol reducing sugars per min. The protein

content was measured according to Sedmak and Grossberg (1977) using bovine serum albumin as standard.
Endo-galactanase activities were measured by incubating 0.1 ml of a 0.5% w/v potato AG solution in distilled water with 0.4 ml of a diluted enzyme solution in 0.05 M sodium acetate buffer, pH 5.0. The increase in reducing end groups was measured after 1 h incubation at 30 °C by the method of Nelson-Somogyi (Somogyi, 1952), unless stated otherwise. Galactose was used as standard.
Activities on p-nitrophenyl derivatives, MHR, 1,5-α-l-arabinan, polygalacturonic acid, CM-cellulose and arabinoxylan were measured as described by Rombouts et al. (1988).

PURIFICATION OF ENDO-GALACTANASES FROM A. NIGER AND A. ACULEATUS

The original chromatographic procedure consisted of gel permeation chromatography on Bio-Gel P10 (100-200 mesh, column 30 x 950 mm) for desalting, anion exchange chromatography on DEAE Bio-Gel A (column 30 x 200 mm) and adsorption chromatography on Bio-Gel HTP (column 20 x 140 mm) (Bio-Rad Laboratories, Richmond, California, USA) as described by Rombouts et al. (1988). In the optimisation of this procedure an additional step, gel permeation on Bio-Gel P100 (100-200 mesh, column 20 x 900 mm) (Bio-Rad Laboratories, Richmond, California, USA) was added, using 0.01 M NaOAc pH 5.0 as eluent.
Purification of endo-galactanase from A. aculeatus was performed by identical chromatographic steps. Chromatography on HTP and Bio-Gel P100 could be effectively replaced by anion exchange chromatography on the Mono Q column HR 5/5 with the FPLC system of Pharmacia-LKB Biotechnology (Uppsala, Sweden). Gradient elution was carried out as described by Rombouts et al. (1988).
All purification steps were carried out at 4 °C. All buffers contained 0.01% w/w sodium azide to prevent microbial growth.

SDS-GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSING

SDS-gel electrophoresis, isoelectric focusing and titration curves were performed with the Pharmacia Phastsystem. Experimental details are given by Rombouts et al. (1988).

INFLUENCE OF pH, TEMPERATURE, VARIOUS BUFFERS AND CHEMICALS ON ACTIVITY AND STABILITY OF ENDO-GALACTANASES

Effect of pH on the activity was measured by incubation of 0.1% w/v potato AG in McIlvaine buffer in a pH range of 2.5 up to 8.0 with endo-galactanase for 1 h at 30 °C.
The pH stability was investigated by pre-incubation of endo-galactanase in the McIlvaine buffers for 1.5 h at 30 °C. Subsequently potato AG was added to a final concentration of 0.1% w/v and the mixtures were incubated for 1 h at 30 °C.
The optimum temperature was determined by incubation of 0.1% w/v potato AG in 0.05 M sodium acetate buffer pH 5.0 with endo-galactanase at temperatures in the range of 5 to 70 °C for 1 h. The temperature stability was studied by pre-incubation of the enzyme in 0.05 M sodium acetate buffer pH 5.0 at these temperatures for 1.5 h. Subsequently potato AG was added to a final concentration of 0.1% w/v and the mixtures were incubated for 1 h.
The influence of various 0.05 M buffers and chemicals (1 mM, or 2 mM in case of $CaCl_2$ and EDTA) on the activity and stability was studied at 55 °C, pH 5.0. Activities are expressed as percentage of the activity in sodium acetate buffer. Stability is calculated as percentage of the activity in sodium acetate buffer.

ANALYSIS OF REACTION PRODUCTS BY HPLC

Reaction products formed during incubation of (arabino)galactans, tetragalactose and other substrates with endo-galactanase were analysed by HPLC. For the determination of the shift in the apparent M_w distribution of polymeric

substrates digests were analysed by high performance size exclusion chromatography (HPSEC), as described in chapter 3. Mono- and oligomeric reaction products were analysed by an Aminex HPX 87P (Voragen et al., 1986) or by an Aminex HPX 42A column (Biorad Lab, Richmond, California, USA), as indicated. Analysis using the HPX 42A column was carried out as described for the HPX 87P column.

KINETIC PROPERTIES

Lineweaver-Burk plots of endo-galactanase action on potato and citrus AG were determined by incubation with endo-galactanase in 0.05 M sodium acetate, pH 4.0, for 1 h at 55 °C.
Data analysis for calculation of kinetic parameters, using non-linear regression, was performed by a program called "Enzfitter" (Leatherbarrow, 1987).

RESULTS

SUGAR COMPOSITION OF SUBSTRATES

Characterization of the various substrates was required for a proper interpretation of the action of the enzymes. Their sugar compositions are given in table 1. Potato galactan was a fairly pure polysaccharide with total sugar content 89% (w/w) and a molar galactose/arabinose ratio of 38. Citrus and apple AG both had a neutral sugar content of 69 % (w/w) and a molar galactose/arabinose ratio of 3.7 and 1.5, respectively. MHR had a total sugar content of 90% (w/w) and a molar galactose/arabinose ratio of 0.18. Total sugar composition and glycosidic linkage composition of potato AG is given in chapter 3. Methylation analysis revealed that a arabinose reduced citrus AG, obtained by treatment with arabinofuranosidase B (Rombouts et al., 1988), was substituted to a low extent at C_6 with 1.5-linked arabinose residues. In the citrus AG the 1,4-linked 1,4-galactan backbone was substituted with short arabinose side-chains or highly

branched side-chains (results not shown).

Table 1: Sugar composition of substrates[a]

Sugar	potato[b] galactan	citrus[b] AG	apple[b] AG	MHR[c]
Rhamnose/ Fucose	0.36	0.3	0	5.6
Arabinose	2.5	20	32	55
Xylose	0.31	0	0.46	8.4
Mannose	0	1.9	0	0
Galactose	94	74	47	9.9
Glucose	0	4.5	21	0
AUA	2.9	nd	nd	21
Total sugar content[d]	89	69[e]	69[e]	90
Gal/Ara ratio	38	3.7	1.5	0.18

[a] The sugar composition is expressed in mole percentages with the total sugars set at 100 mole%. nd= not determined.
[b] Analysis according to Englyst and Cummings (1984).
[c] Analysis according to Albersheim et al. (1967).
[d] Total sugar content is expressed as weight percentage (% w/w) of the freeze dried extract.
[e] Total content of neutral sugars.

PURIFICATION OF ENDO-GALACTANASES FROM ASPERGILLUS NIGER AND ASPERGILLUS ACULEATUS.

The procedure used initially for the isolation of endo-galactanase from Aspergillus niger was based on the procedure used for the isolation of arabinanases by Rombouts et al. (1988) and is shown in figure 1. Fractions containing galactanase activity were obtained by chromatography on DEAE Bio-Gel A. They were combined in pools II_5, III_1, III_4 and III_5 and were further purified on a Bio-Gel HTP column. Contaminating arabinofuranosidase, endo-arabinanase and a substantial amount of polygalacturonase activity could be removed in this last step. The various fractions (F, J, L and M) obtained were found to be very similar in their properties.

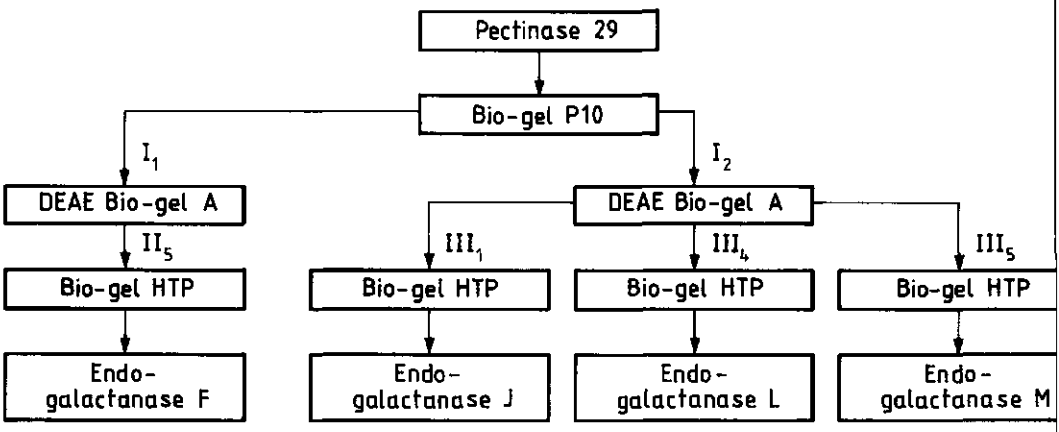

Figure 1: Flow sheet of the purification of endo-galactanases F, J, L and M from an enzyme preparation of Aspergillus niger. The Roman numerals with Arabic subscripts refer to enzyme pools.

The galactanase isolation could be simplified and optimised by a similar procedure consisting of four steps (figure 2A). HPLC analysis of incubation mixtures of enzyme fractions and potato AG revealed that all endo-galactanase activity could be found in pool II_6. Contaminating protein in pool III_4 could be removed by gel permeation chromatography on Bio-Gel P100.
The purification of endo-galactanase from A. aculeatus is summarised in figure 2B. Endo-galactanase in pool II_3 eluted at a similar ionic strength as A. niger endo-galactanase (pool II_6, figure 2A). The enzyme was further purified by anion exchange chromatography on Mono Q. In this step contaminating polygalacturonase and CMC-ase were separated from endo-galactanase.
Some characteristics of endo-galactanase fractions isolated from A. niger and A. aculeatus are presented in table 2. The optimised procedure for the A. niger preparation resulted in

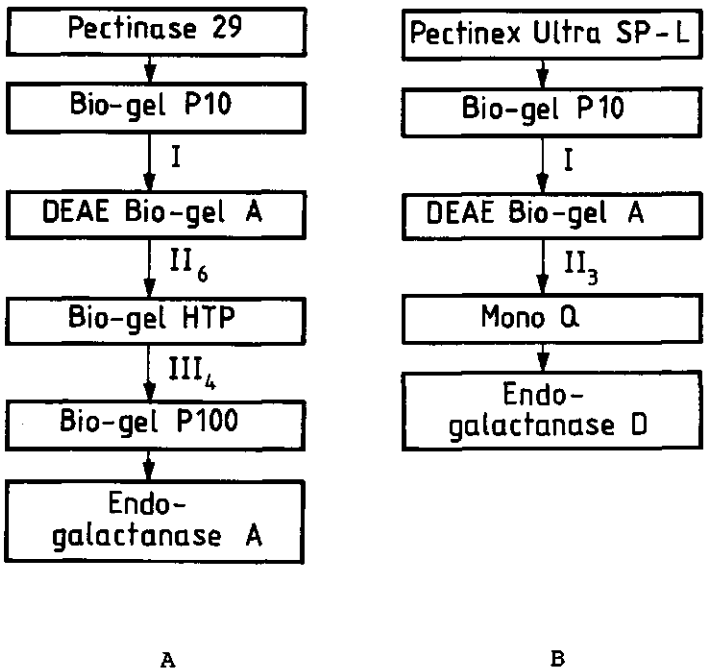

Figure 2: Flow sheet of the optimised purification of endo-galactanase A and D from an enzyme preparation of Aspergillus niger (**A**) and Aspergillus aculeatus (**B**). The Roman numerals with Arabic subscripts refer to enzyme pools.

an endo-galactanase fraction with a high specific activity on potato AG. The endo-galactanase was obtained in a higher yield than endo-galactanase F. Endo-galactanase D from A. aculeatus showed a higher specific activity than F and A (244 U/mg versus 95.2-158 U/mg at pH 5.0, 30 °C). Enzymes A and F showed the same mobility on SDS-electro-phoresis. Enzyme D also moved as one band and showed a similar mobility on SDS-electrophoresis.

Table 2: Comparison of purification of endo-galactanases from A. niger and A. aculeatus

Enzyme	Specific activity pH 5.0, 30 °C (U/mg)	yield (%)	total activity (U)
From A. niger			
F	95.2	5.2	178
A	158	21	404
From A. aculeatus			
D	244	22	391

PHYSICO-CHEMICAL PROPERTIES

The A. niger (M_r=43 kD) and A. aculeatus (M_r=42 kD) endo-galactanases differed with respect to their specific activities, especially at pH 5.0 and 30 °C and their temperature stability (tables 2 and 3). A. niger endo-galactanase was stable up to 60 °C as opposed to the A. aculeatus galactanase which was only stable below 35 °C. Both enzymes showed the highest activity at pH 4.0-4.3 and 50-55 °C.

The isoelectric points of these enzymes could not be determined. Titration curves of endo-galactanase A and D showed that in the pH range 4 to 6 the enzymes did not migrate but remained at the site of application. This aberrant behaviour was also found by Rombouts et al. (1988) for A. niger arabinanases. Endo-galactanase A was fairly resistant to freezing and thawing. In the presence of 0.1 and 0.01% w/v thiomersal as preservative, instead of 0.01% w/v NaN_3, in the reaction mixture 80 and 108% of the activity under standard conditions was measured. The deviation of the latter figure is within experimental error.

The effects of various incubation buffers on the activities activities and stabilities of both enzymes are listed in table 4. Both enzymes displayed high activities (and stability in case of the A. niger galactanase) in a sodium acetate buffer.

Table 3: Physico-chemical properties of endo-galactanases from A. niger and A. aculeatus

	A	D
Specific activity (pH 4.0, 55 °C) on potato AG (U/mg)	223	287
M_r (SDS-electrophoresis)	43000 D	42000 D
Glycoprotein	-	nd
Isoelectric point	4-6	4-6
Optimum pH	4.0	4.3
pH Stability	5-7	5-7
Optimum temperature	50-55 °C	50 °C
Temperature stability	≤60 °C	≤35 °C
Kinetic parameters (potato AG, 0.005 µg protein/ml)		
V_{max} (x 10^3 min^{-1})	55.5	58.4
K_m (g/l)	0.77	0.31
V_{max}/K_m (x 10^3 l min^{-1} g^{-1})	72	188
Freezing and thawing (5 x)	93%	
Freezing and thawing (10 x)	78%	
0.1% w/v Thiomersal in buffer	80%	
0.01% w/v Thiomersal in buffer	108%	

Similar activities were found using a citric acid/NaOH buffer; because the stability of the A. niger galactanase was decreased the sodium acetate buffer is preferred. A. aculeatus galactanase was not stable in both buffers. Due to analytical problems with the Nelson-Somogyi assay of digests in citric acid/NaOH buffer (the assay gave precipitates), these digests were analysed by HPLC and compared with digests in sodium acetate buffer.

For determination of the effect of chemicals on the activities and stabilities of both galactanases an excess of the chemicals listed in table 4 was present in reaction mixtures. Pb^{2+} and to a lesser extent Ag^{+} and Zn^{2+} ions in the sodium acetate buffer had a negative effect on the activities of both enzymes and stability of the A. niger enzyme. Ca^{2+} ions and EDTA did not have any effect on activity at optimum temperature and thermal stability of both enzymes.

SUBSTRATE SPECIFICITY AND MODE OF ACTION

The data presented in table 5 and figure 3 clearly show that enzymes A, D and F are only active on substrates with a backbone of 1,4-ß-D-galactopyranose residues. Small amounts of pectin lyase in fractions A and F and polygalacturonase in

Table 4: Effects of various incubation buffers and chemicals on the activity and stability of endo-galactanases from A. niger and A. aculeatus.

Buffer/Chemical	A		D
	activity	stability	activity
Naacetate	100	100	100
Nasuccinate/HCl	81	75	78
Oxalic acid/NaOH	77	69	68
Citric acid/NaOH	103	62	102
Nasuccinate/ Oxalic acid	95	93	93
KCl	97	92	93
$AgNO_3$	41	2.1	9.0
$MgCl_2$	88	79	100
$CaCl_2$	97	93	98
$MnCl_2$	103	84	77
$CoCl_2$	80	74	89
$NiCl_2$	73	57	70
$ZnSO_4$	52	43	58
$BaCl_2$	90	86	93
$Pb(NO_3)_2$	14	0	0
DTT	105	101	109
EDTA	102	99	106

fractions A and D could be detected. Fraction F showed some activity on polygalacturonic acid. HPLC analysis of reaction products, however, showed that galactose and galactobiose were released, presumably from galactan impurities in the polygalacturonic acid preparation.

Table 5: Substrate specificity of endo-galactanases from A. niger and A. aculeatus[a]

Substrate \ activity	Activities expressed as percentage of activity on potato AG: Enzymes A. niger A	Enzymes A. niger F	Enzyme A. aculeatus D
Potato AG	100	100	100
PNP-α-D-Gal	-	-	-
PNP-ß-D-Gal	-	-	-
PNP-α-L-Ara	-	-	-
PNP-α-D-Xyl	-	-	-
PNP-ß-D-Glc	-	-	-
PNP-ß-D-Xyl	-	-	-
Arabinoxylan	-	-	-
1,5-α-L-arabinan	-	-	-
MHR	-	-	-
Stractan	-	-	-
Polygalacturonic acid	0.4	0.06	0.4
Pectin lyase	0.11	0.16	-
Pectate lyase	-	-	-

[a] Incubation: 10 μg enzyme (6.0 μg of F) protein per ml 0.05 M sodium acetate buffer pH 5.0, 20 h at 30 °C.

The release of oligomers from potato AG by endogalactanase A is presented in figure 3A. Similar patterns were obtained when apple or citrus AG was used as substrate. It can be seen that initially tetramer galactose is formed and to a lesser extent the trimer, maximum amounts were reached after 0.5 h and 1.5 h, respectively. Also dimeric galactose was released from the very beginning. After 2 h its production gradually levelled off. Monomeric galactose accumulated at a low rate in the course of the reaction. After 24 h an extent of hydrolysis of 52% was reached. These values were derived from the time curves in figure 3B. This action pattern is typical for all endo-galactanase fractions of the A. niger preparation. The degradation of tetragalactose by the A. niger and A. aculeatus endo-galactanase was demonstrated by HPLC analysis of digests of tetragalactose (figure 3C).

The mode of action of galactanase D differed only very

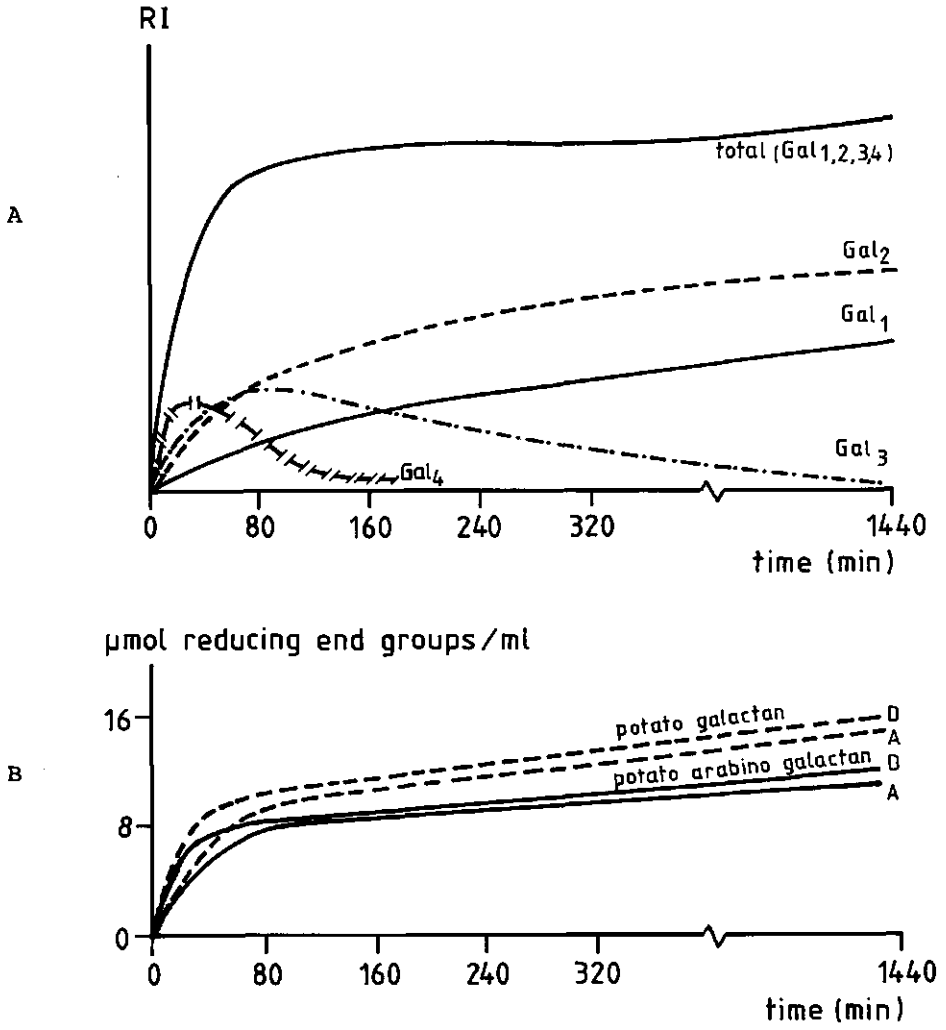

Figure 3: Time course studies of the degradation of potato AG by endo-galactanase from A. niger **(A)** analysed by HPLC using a HPX 42A column. Time curves of action of A. niger and A.aculeatus endo-galactanases on potato AG and potato galactan **(B)**. Incubation: 0.60 µg protein and 4 mg substrate per ml 0.05 M sodium acetate buffer, pH 5.0 at 30 °C. Reaction products released from tetragalactose by endo-galactanase from A. niger and A. aculeatus **(C)** analysed by HPLC using a HPX 87P column. Incubation: 1 µg protein and 2 mg substrate per ml 0.05 M sodium acetate buffer, pH 5.0, ½ h at 30 °C.

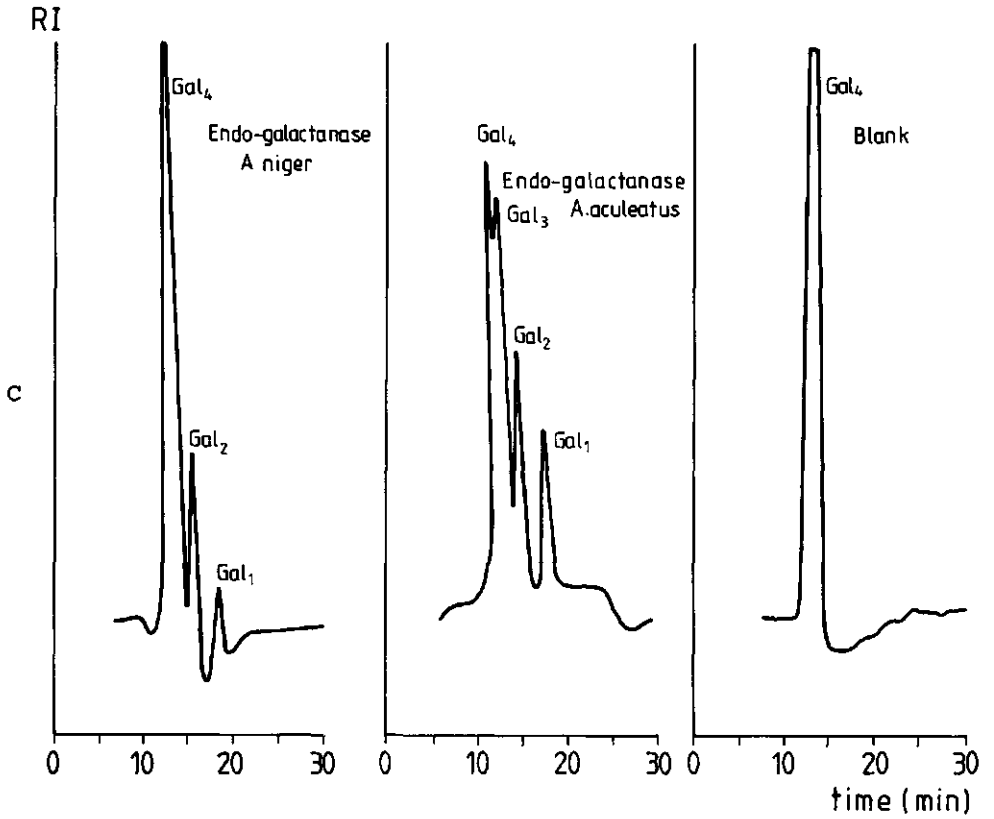

Figure 3 - continued

slightly from galactanase A. Enzyme D degraded potato AG more rapidly. Very small amounts of oligomers of DP ≥9, which were hydrolysed by enzyme A, were not completely degraded by enzyme D (results not shown). The ratio of released galactose/galactobiose was found to be higher for enzyme D.
The mode of action of enzymes A and D on potato galactan was similar to the pattern in figure 3A. This galactan was degraded more rapidly and to a further extent by both enzymes, viz to a level of 66% and 71% hydrolysis after 24 h incubation with A and D (figure 3B).

KINETIC PARAMETERS

Kinetic parameters of both enzymes are presented in table 3 and figure 4 and 5. Because V_{max}/K_m is considered to be a measure of the catalytic efficiency and specificity, these values were compared. For K_m in g/l and V_{max} in min^{-1} the V_{max}/K_m values of endo-galactanase F from A. niger (0.058 µg protein/ml reaction mixture) were 8.46×10^3 and 13.3×10^3 on

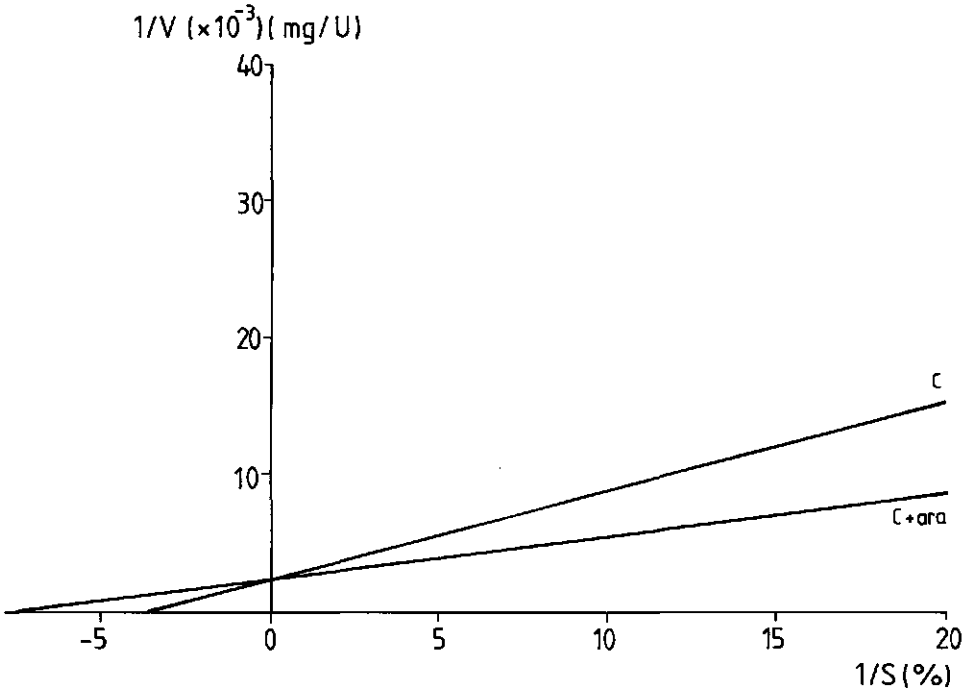

Figure 4: Lineweaver-Burk plots of endo-galactanase F (A. niger) on citrus AG (C) and linearised citrus AG (C+Ara).

citrus AG and citrus AG linearised with arabinofuranosidase B, respectively. The Lineweaver-Burk plots in figure 4 show that lowering the degree of branching did not affect V_{max} but had a

clear effect on the K_m values.

Data in table 3 show that the enzyme from A. aculeatus differed from the enzyme of A. niger. Enzymes A and D had similar V_{max} values but differed with respect to their affinity for potato AG. Enzymes A and D were characterized by the following V_{max}/K_m values on potato AG; 72.0 x 10^3 and 188 x 10^3 l min^{-1} g^{-1}, respectively.

Analysis of digests of high concentrations of potato AG for determination of kinetic parameters could not be performed with the Nelson-Somogyi assay (Somogyi, 1952). The assay gave precipitates with this substrate, resulting in poor reproducibility. The automated neocuproine assay reaction (Stephens et al., 1974) gave more reproducible absorbance readings (Kormelink et al., 1990).

ACTION OF ENDO-ARABINANASE AND ENDO-GALACTANASES FROM A. NIGER AND A. ACULEATUS ALONE AND IN COMBINATION ON POTATO AG

The interaction between galactanases and arabinanases from A. niger (Rombouts et al., 1988)) for the breakdown of potato AG was studied. Arabinofuranosidase B (EC 3.2.1.55) showed very little activity on potato AG and did not enhance the degradation when it was used together with endo-galactanases under conditions used. Endo-1,5-α-L-arabinanase (EC 3.2.1.99), however, was found to be active on potato AG. This activity was calculated to be 20% of the sum of single activities of endo-arabinanase and endo-galactanase A. Synergism exerted by endo-arabinanase and endo-galactanases A and D was investigated. The results are summarised in table 6A and from these data it can be seen that endo-arabinanase in combination with both endo-galactanase preparations accelerates the breakdown of the polymer. The reaction products formed during incubation were also analysed by HPSEC and HPLC for changes in apparent M_w distribution (figure 5) and formation of oligomeric reaction products (table 6B). The chromatograms in figure 5 show that AG degradation is strongly enhanced by the

Table 6A: Relative activities[a] of endo-arabinanase and endo-galactanases from A. niger and A. aculeatus on potato AG

	A	D	endo-ara
endo-ara	130	121	52
D	88	85	
A	92		

Table 6B: Relative contents of galactose, galactobiose and galactotriose in digest of potato AG[b]

	A	D	A + endo-ara	D + endo-ara
Galactose	100	100	128	119
Galactobiose	100	100	135	132
Galactotriose	100	100	127	132

[a] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Incubation: 0.05 μg galactanase and 1.1 μg endo-arabinanase protein and 1 mg substrate per ml 0.05 M sodium acetate buffer, pH 4.0, 1 h at 55 °C.
[b] Analysis by HPLC using the HPX 87 P column.

combined action of endo-arabinanase and endo-galactanase from A. niger and A. aculeatus. The data of table 6B revealed that the combined action of endo-arabinanase and endo-galactanase from A. niger or A. aculeatus resulted in an increased release of galactose and galactobiose and galactotriose. An increase in the content of tetramer in the digest could not be detected.

DISCUSSION

PURIFICATION

The purification scheme as designed by Rombouts et al. (1988) for the isolation of arabinanases from the experimental A. niger enzyme preparation, could also be used for the isolation of endo-galactanases. It resulted, however, in a number of

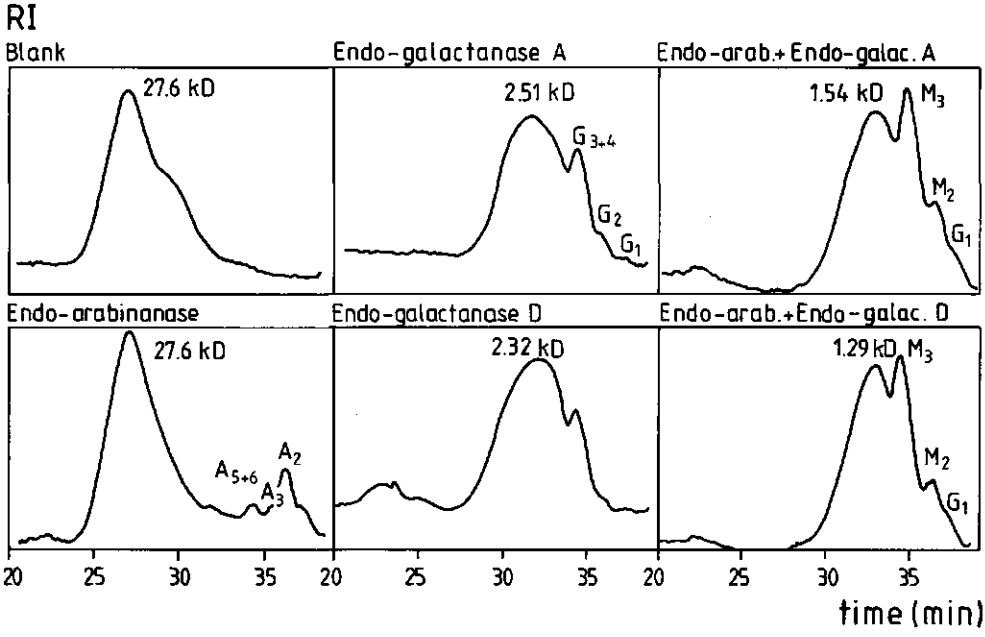

Figure 5: HPSEC analysis of reaction products of digests of potato AG with endo-arabinanase and endo-galactanases from A. niger and A. aculeatus. Experimental details in the legend are described in table 6. G_1, monomeric galactose; G_2, dimeric galactose, etc.. A_2, dimeric arabinose; A_3, trimeric arabinose, etc.. M_2 mixture of A_2, G_2 and A_3; M_3, mixture of G_3, G_4, A_5 and A_6.

endo-galactanase fractions which were found to have similar properties. The fact that a number of endo-galactanase fractions was obtained is obviously caused by the procedure followed. Therefore, this procedure was optimised and successfully applied in the purification of endo-galactanases from A. niger and A. aculeatus enzyme preparations. In the purification procedure of endo-galactanase from A. aculeatus the last two steps were successfully replaced by chromatography on Mono Q.

PROPERTIES

The two endo-galactanases were similar in a number of properties. They only degraded substrates with a backbone of 1,4-ß-D linked galactopyranose residues. Potato galactan was the best substrate for these enzymes, as shown in figures 3B and 4. During the action of the endo-galactanases on (arabino)-galactans low galacto-oligomers were formed, mainly of DP ≤4 and a very rapid change in the molecular weight distribution of (arabino)galactan was observed. Galactose and galactobiose accumulated as end products. These data suggest that the enzymes from both fungi are endo-1,4-ß-D-galactanases (EC 3.2.1.89) which hydrolyse according to a multiple attack mechanism (Robyt and French, 1967). Galactanases from various strains of Bacillus subtilis differ in their action pattern from these endo-galactanases. Endo-galactanases from B. subtilis var amylosacchariticus released mainly galactotriose and to a lesser extent galactobiose and galactose as final hydrolysis products (Yamamoto and Emi, 1988). Galactanases from two other strains, viz K-50 (Emi et al., 1971) and WT 168 (Labavitch et al., 1976) showed both exo- and endo-activity, respectively galactobiose and galactotetraose were formed as major reaction products. The galactanase purified from another B. subtillis strain appeared to be an exo-galactanase which released galactobiose as major reaction product (Nakano et al., 1990). The action pattern of the endo-galactanases from P. citrinum (Nakano et al., 1985) and from Bacillus sp. S-2 and S-39 (Tsumura et al., 1991) was very similar to the A. niger and A. aculeatus enzymes.

The latter galactanases did not show glycosyl transferase activity, as described for endo-galactanases I and II from Penicillium citrinum (Nakano et al., 1985, 1986, 1988). Endo-galactanases purified from A. niger and A. aculeatus showed optimal activity at 50-55 °C and pH 4.0-4.3, which is similar to previous reported data for the enzyme complex of A. niger (Tavobilov et al., 1986) and the endo-galactanases from P. citrinum (Nakano et al., 1985). The pure S-2 and S-39 endo-

galactanases were reported to show activities at pH 10 and at both pH 4 and 9, respectively (Tsumura et al., 1991).
The endo-galactanase from A. niger was most stable in a sodium acetate buffer, therefore this buffer was used in the experiments. The endo-galactanases from A. niger and A. aculeatus differed in their thermal stability, kinetic properties and specific activity. Endo-galactanase from A. niger was stable up to 60 °C, the endo-galactanase from A. aculeatus, however, was only stable up to 35 °C. EDTA or Ca^{2+} ions did not have any effect on the activity at 55 °C and thermal stability of both enzymes. This in contrast to the endo-galactanases of B. subtilis var amylosachariticus which were found to be sensitive to EDTA and could be stabilized by Ca^{2+} ions (Yamamoto and Emi, 1988). The Lineweaver-Burk plots show that the A. aculeatus endo-galactanase has a higher affinity for potato AG than the A. niger enzyme.
Determination of kinetic properties is strongly influenced by the methods (Kormelink et al., 1990) and substrates used. Therefore, these properties could not be compared with values reported in literature (Nakano et al., 1985; Yamamoto and Emi, 1988). The specific activities of A. niger and A. aculeatus on potato AG were found to be respectively 158 and 244 U/mg in a sodium acetate buffer pH 5.0 at 30 °C.

DEGRADATION OF POTATO AG

Combined action of arabinanases and endo-galactanase was studied for degradation of potato AG. Endo-arabinanase exerted synergistic effects with both endo-galactanases. The action of these enzyme combinations resulted in an acceleration of the enzymic hydrolysis of the AG. Also an increase in the shift of the molecular weight distribution of the digest was observed and galactose, galactobiose and galactotriose were formed in increased amounts. The results also revealed that galactanase action was hindered by the presence of arabinan side-chains. Arabinofuranosidase B showed very little activity on the substrate and therefore did not exert a detectable synergistic

effect with endo-galactanase under conditions used. These data indicated that the substrate is an AG with linear 1,5-α-L-arabinans substituted on the galactan backbone. This was also indicated by the results of methylation analysis given in chapter 3.

A study of synergistic effects exerted by galactosidases, endo-galactanases, endo-arabinanase and arabinofuranosidases will be a subject of a future communication.

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CHAPTER 5

ß-D-GALACTOSIDASE FROM ASPERGILLUS NIGER AND ITS ROLE IN DEGRADATION OF TYPE I ARABINOGALACTANS

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ABSTRACT

ß-D-galactopyranosidase was purified from an experimental enzyme preparation derived from Aspergillus niger to almost complete homogeneity. The enzyme had a M_r of 116 kD, a pI of 4.4 and showed highest activity at pH 5.0 and 50 °C towards PNP-ß-D-Gal as substrate. The enzyme was stable up to 50 °C at pH 5.0 and in the pH range of 3.5 to 7 at 30 °C. In the presence of Ag^+ ions the enzyme activity was decreased. This ß-D-galactosidase was also active towards arabinogalactans having a 1,4-ß-D-galactan backbone, from which it released terminal linked galactose residues. The V_{max} and K_m values for PNP-ß-D-Gal, for soy AG and onion F40 measured at pH 5.0 and 50 °C were 1.7 x 10^3 min^{-1} and 1.9 x 10^{-3} M; 1.1 x 10^3 min^{-1} and 0.87 10^{-3} M; 38 10^{-3} M and 0.413 M anhydrogalactose, respectively. The enzyme preferentially attacked 1,4-ß-linked D-galactose residues but was also able to release 1,6-ß-linked D-galactose residues, which were present as single-unit side-chains. However, no activity of ß-D-galactosidase was found on type II arabinogalactans.

[1] TO BE SUBMITTED

INTRODUCTION

Arabinogalactans have been found in many higher plants. This group of polysaccharides is subdivided in two types: arabinogalactans which in general consist of a linear backbone of 1,4-ß-linked D-galactopyranose residues with single-unit or 1,5-α-linked L-arabinofuranose residues connected to O-3 or O-6 of the galactose residues (type I), and highly branched arabinogalactans with a galactan backbone with 1,3-ß-linked D-galactopyranose units and 1,6-ß-D-galactopyranose units mainly in exterior chains (Clarke et al., 1979 and Stephen, 1983). These exterior chains carry short side-chains of predominantly α-L-arabinofuranose residues, which are also present as branches on the 1,3-linked chain. The arabinose content of type I and type II arabinogalactans can be as high as 43 and 80% (w/w), respectively.

Degradation of type I arabinogalactans by endogenous ß-galactosidase appears to be important in cell growth and differentiation (Konno et al., 1986) and fruit ripening (Bartley, 1974, Pressey , 1983). It has been proposed that in lupin cotyledons ß-galactosidases play a role in germination by degrading both types of arabinogalactans (Matheson and Saini, 1977). A ß-galactosidase able to degrade partly de-arabinosylated type II arabinogalactans was found in imbibed radish seeds (Sekimata et al., 1989).

Complete saccharification of type I arabinogalactans is of interest for economical conversion of biomass to chemicals and fuels. This saccharification can not be completed by an enzyme mixture consisting of endo-galactanase and endo-arabinanase and arabinofuranosidases, as galactobiose in a digest is resistant to endo-galactanase (chapter 2).

ß-Galactosidase, first discovered by Beijerinck (1889) is ubiquitous in nature (Wallenfels and Weil, 1972). Microbial ß-galactosidases, relevant to our research, have been purified from Aspergillus oryzae (Akasaki et al., 1976 and Ogushi et al., 1980), A. niger (Bahl and Agrawal, 1969, Greenberg and Mahoney, 1981, Lee and Wacek, 1970, Widmer and Leuba, 1979),

Corticium rolfsii (Kaji et al., 1972), Penicillium citrinum (Watanabe et al., 1979), P. multicolor (Takenishi et al., 1983), Macrophomina phaseoli (Sugiura et al., 1976b), Slerotium tuliparum (Sugiura et al, 1976a). ß-Galactosidases have also been purified from Bacillus subtilis (Anema, 1964), B. circulans (Mozaffar et al., 1984) and B. macerans (Miyazaki, 1988).
This study focuses on the purification and characterization of a ß-D-galactosidase (EC 3.2.1.23) from an experimental enzyme preparation derived from A. niger and its role in the enzymic degradation of type I arabinogalactans isolated from various plant sources.

MATERIALS AND METHODS

SUBSTRATES

p-Nitrophenyl-ß-D-galactopyranose (PNP-ß-D-Gal) (Koch and Light, Colnbrook, Bucks, UK) was used to measure ß-D-galactosidase activity. Other glycosidase activities were measured using PNP derivatives of α-D-galactopyranose (Koch and Light), α-L-arabinofuranose (Sigma Chemical Company, St. Louis, Missouri, USA), ß-D-xylopyranose and ß-D-glucopyranose (Koch and Light). Also lactose (Merck, Darmstadt, Germany) and lactulose (Merck) were used as substrate.
Type I and type II arabinogalactans were also tested as substrates. Type I arabinogalactans were prepared from soy meal, potato fibre and onion powder, as described in chapter 3. They are denoted as soy AG, potato AG and onion F40, repectively. Type II arabinogalactans used in our studies were from larch wood ("stractan"), purchased from St. Regis Company (Tacoma, Washington, USA) and from green coffee beans (denoted as coffee AG). The latter was isolated according to a procedure described in chapter 3. Other substrates used were 1,5-α-L-arabinan, an arabinose-rich ramified pectin fraction from apples (designated as MHR, Schols et al, 1990), arabinoxylan ex oat spelts (Koch and Light, Colnbrook, Bucks,

UK), polygalacturonic acid (ICN, Cleveland, Ohio, USA) and CM-cellulose (Akucell AF type 0305, Akzo, Arnhem, The Netherlands). Highly esterified pectin, DE 93% was from the laboratory collection.

METHYLATION ANALYSIS

Glycosidic linkage composition was established by methylation analysis according to Hakomori (1964) as modified by Sanford and Conrad (1966). Care was taken to ensure total methylation, by repeating the procedure if after methylation, any material remained insoluble in $CHCL_3$/MeOH (50/50% v/v). Methylated polysaccharides were purified by dialysis, freeze dried and subsequently subjected to two different methods of hydrolysis, converted to partially methylated alditol acetates and analysed, as described in chapter 3.

ENZYME PREPARATION

Pectinase 29, an experimental enzyme preparation derived from Aspergillus niger (kindly provided by Gist Brocades, Seclin, France) was used as source for ß-D-galactosidase.

ENZYME ASSAYS

All enzyme activities are expressed in International Units (U). One unit of enzyme activity is defined as the amount which liberates 1 µmol reducing sugars per min. Protein content was measured according to Sedmak and Grossberg (1977). Determination of glycosidase activity on PNP-ß-D-Gal and other p-nitrophenyl glycosides as described by Rombouts et al. (1988), was slightly modified by using micro-titer plates (PS-Mikrotiterplate 96K F-Form, purchased from Greiner Labortechnik, Germany). Incubation mixtures of 125 µl 0.05 M sodium acetate buffer pH 5.0 containing 0.02% w/v substrate and enzyme aliquots were incubated for 1 h at 30 °C. The extinction coefficient (13700 M^{-1} cm^{-1}) was corrected by

multiplication by 1.388 for use of titer plate and measurement at 405 nm.

PURIFICATION OF ß-D-GALACTOPYRANOSIDASE FROM A. NIGER

Purification of ß-D-galactosidase from A. niger was performed by a procedure, which comprised a desalting step by gel permeation chromatography on Bio-gel P10 (100-200 mesh, column 30 x 950 mm), anion exchange chromatography on DEAE Bio-gel A (column 30 x 200 mm), chromatography on cross-linked alginate (column 20 x 150 mm, Rombouts et al., 1988), adsorption chromatography on Bio-gel HTP (column 20 x 160 mm, Biorad Laboratories, Richmond, California, USA) and gel permeation chromatography on Ultrogel AcA54 (column 25 x 875 mm, IBF, Villeneuve-la-Garenne, France). An additional step consisted of chromatofocusing on Mono P HR 5/20 equilibrated with 0.025 M bis-tris pH 6.0 and eluted with polybuffer 74 (10 times diluted), pH 4.0 with the FPLC system (Pharmacia-LKB Biotechnology, Uppsala, Sweden).
All purification steps were carried out at 4 °C. All buffers contained 0.01% w/v sodium azide to prevent microbial growth.

SDS-GEL ELECTROPHORESIS

SDS-gel electrophoresis was performed with the Phastsystem (Pharmacia-LKB Biotechnology). Experimental details are given by Rombouts et al. (1988).

INFLUENCE OF pH, TEMPERATURE, VARIOUS BUFFERS AND CHEMICALS ON ACTIVITY AND STABILITY OF ß-D-GALACTOSIDASE

Effect of pH and temperature on ß-D-galactosidase activity and stability was measured as described in chapter 4.
The influence of various buffers (0.05 M pH 5.0) and chemicals on ß-D-galactosidase activity was studied at 30 °C. Activities are expressed as percentage of the ß-D-galactosidase activity measured under standard conditions.

HPLC ANALYSIS OF REACTION PRODUCTS

Monomeric and oligomeric reaction products formed during incubation of lactose, lactulose and (arabino)galactans with ß-D-galactosidase were analysed by HPLC using a CHPb column (Merck) as described by Voragen et al. (1986). The shifts in the apparent M_w distribution of polymeric substrates as result of enzyme action were analysed by high performance size exclusion chromatography (HPSEC), as described in chapter 3.

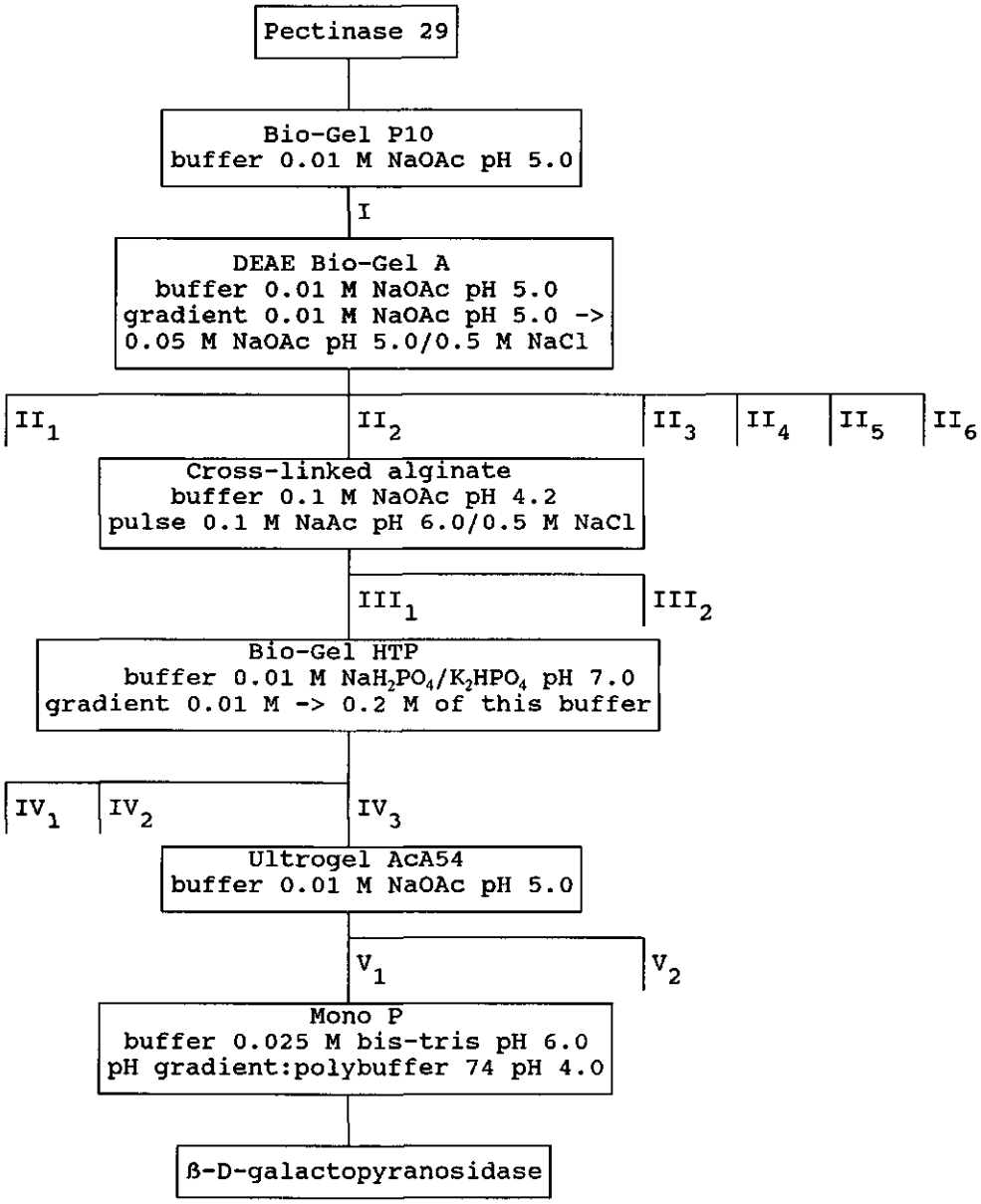
KINETIC PROPERTIES

Lineweaver-Burk plots of ß-D-galactosidase action on PNP-ß-D-Gal soy AG and onion F40 were determined by incubating these substrates with ß-galactosidase in 0.05 M buffer, pH 5.0, for 1 h at 50 °C and measuring release of galactose by HPLC.
Data analysis for calculation of kinetic parameters, using non-linear regression, was performed by a program called "Enzfitter" (Leatherbarrow, 1987).

RESULTS

PURIFICATION OF ß-D-GALACTOSIDASE FROM A. NIGER

The procedure used for the isolation of ß-D-galactosidase from A. niger is outlined schematically in figure 1. After chromatography on DEAE Bio-Gel a pool (II_2) containing ß-D-galactosidase was collected. Pool II_2 was run over a cross-linked alginate column to remove polygalacturonase activity and the pooled ß-D-galactosidase containing fractions (III_1) were further purified on a Bio-Gel HTP column (pool IV_3), followed by gel permeation chromatography on Ultrogel AcA54 resulting in pool V_1. By gel permeation chromatography α-galactosidase was removed from the ß-D-galactosidase present in pool IV_3 (figure 2). Chromatography on Mono P was carried out to remove a substantial amount of contaminating ß-D-xylosidase present in fraction V_1. The enzyme was obtained in a



'igure 1: Flow sheet of the purification of ß-D-galactosidase from an enzyme preparation of A. niger. The Roman numerals with Arabic subscripts refer to enzyme pools.

yield of 16%, the overall purification was 41-fold. It showed a specific activity of 6.1 U/mg on PNP-ß-D-Gal at pH 5.0, 30 °C. SDS-electrophoresis revealed that a major diffuse band and another faint band were present in the ß-D-galactosidase preparation.

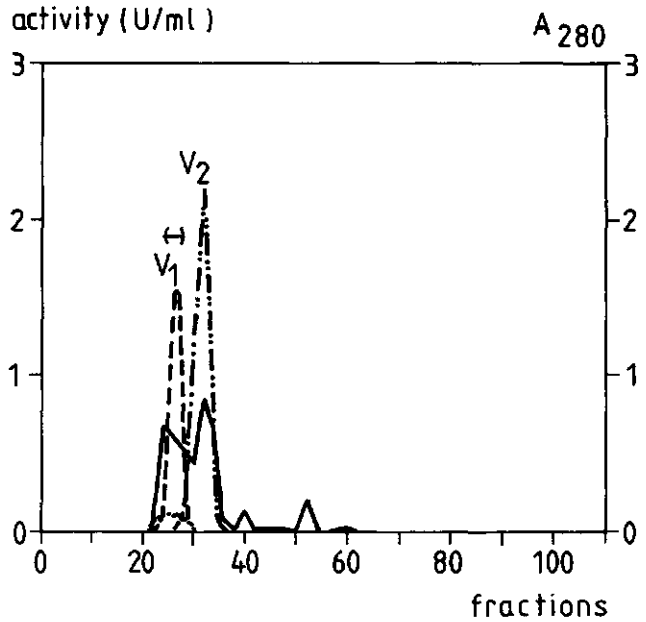


Figure 2: Gel permeation chromatography on Ultrogel AC A54 of pool IV_3 from the HTP Bio-gel A column. Fractions of 5 ml were collected at a flow rate of 20 ml/hr.
— — ß-D-galactosidase; —..— α-D-galactosidase;
..... ß-D-xylosidase; ——— protein.

PHYSICO-CHEMICAL PROPERTIES

Some characteristics of ß-D-galactosidase are presented in table 1. The enzyme showed highest activity at 50 °C, pH 5.0. Optimal stability was observed in the pH range of 3.5 to 7 at 30 °C. At pH 5.0 it was stable up to 50 °C, using PNP-ß-D-Gal as substrate. The isoelectric point, measured by chromatofocusing, appeared to be at pH 4.4.

Table 1: Physico-chemical properties of ß-D-galactosidase from A. niger

Characteristic	ß-D-galactosidase
Specific activity (U/mg, pH 5.0, 30 °C)	
on PNP-ß-D-Gal	6.1
soy AG	0.41
M_r (SDS-electrophoresis) (kD)	116[a]
Isoelectric point	4.4
Optimum pH	5.0
pH Stability	3.5-7
Optimum temperature (°C)	50
Temperature stability (°C)	≤50

[a] Estimated value

Table 2: Effects of various incubation buffers and chemicals on the activity ß-D-galactosidase

Buffer/Chemical	activity (%)
Naacetate	100
Nasuccinate	108
Naoxalate	107
Nacitrate	105
Nasuccinate/ oxalic acid	99
KCl	97
$AgNO_3$	34
$MgCl_2$	98
$CaCl_2$	100
$MnCl_2$	99
$CoCl_2$	97
$NiCl_2$	98
$ZnSO_4$	93
$BaCl_2$	98
$Pb(NO_3)_2$	107
DTT	104
EDTA	103

Concentration of EDTA and $CaCl_2$ present in reaction mixtures: 2 mM. Other additives 1 mM.

The effects of various incubation buffers (0.05 M; pH 5.0) on the activity of ß-D-galactosidase are listed in table 2. No significant differences were observed when other buffers were used for incubations to determine the activity on PNP-ß-D-Gal.

For determination of the effect of various cations, DTT and EDTA on the activity of ß-D-galactosidase an excess of these substances was present in reaction mixtures. ß-D-galactosidase showed lower activity in the presence of Ag+ ions. Other ions including Pb^{2+} and Zn^{2+}, did not have any effect on the activity, measured under standard conditions. Also no effect was observed when $CaCl_2$ or EDTA were added to the reaction mixture.

SUBSTRATE SPECIFICITY AND MODE OF ACTION

ß-D-Galactosidase showed highest activity on PNP-ß-D-Gal (table 3) and showed substantial lower activity towards lactulose and only very little activity towards lactose.

Table 3: Substrate specificity of ß-D-galactosidase from A. niger

Substrate \ activity	percentage of activity on PNP-ß-D-Gal
PNP-ß-D-Gal	100
PNP-α-D-Gal	-
PNP-α-L-Ara	0.7
PNP-α-D-Xyl	-
PNP-ß-D-Glc	-
PNP-ß-D-Xyl	1.4
Lactose	0.6
Lactulose	5.9
Arabinoxylan	-
1,5-α-L-arabinan	-
MHR	1.0
Potato AG	3.3
Soy AG	6.7
Onion F40	1.2
Stractan	-
Coffee AG	-
CMC	-
Polygalacturonic acid	1.5
Pectin (lyase activity)	-
Polygalaturonic acid (lyase activity)	-

Incubation: 0.5 µg enzyme protein per ml 0.05 M sodium acetate buffer pH 5.0, 20 h at 30 °C.

Small amounts of contaminating polygalacturonase, xylosidase

and arabinofuranosidase were shown to be present in the ß-D-galactosidase preparation. However, the activity of ß-D-galactosidase on polygalacturonic acid can be ascribed to release of galactose by activity on galactose residues present in this preparation (chapter 4). The results summarised in table 3 and figure 3 also show that ß-D-galactosidase was able to attack type I arabinogalactans. Highest activity was found on an arabinogalactan present in the alkali extract from soy meal. Less activity of ß-D-galactosidase was observed towards potato AG and onion F40. HPLC analysis of the digests of soy and potato AG showed that galactose was released as sole reaction product and that no shift in the molecular weights distribution of the bulk of the polymers occurred (figure 4). No activity was found on type II arabinogalactans from larch wood and green coffee beans.

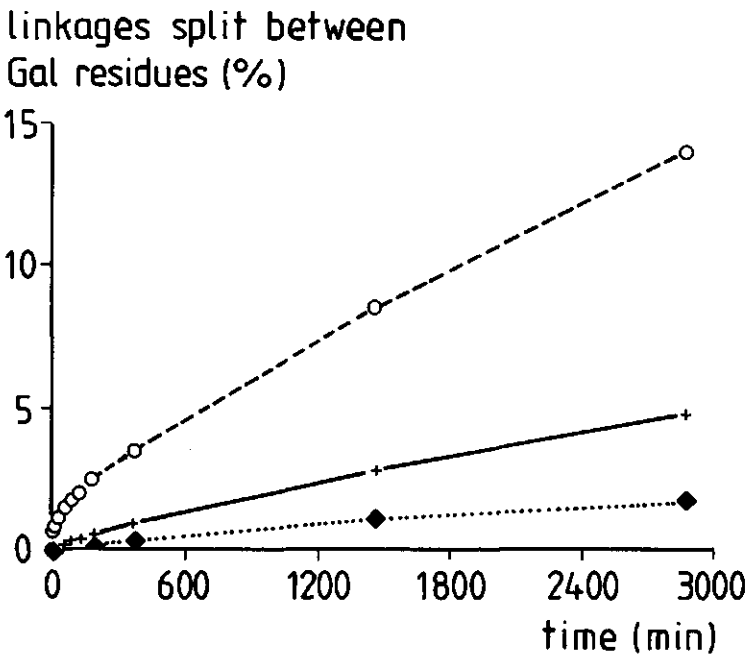


Figure 3: Time course experiments of action of ß-D-galactosidase on soy AG (o), potato AG (+) and onion F40 (♦). Incubation: 1.25 µg protein and 4 mg substrate per ml 0.05 M sodium acetate buffer pH 5.0, 30°C.

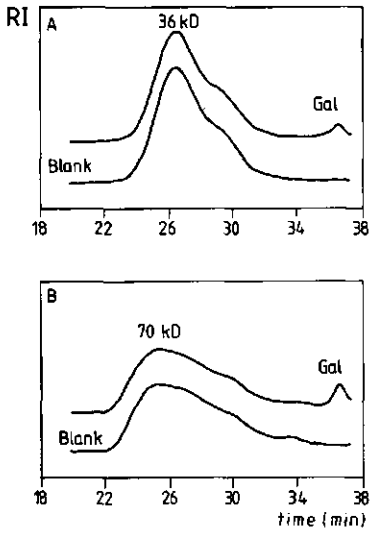


Figure 4: HPSEC analysis of reaction products released by ß-D-galactosidase from soy AG (A) and potato AG (B) after 24 h of incubation, under conditions used as indicated in figure 3.

Table 4: Methylation analysis of onion F40 and ß-D-galactosidase-treated onion F40.

Mode of glycosidic linkage (mole %)	untreated	ß-galactosidase-treated
1-Gal*p*	18	13
1,4-Gal*p*	67	76
1,6-Gal*p*	0	0
1,4,6-Gal*p*	11	7.7

Incubation: 1 mg substrate and 2.5 µg protein per ml 0.05 M sodium acetate buffer pH 5.0 for 20 h at 30 °C.

Methylation analysis of ß-D-galactosidase-treated onion F40 showed that the enzyme was able to linearise the polymer, as a decrease in the mole% of terminal galactose side-chains linked to the backbone from 18 to 13 mole% was observed, accompanied by a decrease in mole% of 1,4,6-Gal*p* from 11 to 7.7 (table 4).

KINETIC PARAMETERS

The V_{max}/K_m values of ß-D-galactosidase listed in table 5 show that this enzyme has highest catalytic efficiency for the synthetic PNP-derivative. The V_{max}/K_m values of ß-galactosidase (K_m expressed in mole anhydrogalactose present in the reaction mixture and V_{max} in min^{-1}) were 20 x 10^5, 0.5 x 10^5 and

Table 5: Kinetic parameters of ß-D-galactosidase on PNP-ß-D-Gal, soy AG and onion F40

Substrate	PNP-ß-D-Gal	soy AG	onion F40
Enzyme concentration (µg protein/ml)	0.05	1.0	1.0
V_{max} (10^3 min^{-1})	1.7	1.9	1.1
K_m (10^{-3} M Gal)	0.87	38	413
V_{max}/K_m (10^5 l min^{-1} mol^{-1})	20	0.5	0.032

0.032 x 10^5 on PNP-ß-D-Gal, soy AG and onion F40, respectively. The difference in V_{max} values on soy AG and PNP-ß-D-galactopyranoside were within experimental error. The V_{max} value of the enzyme on onion F40 was lower than the values on PNP-ß-D-Gal and soy AG (table 5 and figure 5).

DISCUSSION

PURIFICATION OF ß-D-GALACTOSIDASE FROM A. NIGER

A rapid three-step procedure, as published by Greenberg and Mahoney (1981), gave no satisfactory results when applied for the purification of ß-D-galactosidase from the A. niger preparation Pectinase 29. Therefore an elaborate six-step procedure had to be used. It appeared that contaminating ß-xylosidase was very persistent. The presence of contaminating protein in the ß-D-galactosidase preparation was confirmed by SDS-gel electrophoresis. A major diffuse band and a very faint band were observed. ß-D-Galactosidase was obtained in 16 % yield.

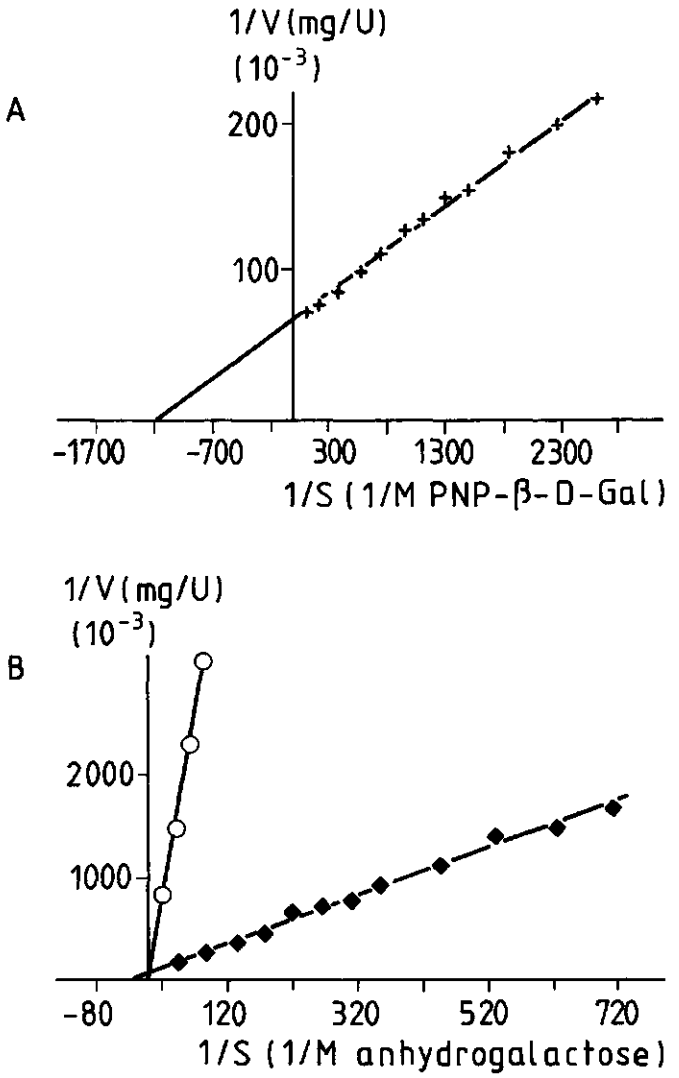


Figure 5: Lineweaver-Burk plots of ß-D-galactosidase on **(A)** PNP-ß-D-Gal and **(B)** soy AG (♦) and onion F40 (o). Incubation was performed as indicated in table 3. Substrate concentration is expressed in mole anhydrogalactose residues present in the reaction mixture.

PROPERTIES OF ß-D-GALACTOSIDASE FROM A. NIGER

ß-D-Galactopyranosidase (EC 3.2.1.23) from the A. niger preparation used in this study differed significantly in its

substrate specificity from other fungal ß-D-galactosidases. The latter ß-D-galactosidases could not degrade type I arabinogalactans (Takenishi et al., 1983, Watanabe et al., 1979).

The ß-D-galactosidase showed highest activity on PNP-ß-D-Gal and was able to release terminal linked galactose from type I arabinogalactans. Galactose was formed directly from the polymeric substrate and not through intermediate reaction products. Kinetic data of ß-D-galactosidase using PNP-ß-D-Gal and soy AG as substrates revealed that affinity was higher on the low M_w substrate than on polymeric substrate, but the V_{max} values were similar. Activity of the enzyme on soy AG was significantly higher than on onion F40, because of differences in structural features (chapter 3). Methylation analysis of onion F40 treated with ß-D-galactosidase revealed that galactose residues 1,6-linked to the main chain were released. Since the enzyme was more active on soy AG it can be concluded that ß-D-galactosidase prefered the non-reducing end of a 1,4-ß-linked galactan backbone and has lower activity on 1,6-linked single-unit galactose side-chains. However, no activity was found on type II arabinogalactans from coffee and larch. This may be caused by the presence of arabinose as a ß-D-galactosidase purified from a A. niger preparation by Bahl and Agrawal (1969), released terminal linked galactose residues from a de-arabinosylated type II arabinogalactan isolated from a red wine (Brillouet et al., 1990).

Degradation of type I arabinogalactans by ß-D-galactosidase has only been demonstrated for plant ß-D-galactosidases in apple (Bartley, 1974), in carrot (Konno et al., 1986), in lupin cotyledon (Matheson and Saini, 1977) and in tomato (Pressey, 1983). Edwards et al. (1988) showed that nasturtium ß-D-galactosidase, which preferentially attacked xyloglucan, only hydrolysed a type I arabinogalactan from onion to a slight extent. Radish seed ß-D-galactosidase, however, attacked 1,6- and 1,3-linkages in galacto-oligomers and the rate of hydrolysis increased with increasing chain lengths (Sekimata et al., 1989).

ß-Galactosidase showed activity on lactulose and no activity on lactose, whereas ß-D-galactosidases purified from A. niger by Widmer and Leuba (1979) were active on lactose. Activity of A. niger ß-D-galactosidase towards lactulose was also found by Harju (1986).

The ß-D-galactosidase used in this study also differed with respect to other physico-chemical properties from A. niger ß-D-galactosidases studied by several other authors. Lee and Wacek (1970) observed two pH optima (2.7-2.9 and 3.8) for A. niger. The latter value was also found by Bahl and Agrawal (1969) for their ß-D-galactosidase. Widmer and Leuba (1979) found three multiple forms of ß-D-galactosidase which showed optimal activity in the pH range of 2.5 to 4.0 and were stable up to 60 °C. The enzymes occurring in three forms, only differed in carbohydrate content. No evidence was found for such differentation in our purified preparation.

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CHAPTER 6

DEGRADATION OF TYPE I ARABINOGALACTANS: SYNERGISTIC EFFECTS EXERTED BY ENDO-GALACTANASE, ß-D-GALACTOSIDASE AND ARABINANASES FROM ASPERGILLUS NIGER

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ABSTRACT

Investigations on the enzymic degradation of (arabino)galactans from various sources, e.g. potato fibre, onion powder, soy meal and citrus pomace revealed that enzyme mixtures containing various arabinogalactan degrading enzymes were required for optimal breakdown in the initial stage. Synergistic effects were observed for a combination of endo-galactanase and ß-D-galactosidase in the degradation of the major (arabino)galactan fractions collected from alkaline extracts from potato and onion by precipitation. It was indicated that ß-D-galactosidase improved the action of endo-galactanase on these substrates by removal of single-unit galactose side-chains. Synergism between endo-galactanase and endo-arabinanase in degradation of the potato F40 fraction indicated that linear arabinan side-chains hinder endo-galactanase action. Synergism between endo-galactanase and

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ß-D-galactosidase in degradation of the citrus TF40 fraction and crude soy AG could be explained by saccharification of galacto-oligomers released by endo-galactanase. Synergism between endo-galactanase and endo-arabinanase or arabinofuranosidase B in degradation of citrus TF40 fraction was also observed. It was indicated that the soy AG preparation, and the citrus, onion and potato F40 fractions contained an arabinan population.

INTRODUCTION

The arabinogalactans present in plant cell walls can be divided into type I having a backbone of 1,4-ß-D-linked galactopyranose residues and type II, arabino-1,3/6-ß-D-galactans (Clarke et al., 1979 and Stephen, 1983). These polysaccharides are important components of the cell wall because they are considered to belong to the pectin matrix which contributes to the rigid structure plant cell walls (Carpita and Gibeaut, 1993).

Degradation of plant cell wall polysaccharides may enhance yields in fruit juice extration and in processing vegetables, improve ultrafiltration of wine (Brillouet, et al., 1990) and improve digestion of animal feed by monogastric animals (Beudeker et al., 1988). Also for bioconversion of plant biomass to fuels cell walls have to be degraded.

It has been observed that synergistic action between pectinases and cellulases greatly enhanced plant biomass conversion (Beldman et al., 1984). For complete bioconversion of biomass not only pectins and cellulose but also hemicelluloses, e.g. type I arabinogalactans have to be degraded.

A study on enzymic degradation of a crude type I arabinogalactan preparation showed that a considerable synergistic effect occurred when endo-galactanase was used in combination with endo-arabinanase (chapter 4). Type I arabinogalactans have also been used in degradation studies with ß-D-galactosidase (chapter 5).

In order to investigate conditions for optimal degradation of various type I arabinogalactans, endo-galactanase (chapter 4) ß-galactosidase (chapter 5) and arabinanases from Aspergillus niger (Rombouts et al., 1988) were used separately and in combinations.

MATERIALS AND METHODS

EXTRACTION OF TYPE I ARABINOGALACTANS

Alkaline extracts containing type I arabinogalactans were prepared from potato fibre, onion powder and citrus pomace by refluxing with an alkali solution containing $NaBH_4$ (Labavitch et al., 1976), as described in chapter 3. An alkaline extract from soy meal was kindly provided by NOVO Nordisk, Dettingen, Switzerland. These crude extracts from potato fibre, onion powder and citrus pomace are denoted as potato AG, onion AG, citrus AG, respectively, and the soy arabinogalactan preparation as soy AG. Details on sugar composition and strutural features are given in chapter 3.

FRACTIONATION OF ALKALI EXTRACTED PREPARATIONS

Potato AG, onion AG and citrus AG were fractionated by graded ethanol precipitation. The fraction precipitating at 40% v/v ethanol contained the major (arabino)galactans in a much purer form (F40 fractions, chapter 3). Sugar and glycosidic linkage compositions of crude extracts and these fractions are presented in chapter 3.

ENZYMES

Endo-1,4-ß-D-galactanase (EC 3.2.1.89) (chapter 4), ß-D-galactosidase (EC 3.2.1.23) (chapter 5), Arabinofuranosidase B (EC 3.2.1.55) and endo-arabinanase (EC 3.2.1.99) (Rombouts et al., 1988) from Pectinase 29, an experimental enzyme preparation derived from Aspergillus niger were used in

degradation studies.

ENZYME ASSAYS

All enzyme activities are expressed in International Units (U). One unit of enzyme activity is defined as the amount which liberates 1 μmol reducing sugars per min. The protein content was measured according to Sedmak and Grossberg (1977). Bovine serum albumin was used as standard.
Aqueous solutions of freeze dried F40 fractions were prepared by stirring overnight at room temperature. Digests of substrates were obtained by incubation of 1 mg substrate per ml 0.05 M sodium acetate buffer pH 4.0 for 1h at 55 °C. Enzymes were used in the following concentrations: 0.05 μg endo-galactanase, 1.0 μg endo-arabinanase, 1.0 μg arabinofuranosidase B and 2.5 μg ß-D-galactosidase per ml reaction mixture. After incubation, the reaction mixtures were inactivated by placing them into a bioling water bath for 5 minutes. Increase in reducing end groups was determined as described by Somogyi (1952).

ANALYSIS OF SUBSTRATES AND DIGESTS OF SUBSTRATES BY HPLC

For determination of shifts in the apparent M_w distribution of (arabino)galactans, reaction mixtures were analysed by high performance size exclusion chromatography (HPSEC), as described in chapter 3. Mono- and oligomeric reaction products were analysed on a CHPb column (Merck, Darmstadt, Germany) as described by Voragen et al. (1986).

RESULTS

DEGRADATION OF (ARABINO)GALACTANS

Potato F40 and potato AG

The action of endo-galactanase on potato F40 resulted in a

shift of the apparent molecular weight distribution and release of low galacto-oligomers (figure 1). No shift was observed after incubation with ß-D-galactosidase or endo-arabinanase. Enzymic degradation of potato F40 by ß-D-galactosidase could only be detected with the CHPb column (table

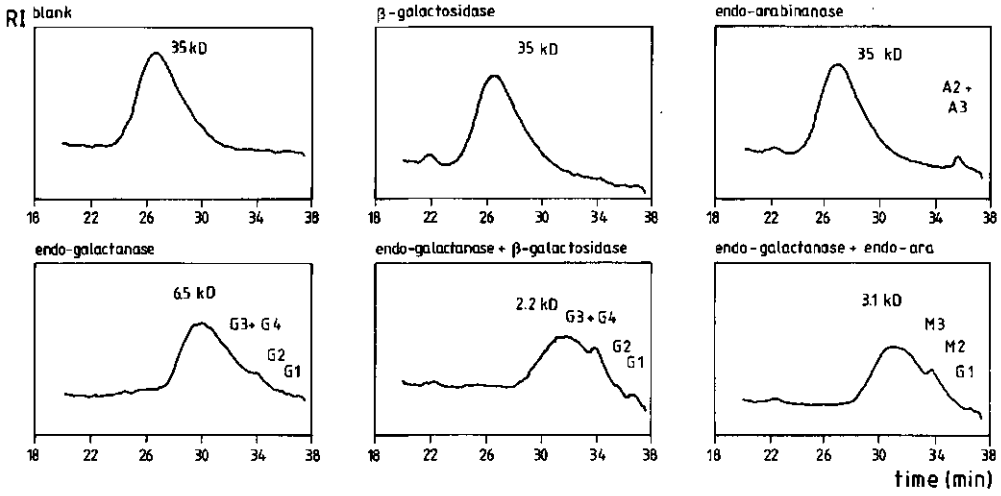

Figure 1: HPSEC analysis of reaction products of digests of potato F40 with endo-galactanase, ß-D-galactosidase and endo-arabinanase from A. niger. G_1, monomeric galactose; G_2, dimeric galactose, etc.; A_2, dimeric arabinose; A_3, trimeric arabinose, etc.; M_2 mixture of A_2, G_2 and A_3; M_3, mixture of G_3, G_4, A_5 and A_6.

1). The activities of ß-D-galactosidase and endo-galactanase on potato F40 were measured to be 0.5 and 6.4 mU per ml, respectively. Activity of arabinofuranosidase B on potato F40 was very small and no synergism occurred with endo-galactanase, nor ß-D-galactosidase (results not given).

Table 1 : Relative activities of ß-D-galactosidase[a] and endo-arabinanase[b] and endo-galactanase[b] on potato F40

	ß-D-gal	endo-ara	endo-gal
ß-D-gal	95	108	220
endo-ara		65	153
endo-gal			96

[a] Analysis by HPLC using the CHPb column.
[b] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Activities were determined using the Nelson-Somogyi assay.

Table 2: Relative amounts of galactose, galactobiose, galactotriose and galactotetraose in digests of potato F40[b]

	ß-D-gal	endo-gal	endo-gal+ ß-D-gal	endo-gal+ endo-ara
galactose	80	100	320	260
galactobiose		100	287	273
galactotriose		100	240	207
galactotetraose		100	145	183

[b] Analysis by HPLC using the CHPb column. Amounts of galacto-oligomers released are expressed as percentage of the amounts formed by action of endo-galactanase alone.

Endo-galactanase was found to act synergistically with ß-D-galactosidase as well as with endo-arabinanase in the degradation of potato F40 (figure 1, table 1 and 2). Although ß-D-galactosidase showed only a low activity towards potato F40 (measured as oligomeric reaction products, table 2), the endo-galactanase/ß-D-galactosidase combination degraded potato F40 to a similar extent as the endo-galactanase/endo-arabinanase combination.

Results obtained with the latter combination were similar to data reported for potato AG previously (chapter 4). No synergistic effects were observed when ß-D-galactosidase was used in combination with endo-arabinanase under conditions

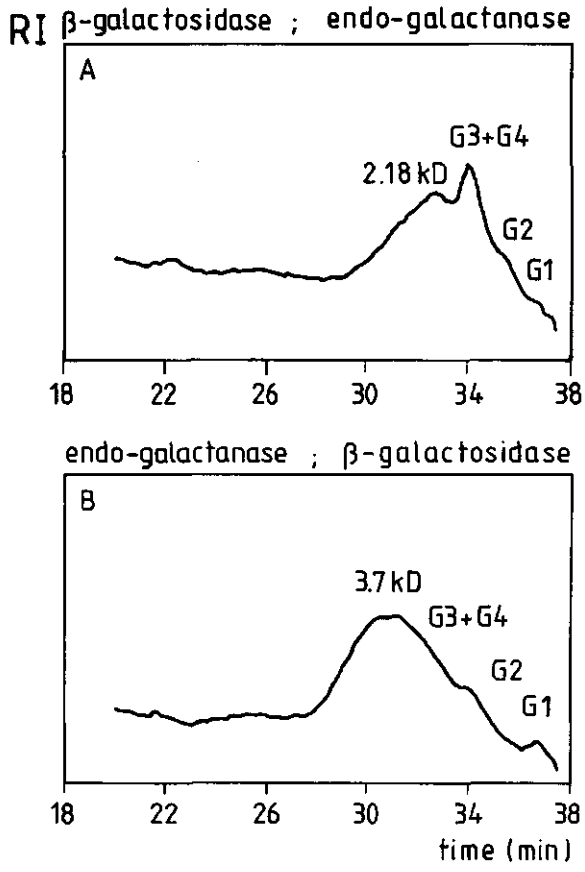

Figure 2: HPSEC analysis of reaction products of potato F40 pretreated with ß-galactosidase followed by action of endo-galactanase (**A**) and pretreated with endo-galactanase followed by action of ß-D-galactosidase (**B**).

used.

As shown in table 3 and figure 2 pretreatment of potato F40 with ß-D-galactosidase followed by action of endo-galactanase resulted in higher degree of hydrolysis than pretreatment with endo-galactanase followed by attack of ß-D-galactosidase. As expected, pretreatment of potato F40 with endo-galactanase followed by action of endo-galactanase did not result in enhanced degradation. The relative activity appeared to be

lower than 100% because of the limited substrate concentration in the reaction mixture.

HPSEC analysis of incubation of the potato F40 substrate blank with endo-galactanase or ß-D-galactosidase alone revealed that the obtained chromatograms were similar to chromatograms designated as endo-galactanase and ß-galactosidase in figure 1, respectively.

Enzyme concentrations in reaction mixtures, temperature and incubation time used in this experiments were identical to the previous experiment.

Analysis of potato AG digests obtained after incubation with these enzymes gave similar results (not shown).

Table 3: Relative activities[a] of endo-galactanase and ß-D-galactosidase on potato F40 pretreated with ß-D-galactosidase and endo-galactanase.

	Pretreated with		
Followed by action of	blank	ß-D-gal	endo-gal
endo-gal	100	203	83
ß-D-gal			116

[a] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Activities were determined using the Nelson-Somogyi assay.

Onion F40 and onion AG

Degradation studies of onion F40 and AG using endo-galactanase and ß-D-galactosidase were performed similarly to experiments conducted with potato F40. Results obtained were similar to the data reported for potato F40 (not shown).

Soy AG

Data presented in table 4 clearly show that endo-galactanase ß-D-galactosidase, arabinofuranosidase B as well as endo-

arabinanase were able to degrade the soy AG preparation. The activities of endo-galactanase, ß-D-galactosidase, arabinofuranosidase B and endo-arabinanase on soy AG were measured to be 12, 1.8, 9.4 and 0,57 mU per ml, respectively. Activity of endo-galactanase on soy AG was higher than on

Table 4 : Relative activities[a] of ß-D-galactosidase, arabinofuranosidase B, endo-arabinanase and endo-galactanase on soy AG

	ß-D-gal	arafur B	endo-ara	endo-gal
ß-D-gal	70	101	102	114
arafur B		59	115	101
endo-ara			69	102
endo-gal				70

[a] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Activities were determined using the Nelson-Somogyi assay.

potato F40 (12 vs 6.4 mU/ml). Reaction conditions were identical to conditions of experiments with potato F40. Synergistic effects were observed when endo-galactanase was used in combination with ß-D-galactosidase. Synergistic effects were exerted also by a combination of arabinofuranosidase B and endo-arabinanase. No synergistic effects were observed for the endo-galactanase/arabinofuranosidase B combination. It appeared that endo-arabinanase showed very little activity on soy AG and did not enhance soy AG degradation in combination with endo-galactanase.

As shown in figure 3 and table 5 pretreatment with endo-galactanase followed by action of ß-D-galactosidase (designated in figure 3 as endo-galactanase;ß-D-gal) resulted in enhanced degradation of the galactan backbone compared to pretreatment with ß-D-galactosidase followed by action of endo-galactanase. Degradation of soy AG by endo-galactanase alone resulted in release of smaller amounts of oligomers

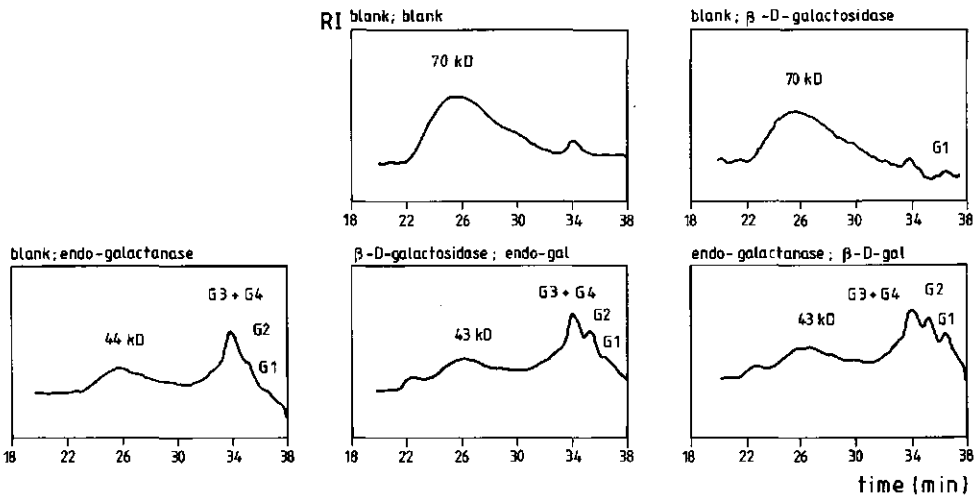

Figure 3: HPSEC analysis of reaction products of digests of soy AG with ß-D-galactosidase, endo-galactanase and of digest pretreated with ß-galactosidase followed by action of endo-galactanase and pretreated with endo-galactanase followed by action of ß-D-galactosidase.

Table 5: Relative activities[a] of endo-galactanase and ß-D-galactosidase on soy AG pretreated with ß-D-galactosidase and endo-galactanase.

	Pretreated with		
Followed by action of	blank	ß-D-gal	endo-gal
endo-gal	100	94	58
ß-D-gal			130

[a] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Activities were determined using the Nelson-Somogyi assay.

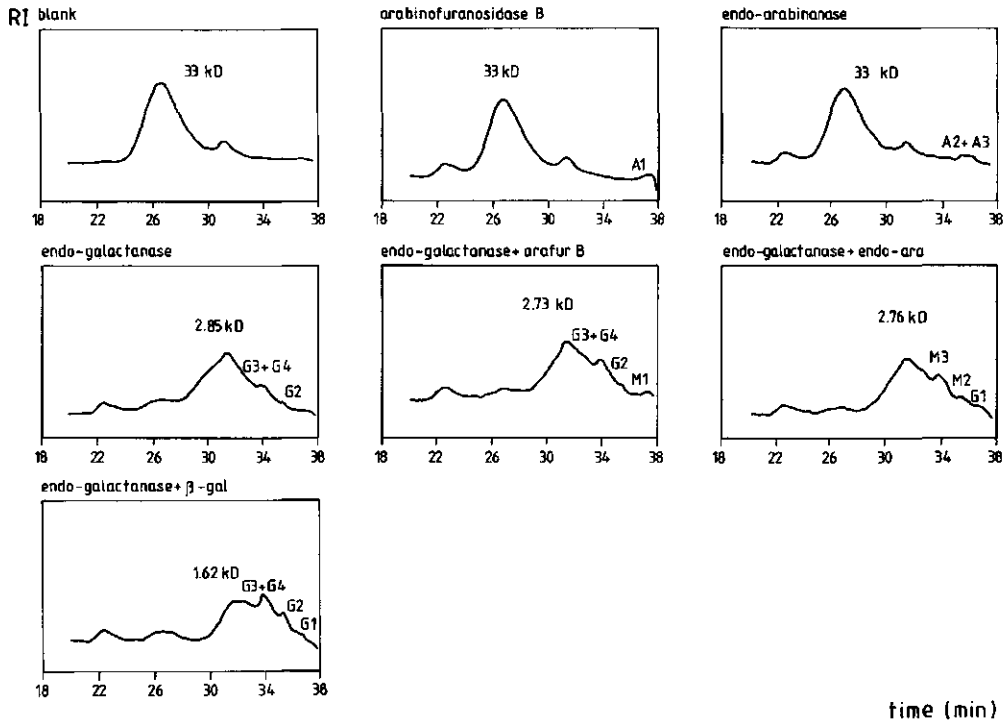

Figure 4: HPSEC analysis of reaction products of digests of citrus TF40 with endo-galactanase, ß-D-galactosidase, arabinofuranosidase B and endo-arabinanase from A. niger. G_1, monomeric galactose; G_2, dimeric galactose, etc.; A_2, dimeric arabinose; A_3, trimeric arabinose, etc.; M_2 mixture of A_2, G_2 and A_3; M_3, mixture of G_3, G_4, A_5 and A_6.

compared to the amounts analysed in the latter two digests.

Citrus TF40

The citrus TF40 fraction was subjected to experiments similar to those described for potato F40 (figure 4, table 6 and 7). It appeared that ß-D-galactosidase did not show any activity towards citrus TF40 (table 6). The activities of endo-galactanase, arabinofuranosidase B and endo-arabinanase on citrus F40 were measured to be 8.2, 3.2 and 2.1 mU per ml, respectively. HPSEC analysis of digests revealed that only action of endo-galactanase resulted in a shift of the apparent M_w distribution (figure 4). Results presented in figure 4 and

table 6 and 7 show that synergistic effects exerted by the endo-galactanase/ß-D-galactosidase combination were found to be larger than for the endo-galactanase/endo-arabinanase or endo-galactanase/arabinofuranosidase B combination. Endo-arabinanase and arabinofuranosidase B were also found to act synergistically.

Table 6 : Relative activities[a] of ß-D-galactosidase, arabinofuranosidase B, endo-arabinanase and endo-galactanase on citrus TF40

	ß-D-gal	arafur B	endo-ara	endo-gal
ß-D-gal	0	96	106	203
arafur B		59	144	127
endo-ara			59	144
endo-gal				92

[a] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Activities were determined using the Nelson-Somogyi assay.

Table 7: Relative contents[b] of galactose, galactobiose, galactotriose and galactotetraose in digests of citrus TF40

	ß-D-gal	endo-gal	endo-gal+ arafur B	endo-gal+ endo-ara	endo-gal+ ß-D-gal
galactose	0	100	190	240	305
galactobiose		100	285	300	445
galactotriose		100	141	184	275
galactotetraose		100	141	158	327

[b]Analysis by HPLC using the CHPb column.

DISCUSSION

DEGRADATION OF TYPE I ARABINOGALACTANS

The results obtained indicated that for optimal degradation in

the initial stage of the various type I arabinogalactans different combinations of enzymes were required. This was observed for the endo-galactanase/ß-D-galactosidase, endo-galactanase/endo-arabinanase and endo-galactanase/arabino-furanosidase B combinations.

It was found that ß-D-galactosidase acted synergistically with endo-galactanase in the initial stage of the degradation of type I arabinogalactan F40 fractions from potato and onion. These results indicated that single-unit galactose side-chains are present. Removal of these appendages by ß-D-galactosidase action resulted in improvement of the accessibility of these substrates by the endo-galactanase. The observed ability of ß-D-galactosidase of removing the galactose side-chains from polymeric substrates is also in agreement with previous results (chapter 5). For optimal degradation of potato F40 the presence of endo-arabinanase in the enzyme mixture is also essential. It caused a further downward shift in the M_w distribution. The presence of arabinan side-chains linked to the main chains of potato F40 is in agreement with results of methylation analysis of these substrates (chapter 3).

Synergism between endo-galactanase and ß-D-galactosidase in degradation of the type I arabinogalactan fractions from soy and citrus TF40 could only be explained by saccharification of oligomeric products released by endo-galactanase. A combination of endo-galactanase and arabinofuranosidase B or endo-arabinanase did not result in enhanced degradation of soy AG. This indicated that the degree of substitution of the galactan backbone by arabinose and galactose side-chains was low as reported in chapter 3 and, therefore, did not hinder action of endo-galactanase.

HPLC analysis confirmed that no activity of ß-D-galactosidase on the citrus arabinogalactan fraction could be detected. Results presented in chapter 3, however, indicate the presence of single-unit side-chains in citrus F40. Lack of activity of ß-D-galactosidase on citrus TF40 may indicate that its structure is more complex than of e.g. potato F40.

A combination of endo-arabinanase or arabinofuranosidase B

with endo-galactanase resulted in an enhanced degradation of citrus TF40. This indicates the presence of short or heavily branched arabinose side-chains, as reported in chapter 3. However, analysis of digest by HPSEC did not reveal significantly increased shifts in apparent M_w values of peak fractions.

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CHAPTER 7

EXO-1,3-ß-D-GALACTANASE FROM ASPERGILLUS NIGER AND ITS ROLE IN THE ENZYMIC CONVERSION OF COFFEE BEAN ARABINOGALACTAN

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ABSTRACT

A galactanase, degrading the backbone of coffee arabinogalactan in an exo-fashion, was purified from an experimental enzyme preparation derived from Aspergillus niger. Hydrolysis of partly de-arabinosylated coffee arabinogalactan yielded mainly galactose and 1,6-ß-D-galactobiose. At the same time only a very slow change in the apparent M_w distribution of the substrate occurred. In the final stage of the degradation galactose accumulated as the main reaction product. Unmodified coffee arabinogalactan was degraded at a slower rate and to a lesser extent. Formation of minor amounts of several arabinogalacto-oligomers during breakdown of coffee arabinogalactan indicated the limited capability of the exo-galactanase of bypassing branching points in this substrate. This exo-1,3-ß-D-galactanase (M_r=62 kD) showed optimal activity at pH 5.0 and 40 °C. The V_{max} and K_m values for the de-arabinosylated coffee arabinogalactan and unmodified coffee arabinogalactan at pH 5.0 and 40 °C were

[1] TO BE SUBMITTED

4.6 x 10^3 min^{-1} and 1.3 g/l, and 3.9 x 10^3 min^{-1} and 4.1 g/l, respectively. At pH 5.0 the galactanase was stable up to 45 °C, at 30 °C it was stable in the pH range of 2.5 to 7.5. Addition of Pb^{2+} and to a lesser extent Mn^{2+} and Zn^{2+} ions to the reaction mixture had a negative effect on the activity. This was also true for dithiothreitol.
A considerable synergistic effect between arabinofuranosidase B and the exo-galactanase in the degradation of coffee arabinogalactan was observed. This indicated that arabinofuranose residues in the side-chains attached to the galactan backbone hinder exo-galactanase action and also that its capability of bypassing branching points was limited.

INTRODUCTION

In higher plants two types of arabinogalactans, designated as type I and II, are present. Type I arabinogalactans consisting of a 1,4-linked ß-D-galactan backbone, are less widely distributed in higher plants than arabinogalactans of type II (Clarke et al., 1979, and Stephen, 1983). Type II arabino-1,3/6-ß-D-galactan is a highly branched polysaccharide with ramified chains of ß-D-galactopyranose residues joined by 1,3- and 1,6-linkages. The 1,3-linkages predominate in the interior chains while the 1,6-linkages are mainly found in the exterior chains. These exterior chains are mainly terminated with α-L-arabinofuranose residues but some have ß-L-arabinopyranose residues (Clarke et al., 1979, Fincher et al., 1983 and Stephen, 1983).
They are present in many plants and their enzymic degradation is of interest in many areas. They may also be of importance in the enzymic saccharification of arabinogalactan-rich biomass to fuels and chemicals, e.g. larch wood can comprise up to 33% w/w arabinogalactan (Clarke et al., 1979). Lately arabinogalactans of grapes have found great interest for their role in wine making. These arabinogalactans are strongly retained by microfiltration membranes. Their enzymic degradation may prevent fouling and plugging of membranes

(Brillouet et al., 1990). Enzymic degradation of the arabinogalactans may also facilitate digestion of animal feed by monogastric animals (Beudeker et al., 1988).
Three different types of galactanases degrading 1,3/6-ß-D-galactan chains have been described in literature; an endo-1,3-ß-D-galactanase from Rhizopus niveus that released arabinogalacto-oligomers containing 1,3-linked galactose residues from coffee arabinogalactan (Hashimoto et al., 1969, 1971), an endo-1,6-ß-D-galactanase from Aspergillus niger active on de-arabinosylated grape arabinogalactan (Brillouet et al., 1991) and an exo-1,3-ß-D-galactanase from Irpex lacteus which showed activity towards radish arabinogalactan-proteins (Tsumuraya et al., 1990).
Up till now these galactanases have only been used in structural studies of coffee arabinogalactan (Hashimoto, 1971), radish arabinogalactan and arabinogalactan-proteins (Tsumuraya et al., 1984, 1987, 1990) and grape arabinogalactan (Saulnier et al., 1992). In the present paper, we report on the purification and characterization of an exo-1,3-ß-D-galactanase from an enzyme preparation derived from A. niger. Synergistic effects exerted between exo-1,3-ß-D-galactanase and arabinanases in the degradation of coffee arabinogalactan are also studied.

MATERIALS AND METHODS

SUBSTRATES

A type II arabinogalactan (denoted as coffee AG) was isolated from green coffee beans (Coffea arabica, kindly provided by Douwe Egberts, Utrecht, The Netherlands) based on a modified method of Wolfrom and Patin (1965) (chapter 3). By treatment with a pure arabinofuranosidase B (Rombouts et al., 1988) a partly de-arabinosylated coffee AG was obtained (denoted as coffee galactan). Larch wood arabinogalactan ("stractan") was purchased from St. Regis Paper company, Tacoma, Washington, USA). Details on sugar and glycosidic linkage compositions are

given in chapter 3.
Other substrates used were onion F40 (chapter 3), 1,5-α-L-arabinan (Voragen et al., 1982), arabinose-rich highly ramified apple pectin fraction (MHR, Schols et al., 1990), arabinoxylan ex oats spelts, polygalacturonic acid, CM-cellulose, highly esterified pectin, DE 93%, p-nitrophenyl derivatives of α-L-arabinofuranose, α/ß-D-galactopyranose, ß-D-glucopyranose and α/ß-D-xylopyranose (chapter 4).

ENZYME PREPARATION

KBP 001 3L, an experimental liquid enzyme preparation derived from Aspergillus niger was kindly provided by Novo Nordisk, Dettingen, Switzerland.

ENZYME ASSAYS

All enzyme activities were expressed in International Units (U). One unit of enzyme activity is defined as the amount which liberates 1 µmol reducing sugars per min. The protein content was measured according to Sedmak and Grossberg (1977). Bovine serum albumin was used as standard.
Exo-1,3-galactanase activities were measured by incubating 0.1 ml of a 0.5% w/v coffee galactan solution in distilled water with 0.4 ml of a diluted enzyme solution in 0.05 M sodium acetate buffer, pH 5.0. The increase in reducing end groups was measured after 1 h incubation at 30 °C by the method of Nelson-Somogyi (Somogyi, 1952). Galactose was used as standard.
Other activities on polymeric substrates and p-nitrophenyl derivatives were measured as described by Rombouts et al. (1988).

PURIFICATION OF EXO-GALACTANASE FROM A. NIGER

From the KPB 001 3L enzyme preparation 30 ml, which contained 732 mg protein was used for purification. Purification of exo-

galactanase from A. niger consisted of a desalting step by gel permeation chromatography on Bio-Gel P10 (100-200 mesh, column 30 x 950 mm), anion exchange chromatography on DEAE Bio-Gel A (column 30 x 200 mm), adsorption chromatography on Bio-Gel HTP (column 20 x 170 mm, Bio-Rad Laboratories, Richmond, California, USA) and gel permeation chromatography on a Superose 12 column HR 16/50 (Pharmacia-LKB Biotechnology, Uppsala, Sweden).
All purification steps were carried out at 4 °C. All buffers contained 0.01% w/v sodium azide to prevent microbial growth.

SDS-GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSING

SDS-gel electrophoresis, isoelectric focusing and titration curves were performed with the Pharmacia Phastsystem. Experimental details are given by Rombouts et al. (1988).

KINETIC PROPERTIES AND INFLUENCE OF PH, TEMPERATURE, VARIOUS BUFFERS AND CHEMICALS ON ACTIVITY AND STABILITY OF EXO-GALACTANASE

Lineweaver-Burk plots of exo-galactanase action on coffee galactan and coffee AG (0.05 M sodium acetate buffer, pH 5.0, for 1 h at 40 °C) were determined. Data analysis for calculation of kinetic parameters, using non-linear regression, was performed by a program called "Enzfitter" (Leatherbarrow, 1987).
Effect of pH and temperature on exo-galactanase activity and stability was measured according to the method described in chapter 4.
The influence of a variety of buffers (0.05 M; pH 5.0) and chemicals on activity was studied at 30 °C, pH 5.0, as described in chapter 4. Activities are expressed as percentage of the activity measured in sodium acetate buffer.

ANALYSIS OF REACTION PRODUCTS BY HPLC

Reaction products formed during incubation of coffee AG and coffee galactan with exo-galactanase were analysed by HPLC. Changes in the apparent M_w distribution of digests of enzyme degraded substrates were analysed by high performance size exclusion chromatography (HPSEC), as described in chapter 3. Mono- and oligomeric reaction products were analysed by a CHPb column (Merck, Darmstadt, Germany) as described by Voragen et al. (1986).

FRACTIONATION OF A COFFEE GALACTAN DIGEST

Reaction products released from coffee galactan by action of exo-galactanase were fractionated according to their molecular weight on a Bio-Gel P2 column (200-400 mesh, 2.6 x 100 cm, Biorad Labs.) as described by Labavitch et al. (1976). Purity of fractions was checked by high performance anion exchange chromatography (HPAEC) using a Dionex BioLc GPM-II quaternary gradient module equipped with CarboPac PA-1 column (4 x 250 mm) in combination with a CarboPac PA guard column at a flow of 1 ml/min (Dionex, Sunnyvale, Ca., USA). Samples (20 µl) were applied onto the column using a Spectra Physics SP8780 autosampler equipped with Tefzel rotor seal in a Rheodyne injector valve (Spectra Physics, San José, California, USA). Elution was performed at 20 °C with a linear gradient of 0-0.4 M sodium acetate in 0.1 M sodium NaOH during 39.9 min, followed by an isocratic gradient of 1 M sodium acetate in 0.1 M NaOH for 4.9 min. The solvents were degassed and stored under helium pressure using a Dionex EDM module. The eluate was monitored in a Dionex pulsed amperometric detector (PAD) (reference electrode Ag/AgCl) with a working gold electrode to which potentials of E_1 0.1, E_2 0.6 and E_3 -0.6 V were applied for duration of T_1 0.5, T_2 0.1 and T_3 0.1 s.

NMR ANALYSIS

Prior to analysis a sample was desalted by adding a mixture of equivalent amounts of dried AG50W-X4 (H^+, 400 mesh) and AG3-X4A (OH^-, 200-400 mesh) (Bio-Rad Labs.) suspended in water. ^1H NMR spectra for solutions in D_2O were recorded as descibed by Colquhoun et al. (1990).

RESULTS

PURIFICATION OF EXO-GALACTANASE FROM ASPERGILLUS NIGER.

The purification of galactanase from A. niger is schematically presented in figure 1. Analysis of reaction products released from coffee galactan by enzyme fractions obtained after chromatography on DEAE Bio-Gel A showed that the bulk of the galactanase activity was present in fractions combined in pool II_4. Substantial amounts of contaminating arabinofuranosidase in pool II_4 were removed by chromatography on HTP Bio-Gel as shown in figure 2. Polygalacturonase activity collected in pool III_2 and III_3 and ß-galactosidase activity found in pool III_4 were almost completely removed from pool II_4 (not shown). Further purification of galactanase was performed by gel permeation chromatography on Superose 12.
The enzyme was obtained in 9.6 % yield. It was purified 31 fold, moved as one band on SDS-gel electrophoresis. The enzyme showed a specific activity of 19.8 U/mg on coffee galactan at pH 5.0, 30 °C.

PHYISCO-CHEMICAL PROPERTIES

A. niger galactanase appeared to have a molecular weight of 62 kD (determined with SDS-gel electrophoresis) and showed highest activity on coffee galactan at pH 5.0 and 40 °C, viz. 25 U/mg (table 1). The enzyme was stable up to 45 °C at pH 5.0 and showed stability in the pH range of 2.5 to 7.5 at 30 °C.

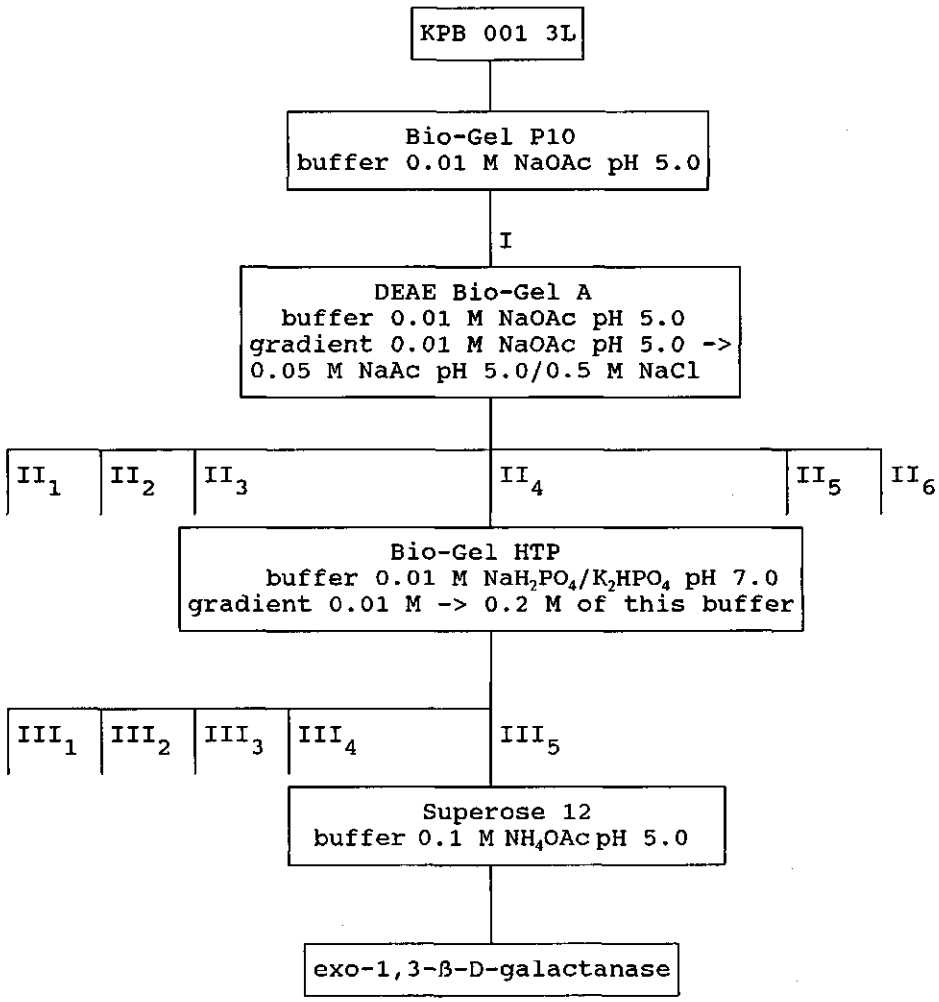

Figure 1: Flow sheet of the purification of exo-galactanase from an enzyme preparation of Aspergillus niger. The Roman numerals with Arabic subscripts refer to enzyme pools.

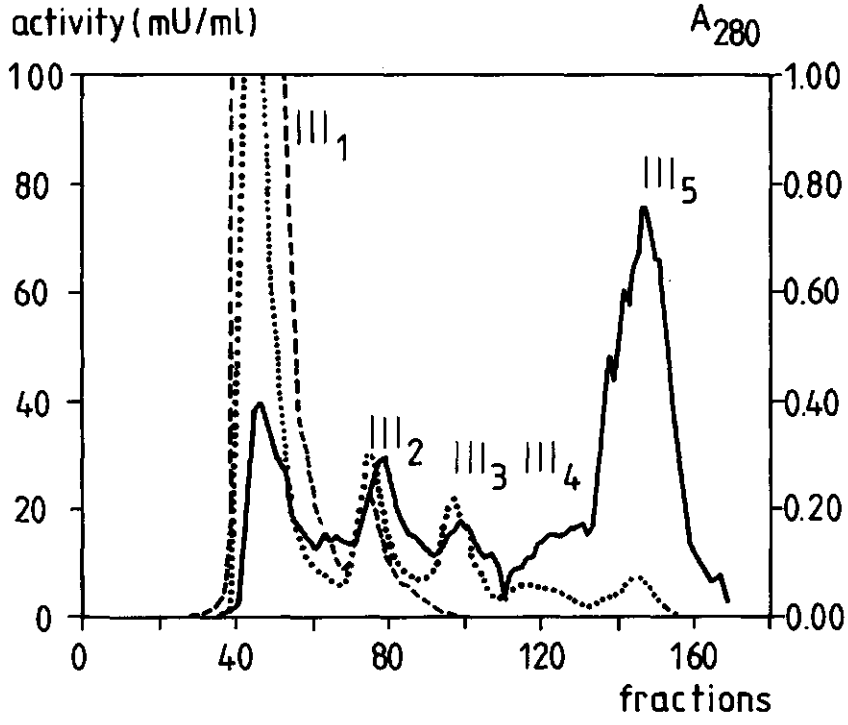

Figure 2: Bio-Gel HTP chromatography of pool II_4 from the DEAE Bio-Gel A column. Fractions of 10 ml were collected at a flow rate of 20 ml/hr. Exo-galactanase activity, ————; arabinofuranosidase activity, ------; protein, ...

The isoelectric point could not be determined, because the enzyme did not migrate on the gel in the pH range of 4 to 6 (table 1). Similar aberrant behaviour was also observed for endo-1,4-ß-D-galactanases (chapter 4) and arabinanases (Rombouts et al., 1988).

The effects of various incubation buffers on the activity of the enzyme are listed in table 2. These results clearly show that for the set of buffers used lowest activity of galactanase was found in a citrate buffer.

For determination of the effect of chemicals on activity of the galactanase an excess of the chemicals listed in table 2 were present in reaction mixtures. Pb^{2+} and to a lesser extent Mn^{2+} and Zn^{2+} ions and DTT inhibited the activity of

Table 1: Some physico-chemical properties of exo-1,3-ß-D-galactanase

Characteristic	exo-galactanase
Specific activity (U/mg, pH 5.0, 40 °C)	
on coffee galactan	25
coffee AG	8.0
larch wood arabinogalactan	0
M_r (SDS-electrophoresis) (kD)	62
Isoelectric point	4-6
Optimum pH	5.0
pH Stability	2.5-7.5
Optimum temperature (°C)	40
Temperature stability (°C)	≤45

Table 2: Effects of various incubation buffers and chemicals on the activity exo-1,3-ß-D-galactanase.

Chemical/ Buffer	activity
Naacetate	100
Nasuccinate	106
Naoxalate	110
Nacitrate	67
Nasuccinate/ oxalic acid	102
KCl	98
$AgNO_3$	102
$MgCl_2$	106
$CaCl_2$	95
$MnCl_2$	79
$CoCl_2$	102
$NiCl_2$	96
$ZnSO_4$	86
$BaCl_2$	105
$Pb(NO_3)_2$	22
DTT	83
EDTA	98

Concentration of chemicals in reaction mixtures: 1 mM, except for EDTA and $CaCl_2$: 2 mM.

galactanase. Inhibition by DTT may indicate a role for cystine close to or in the active site of the enzyme.

SUBSTRATE SPECIFICITY AND MODE OF ACTION

The data presented in table 3 clearly show that galactanase is only active on substrates with a backbone of 1,3-ß-D-galactopyranose residues. Stractan, however, is an exception.

Table 3: Side activities of exo-1,3-ß-D-galactanase from A. niger

Substrate \ activity	percentage of activity on coffee galactan
PNP-α-D-Gal	0.5
PNP-ß-D-Gal	1.4
PNP-α-L-Ara	1.2
PNP-α-D-Xyl	0
PNP-ß-D-Glc	0.01
PNP-ß-D-Xyl	0.01
onion F40	0.6
Stractan	0
Arabinoxylan	0.1
1,5-α-L-Arabinan	0
MHR	0.1
Polygalacturonic acid	1.5
CMC	0
Pectin (lyase activity	0
Polygalacturonic acid (lyase activity)	0

Incubation: 8 µg enzyme protein per ml 0.05 M sodium acetate buffer pH 5.0, 20 h at 30 °C.

Small residues of contaminating polygalacturonase, arabinofuranosidase, ß- and α-galactosidase and xylanase activities were found.

Highest activity was found on coffee galactan as 36% of the linkages between galactose residues were hydrolysed by exo-galactanase after 24 h vs 23% for coffee AG (figure 3).

Analysis of reaction products released after 1 h from coffee galactan by galactanase revealed that galactose and an unknown oligomer were the major reaction products. Chromatography on Bio-Gel P2 showed that this oligomer is a dimer since its elution volume corresponds with the elution volume of maltose

(not shown). Analysis of reaction products obtained by TFA hydrolysis of the dimer revealed that it consisted of

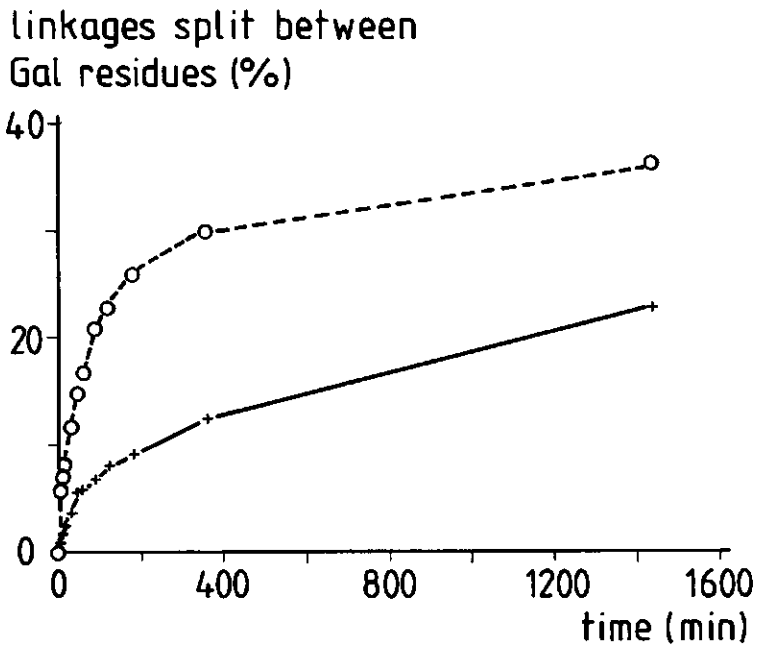

Figure 3: Time curves of action of exo-galactanase on coffee galactan (o) and coffee AG (+). Incubation: 0.45 µg protein and 1 mg substrate per ml 0.05 M sodium acetate buffer pH 5.0, at 30 °C.

galactose (figure 4). Results of ^{1}H NMR analysis of the galactobiose indicated that the galactopyranose residues were 1,6-ß-D-linked, as the characteristic signal for H-4 of this dimer was absent (Van Halbeek et al., 1982; Michalski et al., 1991).

Figure 4A and 5 show the release of oligomers from coffee galactan and coffee AG by galactanase, respectively. In addition, figure 5 shows the changes in the apparent molecular weight of the peak fraction in coffee AG. Galactose and galactobiose which were released from the very beginning of the degradation of coffee galactan, accumulated as end products. A gradual shift in the apparent M_w distribution of this substrate was only visible after 6 h of incubation.

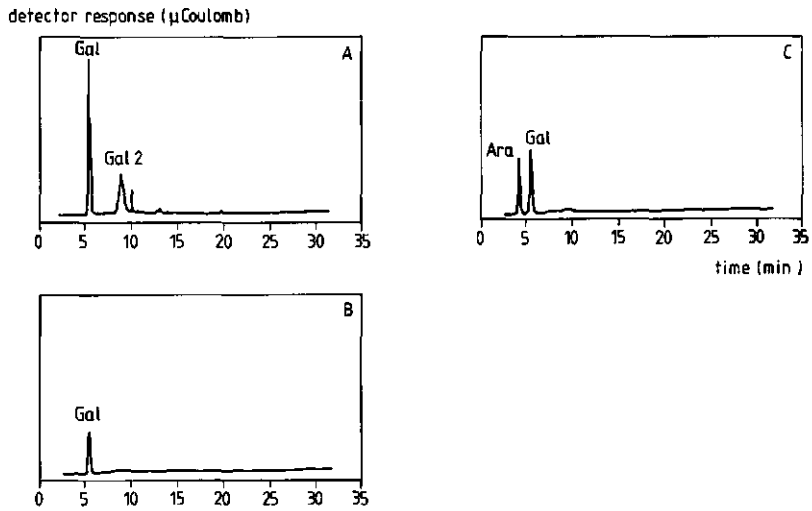

Figure 4: HPAEC analysis of (**A**) coffee galactan digest obtained by action of exo-galactanase. (**B**) sugar released from dimer after hydrolysis with 2 M TFA. (**C**) standard containing arabinose and galactose.

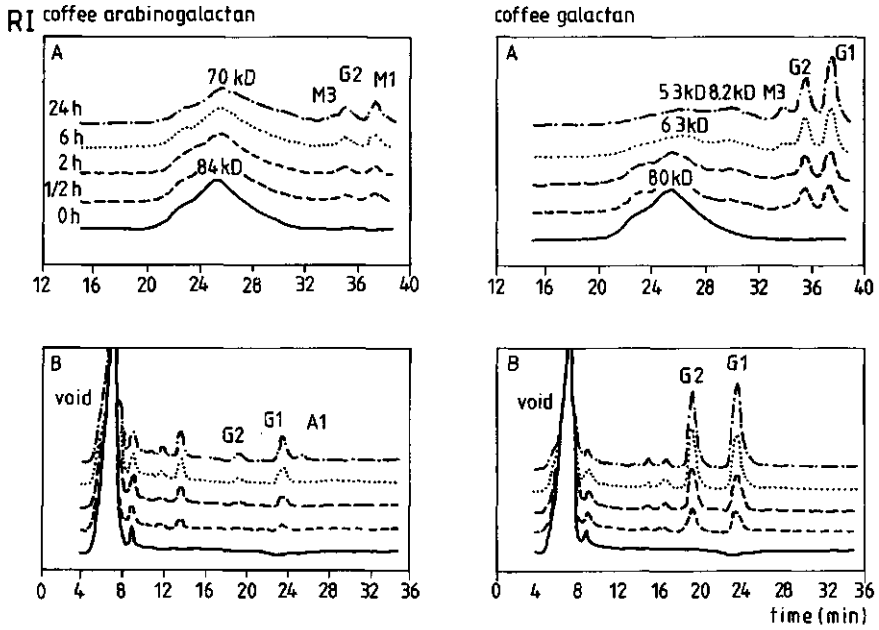

Figure 5: Time course studies of the degradation of coffee galactan and AG by exo-galactanase analysed by HPSEC (**A**) and HPLC using a CHPb column (**B**). A_1, arabinose; G_1, galactose; G_2, galactobiose; M_1, mixture of A_1 and G_1; M_3, mixture of higher oligomers. Reaction times are indicated in the HPSEC chromatograms of digests of coffee arabinogalactan.

Comparison of digests of coffee galactan and AG revealed that for the latter higher oligomers were released in the initial stage. These oligomers accumulated in the course of the reaction. Galactose and galactobiose accumulated at lower rates. A small change in the apparent M_w distribution of the peak fraction of coffee AG was only observed after 24 h.

KINETIC PARAMETERS

Kinetic parameters of the exo-galactanase are presented in table 4 and figure 6. These data clearly show that a decrease

Table 4: Kinetic parameters of exo-galactanase from A. niger on coffee galactan and coffee AG

Kinetic parameter	coffee galactan	coffee AG
V_{max} (10^3 min^{-1})	4.6	3.9
K_m (g Gal/l) 0.87	1.3	4.1
V_{max}/K_m (10^3 l min^{-1} g^{-1})	3.5	0.95

in the catalytic efficiency (expressed as V_{max}/K_m) with increasing arabinose content was found. The Lineweaver-Burk plots show that lowering the degree of substitution with arabinofuranose residues did not affect the V_{max} values but had a clear effect on the K_m values. The observed differences for these V_{max} values were within experimental error. The affinity of the galactanase for the substrate was increased. For K_m expressed in gram per litre anhydrogalactose present in the reaction mixture and V_{max} in min^{-1} the V_{max}/K_m values of exo-galactanase were 0.95 x 10^3 and 3.5 x 10^3 on coffee AG and galactan, respectively.

ENZYMIC DEGRADATION OF COFFEE AG

The synergism exerted between exo-galactanase and arabinanases for breakdown of coffee AG was studied (table 5 and 6). As can

be seen from the relative activity as well as from the release of reaction products (table 5 and 6) combined action of arabinofuranosidase B and exo-galactanase resulted in acceleration of the breakdown of the polymer. Endo-arabinanase showed only little activity on coffee AG and was therefore not used in combination with exo-galactanase.
Combined action of galactanases and arabinanases on stractan was not studied, because these arabinanases were not active towards stractan (Rombouts et al., 1988).

Table 5: Relative activities[a] of arabinofuranosidase B and exo-1,3-ß-D-galactanase from A. niger on coffee AG

	arafur B	Exo-gal
arafur B	86	159
exo-gal		60

[a] Activities of combinations are expressed in percentage of the summations of the activities of the single enzymes, which are set at 100%. Activities were determined using the Nelson-Somogyi assay. Incubation: 1.8 µg galactanase and 1.0 µg arabinofuranosidase B protein and 1 mg substrate per ml 0.05 M sodium acetate buffer, pH 5.0, 1 h at 30 °C

Table 6: Relative contents[b] of arabinose, galactose and galactobiose in digests of coffee AG

	exo-gal	arafur B	arafur B + exo-gal
arabinose	0	100	90
galactose	100	0	207
galactobiose	100	0	801

[b] Analysis by HPLC using the CHPb column, underlined figures as reference.

DISCUSSION

PROPERTIES OF THE EXO-GALACTANASE FROM A. NIGER

The exo-galactanase purified in this study was active on coffee AG but not on stractan and type I arabinogalactans.

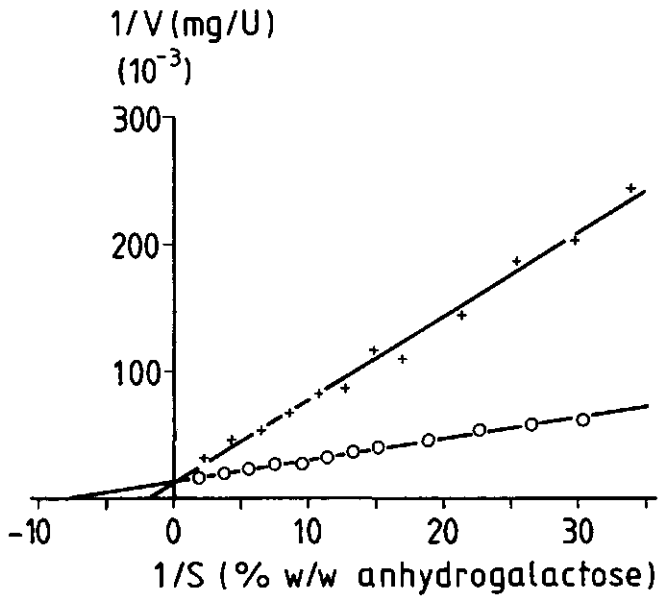

Figure 6: Lineweaver-Burk plots of exo-galactanase from A. niger on coffee galactan (o) and coffee AG (+).

Specific activity on coffee AG by exo-galactanase was increased after partial removal of arabinose side-chains (25 vs. 8 U/mg for coffee AG). Predominantly galactose and 1,6-ß-D-galactobiose were formed in increased amounts and a very slow change in the elution volume of the bulk of the galactan on HPSEC analysis occurred. Also higher oligomers were released, although in smaller amounts. The latter oligomers were presumably galactose and galactobiose residues substituted with arabinose residues. The data obtained suggest that the purified enzyme is an exo-1,3-ß-D-galactanase (EC 3.2.1.90) which is able to bypass branching points in this substrate to a limited extent. Its mode of action is similar to the action pattern of exo-1,3-ß-D-galactanase purified from a Brown rot basidiomycete, I. lacteus (Tsumuraya et al., 1990). A similar mechanism has been reported for exo-1,3-ß-D-glucanase purified from the basidiomycete QM 806 (Nelson et al., 1969).

The modes of action of a partly purified Rhizopus niveus 1,3-

galactanase that released arabinogalacto-oligomers containing 1,3-linked galactose residues from coffee AG (Hashimoto et al., 1969 and Hashimoto, 1971), and of A. niger endo-1,6-ß-D-galactanase that released galactobiose as main reaction product from arabinose-reduced grape arabinogalactan (Brillouet et al., 1991), were clearly different from the one described in this study.

DEGRADATION OF COFFEE AG

A considerable synergistic effect between the exo-galactanase and arabinofuranosidase B was visible from the initial reaction rate, the extent of degradation and the reaction products released from coffee AG. However, treatment of coffee AG with arabinofuranosidase B did not result in complete removal of arabinose residues. This may indicate the presence of arabinofuranose residues in the main chain as suggested by Hashimoto (1971) and Bradbury and Halliday (1990) or a more complex structure of the polysaccharide (chapter 3).
In conclusion it can be stated that for complete degradation of native type II arabinogalactans a mixture of enzymes consisting of exo-1,3-, endo-1.6-ß-D-galactanase, ß-D-galactosidase (Sekimata et al., 1989 and Tsumuraya et al., 1990) and arabinofuranosidases is needed.

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CHAPTER 8

CONCLUDING REMARKS

The aim of this research was to study the enzymic saccharification of type I and II arabinogalactans. Enzymes involved in degrading galactan structures of various type I and II arabinogalactans to galactose are galactanases and ß-D-galactosidases (chapter 2).

ISOLATION OF ARABINOGALACTANS

Type I arabinogalactans, found in several plants, differ in their structural features (Clarke et al., 1979; Fincher et al., 1983; Stephen, 1983). To cover a variety of arabinogalactans occurring in nature, arabinogalactans were extracted from potato fibre (a byproduct from potato starch factories), onion powder, citrus pomace (a byproduct from citrus industries) and green coffee beans. A crude type I arabinogalactan extract from soy meal was made available to us. A type II arabinogalactan from larch wood was purchased.
In general, mild isolation procedures to obtain homogeneous type I arabinogalactans are very laboriously (Stephen, 1983). Extraction by refluxing with 4 N NaOH in the presence of 0.1 M $NaBH_4$ for 20 h at 90 °C (Labavitch et al., 1976), followed by graded ethanol precipitation of the freeze dried crude AG extracts, proved to be a quick procedure for isolation of fairly pure type I arabinogalactans (chapter 3). This extraction procedure with alkali is not a mild method and therefore it is not aimed at preserving the *in vivo* state of the cell wall type I arabinogalactans. Structural artefacts can be formed.
Graded ethanol precipitation of alkaline extracts from onion and citrus was necessary to remove substantial amounts of contaminating polymeric material. However, a xyloglucan population was still present in the major arabinogalactan

fraction collected from the citrus preparation. Therefore, this fraction was treated with an endo-glucanase. A very pure galactan could be obtained by graded ethanol precipitation of the onion alkaline extract.
The crude potato alkaline extract could also be used as such in enzymic degradation studies (chapter 4 and 5), as the major F40 fraction comprised 64% w/w of this preparation (chapter 3).
It appeared that major proportions of (arabino)galactans with high molar galactose/arabinose ratios were collected in the precipitation step with the 40% v/v aqueous ethanol solution (denoted as fraction F40). The presence of arabinan populations in potato and citrus F40 fractions could, however, not be excluded. The presence of an arabinan population in soy AG was indicated (chapter 3).
A fairly pure type II arabinogalactan from green coffee beans was extracted by a laborious procedure based on the method of Wolfrom and Patin (1965). This arabinogalactan may be more complex than reported by Hashimoto (1971) and Wolrom and Patin (1965) (chapter 3). Although removal of lignin prior to extraction of coffee arabinogalactan may affect the yield of this arabinogalactan (Wilkey, 1985), it appeared that the yield of the arabinogalactan obtained by the Wolfrom and Patin procedure (1965) was satisfactory.

PURIFICATION AND CHARACTERIZATION ENZYMES DEGRADING GALACTAN BACKBONES

ENZYMES DEGRADING THE GALACTAN BACKBONE OF TYPE I ARABINOGALACTAN

Endo-1,4-galactanases involved in the bioconversion of type I arabinogalactans were purified from experimental enzyme preparations derived from Aspergillus niger and A. aculeatus. A rapid purification procedure consisting of conventional gel permeation, anion-exchange chromatography and anion-exchange chromatography by using FPLC for purification of an endo-

galactanase was developed (chapter 4). These endo-galactanases were indicated to show highest activity on linear galactans. A multiple attack mechanism (Robyt and French, 1967) was suggested as in the initial stage of degradation both enzymes accumulated mainly tetragalactose and trigalactose accompanied by a big shift in the apparent M_w value of the peak fraction of the bulk of the polymer. Monomer and dimer galactose were accumulated as end products by both endo-galactanases. The most striking difference between the A. niger and A. aculeatus was the fact that the former was stable up to 60 °C and the latter up to 35 °C, under conditions used. The action pattern of the endo-galactanases from P. citrinum (Nakano et al., 1985) and from Bacillus sp. S-2 and S-39 (Tsumura et al., 1991) was very similar to the A. niger and A. aculeatus enzymes (chapter 4). Our enzymes differed from P. citrinum endo-galactanase with respect to glycosyltransferase activity indicated for the latter enzyme.
In order to study a possible role of ß-D-galactosidase in degradation of type I arabinogalactans, a ß-D-galactosidase was purified from the same A. niger preparation (chapter 5). This enzyme showed highest activity on PNP-ß-D-galactopyranose and was able to release galactose residues in an exo fashion from type I arabinogalactans. It was found that the ß-D-galactosidase was able to split 1,4- and 1,6-linkages between galactose residues with preference for the 1,4-linkage. This ß-D-galactosidase differed significantly from other fungal ß-D-galactosidases as these enzymes could not degrade type I arabinogalactans (Takenishi et al., 1983; Watanabe et al., 1979).

A GALACTANASE DEGRADING THE 1,3-LINKED BACKBONE OF TYPE II ARABINOGALACTAN

An exo-1,3-ß-D-galactanase was purified from an experimental enzyme preparation derived from A. niger (chapter 7). The mode of action was found to be very similar to the exo-1,3-ß-D-galactanase from Irpex lacteus, as described by Tsumuraya et

al. (1990). The A. niger exo-galactanase released galactose as main reaction product and it was indicated that highest activity would be found on a linear 1,3-linked galactan. This enzyme was able to bypass branching points in coffee bean arabinogalactan (i.e. side-chains containing galactose and arabinose residues linked at C_6 of 1,3-linked galactose residues) to a limited extent (chapter 7).

ENZYMIC CONVERSION OF ARABINOGALACTANS

TYPE I ARABINOGALACTANS

As pointed out in chapter 3 type I arabinogalactans from various sources differ in their structural characteristics. The enzymic degradation studies showed that for optimal degradation of the various type I arabinogalactans in the intitial stage different combinations of enzymes were required. This was observed for the endo-galactanase/ß-D-galactosidase, endo-galactanase/endo-arabinanase and endo-galactanase/arabinofuranosidase B combinations (chapter 6).
In the initial stage of degradation of potato F40 synergism occurred for a combination of endo-galactanase and ß-D-galactosidase and a combination of endo-galactanase and endo-arabinanase (chapter 6). This was consistent with the structural features of the arabinogalactan present in this fraction; a 1,4-linked galactan backbone substituted at C_6 with single unit galactose side-chains and by 1,5-arabinans (chapter 3). The synergistic effects exerted by a combination of endo-galactanase and ß-D-galactosidase in the degradation of potato F40 and also of onion F40 could be explained by removal of galactose side-chains resulting in higher affinity of endo-galactanase towards the polymeric substrate. Enhancement of degradation of these substrates was also caused by ß-D-galactosidase action on the oligomeric reaction products released by the endo-galactanase. This latter form of synergism occurred only in degradation of type I arabinogalactans found in the alkaline extract from soy meal

and in the citrus TF40 preparation. These data might by explained by the absence of single unit galactose side-chains in the latter two substrates. However, results obtained from methylation analysis of soy AG and citrus TF40 indicated that the presence of galactose single unit side-chains can not be excluded (chapter 3). Synergistic effects exerted by a combination of endo-galactanase and arabinofuranosidase B and a combination of endo-galactanase and endo-arabinanase in degradation of the arabinogalactan present in citrus TF40 were in accordance with a structure consisting of a 1,4-linked galactan backbone substituted at C_6 with short or branched arabinan side-chains (chapter 3 and 6).
It is anticipated that in the end stage of degradation of type I arabinogalactans by endo-galactanase (arabino)galacto-oligomers are present. By addition of appropriate amounts of ß-D-galactosidase, endo-arabinanase and arabinofuranosidase to these reaction mixtures an optimal degradation of type I arabinogalactans can be achieved.

TYPE II ARABINOGALACTANS

A combination of exo-1,3-galactanase and arabinofuranosidase B resulted in an enhanced degradation of type II arabinogalactan from coffee (chapter 7). These results were in accordance with a type II arabinogalactan structure consisting of a 1,3-linked galactan backbone substituted at C_6 with side-chains in which arabinose is present as single unit arabinose, branched arabino-oligomeric units or arabinogalacto-oligomeric units (chapter 3).
However, using this combination did not result in activity on larch wood arabinogalactan (chapter 7). This latter result may be explained by a higher degree of ramification of the larch wood arabinogalactan than found in coffee arabinogalactan or to the presence of arabinopyranose residues in the former, or both (chapter 3). It is anticipated that a mixture of enzymes consisting also of endo-1,6-galactanase (Brillouet et al., 1991), ß-D-galactosidase (Sekimata et al., 1989) and

arabinanases is required for optimal saccharification of type II arabinogalactans.

ENZYMIC CONVERSION OF BIOMASS

In order to achieve complete saccharification of type I and II arabinogalactans in biomass proper combinations of enzymes are required. It is anticipated that in addition to galactan degrading enzymes and arabinanases e.g. α-L-rhamnosidase, α-L-fucosidase and α-L-glucuronidase are needed, as in these arabinogalactans these sugar residues may be present (Clarke et al., 1979; Fincher et al., 1983; Stephen, 1983).
Next to their use in enzymic conversion of arabinogalactans in biomass as described in chapter 2 galactan degrading enzymes may also serve other goals: improvement of digestibility of animal feed by monogastric animals (Beudeker et al., 1988), liquefaction of juices (Voragen et al., 1992) and ultrafiltration of wine (Brillouet et al., 1990). For application of galactan degrading enzymes in the food industry other types of strains have to be used than in bioconversion of biomass for chemicals and fuels as for the former use only food-grade enzyme preparations are allowed.

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SUMMARY

Agricultural biomass consisting mainly of cellulose, hemicellulose and lignin, is a renewable source of fuels and chemicals. An interesting option is enzymic conversion of biomass to readily usable material. To improve the overall economics of enzymic conversion of biomass not only cellulose but also hemicelluloses have to be degraded to monomeric sugars (saccharification). The aim of the work presented in this thesis was to study saccharification of arabinogalactans, a subgroup of the hemicelluloses.

Arabinogalactans (AGs) have been found in numerous higher plants. In most plants these arabinogalactans occur only in small amounts, with exception of Larix and Acacia species. Their role in maintaining cell wall rigidity is discussed in chapter 1).

Chapter 2 discusses structural features of AGs, subdivided in arabino-ß(1->4)-galactans (type I) and arabino-ß(1->3,1->6)-galactans (type II), and gives a brief overview of enzymes degrading galactan structures of AGs.

In chapter 3 the alkaline extraction of type I AGs from potato fibre, onion powder and citrus pomace is described. The extracts appeared to be mixtures of various polysaccharides. The presence of arabinan in all of these extracts is likely. By means of graded ethanol precipitation a major fraction enriched in type I AG could be precipitated in 40% v/v ethanol (denoted as F40). Results indicated that the 1,4-linked galactan backbone of onion F40 was substituted at C_6 with single unit galactose side-chains and for potato F40 also with 1,5-arabinans. Citrus TF40, (F40 treated with an endo-glucanase for removal of contaminating xyloglucan), was suggested to contain a 1,4-linked galactan substituted at C_6 with short arabinose or highly branched arabinan side-chains and single unit galactose side-chains. A type I AG extracted from soy meal with alkali may be substituted at C_6 to a small extent with appendages of arabinose and single unit galactose side-chains.

A type II AG from green coffee beans was indicated to consist of 1,3-linked galactan backbone substituted at C_6 with side-chains of arabinofuranose and galactose (chapter 3). The presence of terminal mannose possibly substituted on the side-chains, indicates a more complex structure for this polysaccharide. Analysis of a commercially available type II AG from larch (stractan) showed that the side-chains consisted also of arabinopyranose residues and this AG was a more heavily branched polymer than coffee AG.

Endo-1,4-ß-D-galactanases involved in the bioconversion of type I AG were purified from experimental enzyme preparations derived from Aspergillus niger and A. aculeatus (chapter 4). Their molecular weights were 42-43 kD and maximal activities were measured at pH 4.0-4.3 and 50-55 °C on de-arabinosylated potato AG. In absence of substrate the A. aculeatus endo-galactanase showed less thermal stability than the A. niger endo-galactanase. Both endo-galactanases, which were similar in their mode of action, were suggested to degrade type I AG according to a multiple attack mechanism. It appeared that a combination of endo-galactanase and endo-1,5-α-L-arabinanase exerted synergistic effects in the initial stage of degradation of the potato AG. The action of these enzymes resulted in an increase in the downward shift of the molecular weight distribution of the digest and increased amounts of galactose, galactobiose and galactotriose (chapter 4). No synergism was observed for a combination of endo-galactanase and arabinofuranosidase B.

Chapter 5 describes the purification of a ß-D-galactopyranosidase. This ß-galactosidase showed maximal activity on PNP-ß-D-galactopyranose at pH 5 and 50 °C and was stable up to 50 °C and in the range of pH 3.5 to 7. It released non-reducing terminal galactose residues from type I AGs but not from type II AGs. With respect to polymeric substrates the enzyme showed highest activity towards 1,4-linkages but was also able to release 1,6-linked single unit galactopyranose side-chains.

Chapter 6 describes that the differences in structural

features of type I AGs were reflected in the combinations of enzymes which exerted synergistic effects in degradation. In the degradation of onion and potato F40 synergism occurred in the initial stage of degradation for the endo-galactanase/ß-D-galactosidase combination. In the degradation of potato F40 the endo-galactanase/endo-arabinanase combination exerted also synergistic effects. The ß-D-galactosidase released single unit galactose side-chains from both substrates thereby improving the affinity for endo-galactanase. These results were consistent with the structural features of these substrates reported in chapter 3.

The activity of ß-D-galactosidase on oligomeric reaction products released by endo-galactanase also enhanced degradation of potato and onion F40. This synergism occurred only in degradation of soy AG and citrus TF40. In the degradation of citrus TF40 synergistic effects were exerted also by the endo-galactanase/arabinofuranosidase B and endo-galactanase/endo-arabinanase combinations.

An exo-1,3-ß-D-galactanase purified from an experimental enzyme preparation derived from A. niger preferentially degraded 1,3-ß-D-galactans (chapter 7). Mainly galactose and 1,6-galactobiose were released as reaction products from partly de-arabinosylated coffee AG. Hydrolysis of coffee AG by this exo-galactanase was accompanied by formation of small amounts of several arabinogalacto-oligomers. This indicated a limited capability of this enzyme of bypassing branching points. Optimal activity was measured at pH 5.0 and 40 °C and thermal stability was found in the pH range of 2.5 to 7.5 and up to 45 °C.

In the enzymic degradation of coffee bean AG a combination of exo-galactanase and α-L-arabinofuranosidase B exerted synergistic effects. For a combination of exo-galactanase and endo-arabinanase no enhancement in degradation of this substrate occurred. None of these combinations showed activity towards a type II arabinogalactan from larch wood (chapter 7).

In chapter 8 discusses the isolation of type I and II AGs, the purification procedure of the endo-1,4-ß-D-galactanases, the

properties and mode action of the purified galactan degrading enzymes, their role in saccharification of AGs and other fields of possible application of galactan degrading enzymes.

SAMENVATTING

Biomassa van landbouwkundige oorsprong, met als hoofdbestanddelen cellulose, hemicellulose en lignine, is een hernieuwbare bron van chemicaliën en brandstoffen. Een interessante mogelijkheid is de enzymatische omzetting van biomassa naar een fermenteerbare suikeroplossing. Om de economische haalbaarheid van deze omzetting te verbeteren is het van belang hierbij zowel cellulose als de hemicelluloses tot monomere suikers af te breken (versuikering). Om die reden spitst het hier beschreven onderzoek zich toe op de versuikering van arabinogalactanen, een subgroep van de hemicelluloses.

Arabinogalactanen worden in talloze hogere planten in geringe hoeveelheden aangetroffen. De Lariks en Acacia soorten zijn uitzonderingen met hogere gehaltes. De rol van arabinogalactanen in het handhaven van de rigide struktuur van de celwand wordt ook beschreven in hoofdstuk 1.

Hoofdstuk 2 beschrijft de verschillen in struktuur tussen diverse arabinogalactanen. Binnen deze groep is er een onderverdeling tussen arabino-β(1->4)-galactanen (type I) en arabino-β(1->3,1->6)-galactanen (type II). Dit hoofdstuk wordt afgesloten met een kort overzicht van enzymen die de galactaan-strukturen in arabinogalactanen afbreken.

In hoofdstuk 3 wordt de alkalische extractie van type I arabinogalactanen uit aardappelvezel, ui-poeder en citrustrester beschreven. Deze extracten, die aangeduid worden als aardappel, ui en citrus AG, bleken mengsels van verscheidene polysacchariden te zijn. Uit de meetresultaten bleek dat waarschijnlijk al deze extracten arabinaan bevatten.

Een stapsgewijze ethanol precipitatie van deze extracten leverde hoofdfrakties op die verrijkt waren in type I arabinogalactanen. Deze frakties precipiteerden na toevoeging van ethanol tot 40 v/v % aan de opgeloste extracten en worden aangeduid als F40 fracties. De 1,4-gebonden galactaan hoofdketen in ui F40 bleek op C_6 vertakt te zijn met enkelstandige galactose eenheden. In aardappel F40 werd daarnaast ook vertakking op C_6 met 1,5-arabinanen aangetroffen.

Voor citrus TF40 (F40 waaruit door behandeling met een endoglucanase IV xyloglucan nagenoeg verwijderd was) werden aanwijzingen gevonden voor een 1,4-gebonden galactaan hoofdketen met op C_6 vertakking met korte zijketens van arabinose of sterk vertakt arabinaan en enkelstandige galactose eenheden.
Analyse van een alkalisch extract uit sojameel toonde aan dat het type I arabinogalactaan mogelijkerwijs in geringe mate op C_6 is vertakt is met korte zijketens van arabinose (of met sterk vertakt arabinan) en enkelstandige galactose eenheden.
Het type II arabinogalactaan dat uit groene koffiebonen (koffie AG) geïsoleerd werd bestond uit een 1,3-gebonden galactaan hoofdketen die op C_6 vertakt is met zijketens die bestaan uit arabinofuranose en galactose eenheden. De aanwezigheid van eindstandige mannose eenheden, die mogelijk gesubstitueerd zijn op deze zijketens geeft aan dat dit polysaccharide een meer complexe struktuur bezit. Een commercieel verkrijgbaar type II arabinogalactaan uit lariks (stractaan) bleek ook arabinopyranose in de zijketens te bezitten en een sterker vertakt polymeer te zijn dan koffie arabinogalactaan
Hoofdstuk 4 beschrijft hoe twee endo-1,4-β-D-galactanases voor de afbraak van type I arabinogalactanen gezuiverd werden uit experimentele enzympreparaten afkomstig van Aspergillus niger en A. aculeatus. Hun molmassa was in de orde van 42-43 kD en optimale activiteit werd gemeten op aardappel galactaan bij een pH en temperatuur in de orde van respectievelijk 4.0-4.3 en 50-55 °C. Dit aardappel galactaan werd verkregen na behandeling van aardappel AG met arabinofuranosidase B.
Zonder substraat was A. aculeatus endogalactanase minder hittestabiel dan het A. niger enzym. Het mechanisme van de afbraak van type I arabinogalactanen door deze enzymen leek op een "multiple attack mechanism".
Beide endo-galactanases werden in combinatie met A. niger endo-1,5-α-arabinanase en α-L-arabinofuranosidase B op aardappel AG getest (hoofdstuk 4). De combinatie endo-arabinanase en endo-galactanase veroorzaakte een synergistisch effekt in de beginfase van de afbraak. Behandeling met deze combinatie van enzymen resulteerde in een grotere afname van de molekuulgewichts-

verdeling van het substaat. Ook werden grotere hoeveelheden van galactose, galactobiose en galactotriose in het hydrolysaat aangetoond. De combinatie van endo-galactanase en arabinofuranosidase B leidde niet tot een verhoging van de afbraak van aardappel AG.

Hoofdstuk 5 beschrijft de zuivering van een β-D-galactosidase dat maximaal aktief was op PNP-β-D-galactopyranose bij pH 5 en 50 °C. De aktiviteit van het β-D-galactosidase bleek stabiel te zijn bij een pH van 3.5 tot 7 en bij een temperatuur tot 50 °C. Dit enzym splitste niet-reducerende eindstandige galactose eenheden af van type I arabinogalactanen, maar niet van type II. Hierbij bleek dat het β-D-galactosidase bij voorkeur 1,4-bindingen splitste, maar ook 1,6-bindingen kon splitsen.

Verschillen in de struktuureigenschappen van type I arabinogalactanen vonden hun weerslag in de verschillende combinaties van enzymen die synergisme vertoonden tijdens afbraak (Hoofdstuk 6). Voor aardappel en ui F40 werd synergisme in de beginfase van de afbraak gevonden voor de endogalactanase/β-D-galactosidase combinatie. Een verklaring hiervoor is dat afsplitsen van de enkelstandige galactose zijketens leidde tot een verhoging van de affiniteit van endo-galactanase voor het minder vertakte substraat. Tijdens afbraak van aardappel F40 bleek de endogalactanase/endo-arabinanase combinatie ook synergisme te vertonen. Deze resultaten sluiten aan bij de strukturele eigenschappen van deze substraten (hoofdstuk 3).

Een toename van de afbraak van aardappel en ui F40 werd ook bereikt door de aktiviteit van β-D-galactosidase op oligomere produkten vrijgemaakt door endogalactanase. Deze vorm van synergisme werd uitsluitend gevonden tijdens afbraak van soja AG en citrus TF40.

Bij citrus TF40 afbraak werd ook synergisme gevonden voor de combinaties endo-galactanase/arabinofuranosidase B en endo-galactanase/endo-arabinanase.

Een exo-1,3-β-D-galactanase werd gezuiverd uit een experimenteel enzympreparaat van A. niger (Hoofdstuk 7). Dit enzym splitste bij voorkeur 1,3-β-D-galactanen. Uit gedeeltelijk gede-arabinosyleerd koffie AG werden voornamelijk galactose en 1,6-galactobiose

vrijgemaakt. Nevenprodukten van deze hydrolyse waren kleine hoeveelheden van diverse arabinogalacto-oligomeren, wat aangeeft dat het enzym slechts in geringe mate in staat is om vertakkingspunten te passeren. De optimale aktiviteit werd gevonden bij pH 5.0 en 40 °C. Het exo-galactanase was stabiel tussen pH 2.5 en 7.5 en tot 45 °C.

Bij de afbraak van koffie AG werd er synergisme gevonden tussen exo-galactanase en arabinofuranosidase B. De combinatie exo-galactanase en endo-arabinanase gaf hier geen verhoging in de afbraak. Geen van de genoemde enzym-combinaties vertoonde aktiviteit op type II arabinogalactan uit lariks

Hoofdstuk 8 beschrijft de isolatie van type I en II arabinogalactanen, de zuiveringsprocedure van de endo-galactanases, de eigenschappen en werkingsmechanismen van de gezuiverde galactaan-afbrekende enzymen en hun bijdragen aan versuikering van type I en II AG. Ook worden andere mogelijke toepassingen van deze galactaan-afbrekende enzymen vermeld.

CURRICULUM VITAE

Hans van de Vis werd op 11 november 1959 te Utrecht geboren. In 1979 behaalde hij het diploma Atheneum-B. In dat zelfde jaar werd begonnen met de studie scheikunde aan de toenmalige Rijksuniversiteit Utrecht. Na het kandidaatsexamen (richting S1) in 1983 werd het doctoraalexamen met als bijvakken Analytische chemie (prof. dr. G. Dijkstra), Klinische chemie (prof. dr. J.B.J. Soons en dr. C. van der Heiden) en als keuzevak Bio-organische chemie (prof. dr. J.F.G. Vliegenthart en prof. dr. J.P. Kamerling) behaald in 1987. Vanaf maart 1987 tot september 1990 was hij in dienst bij de sectie Levensmiddelenchemie en -microbiologie van de Landbouwuniversiteit Wageningen, alwaar het onderzoek gedaan is dat beschreven is in dit proefschrift.
Vanaf 1 oktober 1991 tot 1 januari 1993 was hij werkzaam bij de afdeling Visserijprodukten van TNO te IJmuiden. Sedert de fusie per 1 januari 1993 van deze afdeling met het Rijkinstituut voor Visserijonderzoek (RIVO-DLO) te IJmuiden is hij aldaar werkzaam.