CHARACTERISATION AND ENZYMIC DEGRADATION OF NON-STARCH POLYSACCHARIDES IN LIGNOCELLULOSIC BY-PRODUCTS

A study on sunflower meal and palm-kernel meal

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BIBLIOTHEEK
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WAGENINGEN

STELLINGEN

1) Kennis van alleen de suikersamenstelling van een heterogeen substraat is niet voldoende voor een voorspelling van de benodigde enzymen voor de hydrolyse ervan.
dit proefschrift

2) De vertaling, die Thibault en Crepeau (1989) geven van de suikersamenstelling van zonnepitdoppen naar de daarin aanwezige polysacchariden, is gedeeltelijk onjuist.
Thibault J F, Crepeau B (1989) *Sciences des Aliments* **9** 405-412

3) Gezien een aantal methodische tekortkomingen van de methyleringsanalyse is het gerechtvaardigd om in de literatuur vermelde gegevens over bindingstypes en daarvan afgeleide polysaccharidestrukturen met scepsis te lezen.
dit proefschrift, hoofdstuk 3

4) De relevantie van onderzoek naar polysaccharidases voor de enzymatische versuikering van lignocellulose bevattende grondstoffen of bijprodukten zou wezenlijk vergroot kunnen worden door met name hun werking op onoplosbare substraten te bestuderen.
dit proefschrift

5) Succesvolle toepassingen van enzymen in de pulp- en papierindustrie blijken een nauwkeurige 'fine-tuning' op basis van fundamentele inzichten te vereisen. Dit geldt in versterkte mate voor toepassing van enzymen in de veevoederindustrie waar de problematiek aanzienlijk komplexer is en de huidige kennis van de systemen veel geringer.

6) Schönfeld en Behnke (1991) gaan bij de bepaling van de molecuulgrootte van uronzuur-bevattende oligosacchariden m.b.v. gelfiltratiechromatografie op Biogel P2 ten onrechte ervan uit, dat deze bij gebruik van water als elutiemiddel hetzelfde elutievolume hebben als neutrale oligomeren van gelijke polymerisatiegraad.
Schönfeld A, Behnke U (1991) *Die Nahrung* **35** 749-757

7) Als gevolg van een deels onjuiste interpretatie van de morphogenese van strangen in de uiterwaarden alsmede de rivierkundige eisen die aan het huidige winterbed van de grote rivieren gesteld worden, zal het graven van nevengeulen zoals in het kader van het ecologisch herstel van de Rijn voorgesteld wordt wel leiden tot een verrijking aan biotopen maar niet tot een herstel van de oorspronkelijke morphologie van de uiterwaarden.
De Bruin *et al* (1987) *Ooievaar. De toekomst van het rivierengebied.* Sticht. Gelderse Milieufederatie, Arnhem.
Middelkoop H *et al* (1992) Rapport Geopro 1992.07. Inst.Geogr.Research, Universiteit Utrecht

8) Het bedroevend lage percentage deelneemsters aan lange fiets-tourtochten of aan 20/40km hardloopevenementen staat geheel niet in verhouding tot hun fysieke prestatievermogen bij duursport.

9) Het zou de effektiviteit van de samenwerking van industrie en onderzoeksinstellingen bij gemeenschappelijke projekten ten goede komen als de betrokken R&D medewerkers voor de duur van het desbetreffende projekt uitgesloten werden van 'job-rotation'.

10) De door sommige reisorganisaties getoonde aandacht voor nadelige gevolgen van het tourisme (bijvoorbeeld door steun aan natuur- of milieuprojekten in de gastlanden) zal vermoedelijk in verkooptechnisch opzicht meer opleveren dan voor het aangegeven doel.

11) De complimenteus bedoelde uitspraak 'U spreekt beter Nederlands dan Prins Bernhard' zou door een Duitse gesprekspartner wellicht verkeerd opgevat kunnen worden.

Stellingen behorende bij het proefschrift 'Characterisation and enzymic degradation of non-starch polysaccharides in lignocellulosic by-products. A study on sunflower meal and palm-kernel meal' door E.-M.Düsterhöft. Wageningen, 24 februari 1993.

Voorwoord

Net zo als ik het vervelend vind, om alleen te gaan hardlopen of fietsen, zou ik in m'n eentje ook met dit promotieonderzoek niet zo ver gekomen zijn. En net zo als Henriëtte en de Bennekomse Boslopers bij het hardlopen, of Jaap en het 'fietsclubje' van proceskunde bij het fietsen mij enthousiast gemaakt hebben voor 'grootse' sportieve prestaties, zo waren de steun, begeleiding en hulp van collega's, vrienden en familie onontbeerlijk voor nieuwe motivatie en energie om dit onderzoek af te ronden. Diegenen, die het meest bij dit promotieonderzoek betrokken waren, wil ik nog eens de revue laten passeren.

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Doctoraalstudenten Christel Wijers, Annemarie Vonk, Willeke Thürkow en Gerard Kramer. Ook jullie bijdrage is in dit proefschrift verwerkt.

De medewerk(st)ers van tekenkamer en fotolokatie hebben ervoor gezorgd dat figuren en afbeeldingen netjes verwerkt werden.

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Eva

Für meine Eltern

LIST OF ABBREVIATIONS

ACA	alkali/chlorite/alkali
ara	arabinose
ara*f*	arabinofuranose
AUA	anhydro-uronic acid
CBH	cellobiohydrolase
CM	carboxymethyl
CMC	carboxymethylcellulose
COSY	correlated spectroscopy
CWM	cell wall material
Da	Dalton
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
DP	degree of polymerisation
EDTA	ethylenediaminetetraacetic acid
ETAg	enzyme-thiocarbohydrazide-silver
FID	flame ionization detector
fuc	fucose
gal	galactose
galA	galacturonic acid
GC-MS	gas chromatography-mass spectroscopy
glc	glucose
GLC	gas liquid chromatography
glcA	glucuronic acid
HMBC	multiple-bond HMQC experiment
HMQC	heteronuclear multiple quantum coherence experiment
HPAEC	high-performance anion-exchange chromatography
HPSEC	high-performance size-exclusion chromatography
m/z	mass/charge
M	molar
man	mannose
MMNO	4-methylmorpholine N-oxide
n.dt	not determined
NMR	nuclear magnetic resonance
NSP	non-starch polysaccharides
p	precipitate
PAD	pulsed amperometric detection
pnp	para-nitrophenyl-
res	residue
rha	rhamnose
s	soluble
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
tr.	traces
TOCSY	total correlation spectroscopy
U	Units
XG	xyloglucan
xyl	xylose
xyl*p*	xylopyranose

CONTENTS

CHAPTER 1

INTRODUCTION

Background

Large amounts of by-products from plant origin are produced anually by the food and agricultural industries. They constitute those parts of crops that are left after removal of value-giving components. Examples are beet pulp (residue from sugar production), straws, cereal brans (milling by-products), wheat- or maize gluten feed (by-product of the starch industry), spent grain or distiller's grain (residue from brewing or distillery), citrus pulp (from fruit or juice industry) or oil seed meals (residues from vegetable oil production). These residues still contain considerable amounts of energy, present as various proportions of protein or structural carbohydrates. Thus, they represent potentially valuable and renewable resources which find applications in various areas: a) as direct energy carrier (fuel production), b) as feedstock for the chemical industry, c) as functional ingredients in the food industry (e.g. thickening agents, (Voragen and Beldman 1990)) or d) as animal feed compounds (Bisaria 1991, Gacesa and Hubble 1991).

In plant residues, protein and carbohydrates may be present as intracellular compounds or, together with lignin, as cell wall constituents. The utilisation of these resources is only possible after total or partial conversion of the complex plant materials into peptides or amino acids, oligo- or monosaccharides. In comparison with chemical or physical processes (e.g. hydrolysis, extraction, steam-explosion and various combinations), bio-conversion, i.e. the enzymatic conversion of these residues, represents an energy-saving, environmentally advantageous means to modify or degrade the materials. Many studies have been undertaken to investigate requirements for enzymatic processes (Coughlan 1989 and references therein, Vallander and Eriksson 1990) and their economic feasibility (Kosaric and Velayudhan 1991).

Cellulose, hemicellulose and pectic compounds, designated 'non-starch polysaccharides', and lignin are the major constituents of plant residues or, more specifically, of the cell walls they consist of. To effectively attack these structures, cellulolytic, hemicellulolytic, pectolytic and lignolytic enzyme systems or microorganisms producing them are required. As indicated by the term 'lignocellulosics' which is associated with most plant residues, cellulose- and lignin degradation are considered the most important processes in enzymic conversion. Many of the above mentioned by-products, however, contain also considerable amounts of non-cellulosic polysaccharides (e.g. xylans, pectic compounds, mannans) with potential use for various applications.

Two examples of such by-products are sunflower (*Helianthus annuus* L) meal and palm-kernel (*Elaeis guineensis* Jacq) meal, the raw-materials studied in this thesis. These meals are used to limited extents as animal feed compounds. Enzymatic treatment of feed stuffs may improve their nutritional value by different mechanisms (Chesson 1987): a) degradation of cell walls resulting in improved accessibility or release of intracellular nutrients, b) the direct utilisation of hydrolysis products of polysaccharides, like glucose formed upon cellulose hydrolysis, c) elimination of anti-nutritional factors, d) influencing physiological effects exerted by non-starch polysaccharides or by their degradation products (Graham and Åman 1991, Topping 1991, Annison and Choct 1991). Major improvements by supplementation with exogeneous enzymes may be expected in monogastric animals, as these lack the enzyme systems to degrade structural polysaccharides, while ruminants are hosts to a microflora (Akin 1988) known to effectively degrade lignocellulosic materials.

The substrates investigated in this study

Palm-oil ranks second in the world's vegetable edible oil production, next to soy bean oil; sunflower oil ranks third (Mielke 1986). Two types of oil are produced from the oil palm, one deriving from the mesocarp of the fruits (palm-oil, representing about 20% of the harvested fruit bundles) and one from the kernels (palm-kernel oil, representing about 4% of the fruit bundles) (Salunkhe *et al* 1992). Oil seed meals are the residues obtained after pressing and/or extraction of the fruits. In 1991 the world production of sunflower meal amounted to 9.6 million tons, that of palm-kernel meal to 1.8 million tons (ISTA 1992). Major area of utilisation for both meals is in animal nutrition. The macroscopic structure of both oil-bearing fruits is shown in Fig.1.

Depending on the way of production, the hull content of sunflower meals and, consequently, their chemical composition and nutritional value may differ considerably (Salunkhe *et al* 1992). Sunflower hulls contain about 45% crude fibre and thus largely contribute to the limited use of the meal in animal diets. For dehulled meals, crude fibre contents ranging from 20% to 25% have been reported (Carré and Brillouet 1986, Ibrahim and Zubeir 1991, Musharaf 1991). The protein content of meals varies between 44% (dehulled) to 25% (undehulled); lysine is the first limiting amino acid (Boucqué and Fiems 1988). Sunflower meal can be used as animal feed compound in cattle (Kinard 1975, Richardson and Anderson 1981),

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pig (Baird 1981) and poultry diets (Ibrahim and Zubeir 1991, Musharaf 1991) provided adequate supplementation is given to balance energy and amino acid composition.
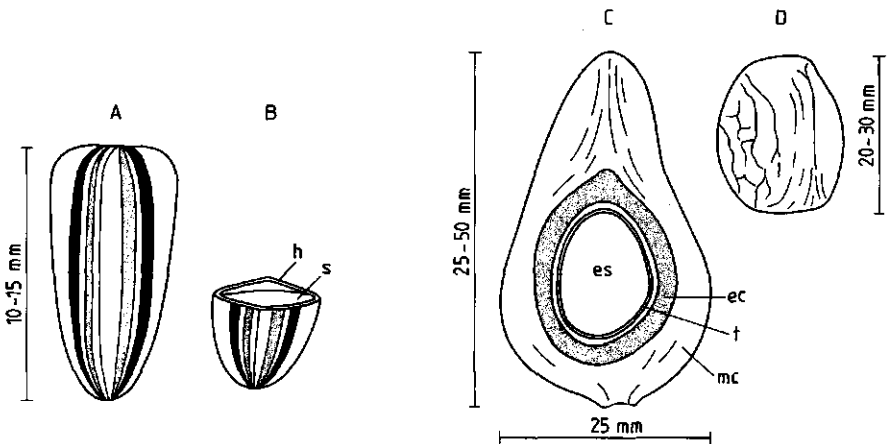
Figure 1: Macroscopic structure of sunflower and palm-kernel fruits. A: sunflower fruit, B: trans-section of fruit, showing hull (h) and seed (s), C: palm-fruit (longitudinal section) showing mesocarp (mc), endocarp (ec), testa (t) and endosperm (es), D: palm-kernel. Adapted from Vaughan (1970), with permission.

Palm-kernel meal contains endosperm, testa and the dark endocarp. It has the lowest protein content of oil seed meals (14% - 20%) and a high proportion of crude fibre (11%-25%). The low digestibility of its nutrients and a low palatability limit the inclusion of palm-kernel meal especially in diets for monogastric animals (Panigrahi and Powell 1991, Onwudike 1986, Ogbonna *et al* 1988, Babatunde *et al* 1975) and requires proper balancing with other feed compounds.

Fine-structure of the substrates - The plant cell wall

For an understanding of the enzymic degradation of plant materials, the composition of their cell walls, the arrangement of constituent polymers within the wall matrix and the morphology of plant tissues must be considered. By using very different approaches numerous researchers have created profound knowledge on the wide field of plant cell wall composition and structure. It is beyond the scope of

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this introduction to give a comprehensive treatise on this subject. In the following only a brief summary of the most relevant aspects concerning this study is given, which does not pretend completeness. The interested reader is referred to comprehensive descriptions and reviews on plant cell walls by Bacic *et al* 1988, Brett and Waldron 1990, Darvill *et al* 1980, Dey and Brinson 1984, Varner and Lin 1989 and the references therein.

Major cell wall polymers

The main component of plant by-products are cell walls. They are composed of carbohydrates (which, in contrast to the reserve polysaccharide starch are called 'structural polysaccharides' or 'non-starch polysaccharides'), protein and lignin. The major types of polysaccharides occurring (Bacic *et al* 1988) are listed below:

Cellulose. This homopolymer is composed of ß-(1→4)linked glucopyranosyl units, which align with a 2-fold screw axis due to intramolecular hydrogen-bonding. Multiple chains can aggregate by intermolecular hydrogen-bonds to form microfibrils. Cellulose occurs in different conformations, the amorphous and crystalline regions varying in proportion according to their origin and tissue type.

Xylans. These polymers have in common a backbone of ß-(1→4)linked xylopyranosyl residues. According to type and amount of substituents, arabinoxylans (varying in amount of single-unit side-chains of α-L-arabinofuranose attached to O-3 or both to O-3 and O-2 of the xylosyl residues), (4-*O*-methyl)-glucuronoxylans (with α-(1→2)linked (4-*O*-methyl)-glucuronosyl substituents) and arabino-glucuronoxylans can be distinguished. The xylosyl residues may additionally be acetylated at position O-2 or O-3.

Mannans. The backbone of this group of polymers is composed of ß-(1→4)linked mannopyranosyl residues or, in glucomannans, of both glucosyl and mannosyl residues. Glucomannans occur acetylated at O-2 or O-3 of the mannosyl residues. Like cellulose, unsubstituted mannans and glucomannans can adopt crystalline conformations. In galacto(gluco)mannans, single-unit side-chains of α-D-galactopyranose are attached to O-6 of the backbone units. Increasing degree of substitution enhances the solubility of these polysaccharides.

Xyloglucans. Like cellulose, xyloglucans possess a backbone of ß-(1→4)linked glucopyranosyl residues. Branching occurs by either single-unit xylosyl residues (α-(1→6)linked to glucose) or by extensions of this xylose side-chain with ß-(1→2)linked galactose, α-(1→2)linked arabinose or the (1→2)linked disaccharide sequence α-L-fucose-(1→2)-ß-D-galactose.

Mixed linked ß-glucans. In contrast to cellulose in this type of glucan ß-(1→3)- and ß-(1→4)linkages occur in ratios of 1:2 or 1:3. Chains of 3 or 4 contiguous ß-

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(1→4)linked glucopyranosyl units are interrupted by ß-(1→3)linkages which lead to a more extended, less ordered conformation than that of cellulose and to relatively higher solubility.

Pectic polysaccharides. This group of cell wall polysaccharides comprises several different neutral and acidic polysaccharides, which occur closely associated with each other in the cell wall. Main building units of pectins are rhamnogalacturonans, chains of α-(1→4)linked galacturonosyl residues in which 'smooth regions', with a low amount of α-(1→2)linked rhamnopyranosyl residues inserted in the chain, and 'hairy' regions, with almost alternating rhamnosyl and galacturonosyl residues, occur in various proportions. Extended neutral sugar side-chains of ß-(1→4)linked (arabino)galactans and α-(1→5)linked (possibly branched through O-3) arabinans are attached mainly through O-4 of rhamnopyranosyl residues giving rise to highly branched structures in the 'hairy' regions. Galacturonosyl residues can be methyl-esterified on position O-6 and carry acetyl-groups on O-2 or O-3.

Lignin. A major non-carbohydrate compound in plant cell walls is lignin, a polymer of three phenylpropanoid alcohols, coniferyl-, sinapyl- and p-coumaryl-alcohol. These building units are linked by a variety of chemical bonds, including alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds and carbon-carbon-linkages, resulting in a very hydrophobic polymer. The relative proportion of the three cinnamyl alcohols and of their linkage-mode varies between plant species and tissue types (Janshekar and Fiechter 1983).

Extensins. These hydroxyproline-rich glycoproteins are major structural wall proteins. Side-chains containing 1 to 4 arabinofuranosyl residues and single galactopyranosyl residues are covalently attached to respectively the hydroxyproline and serine residues of this basic protein (Tierney and Varner 1987).

Interconnections of polymers: Basis for cell wall formation

Evidence for the following types of covalent linkages between different cell wall polymers has been reported (Bacic *et al* 1988, Fry 1986): a) polysaccharide-polysaccharide (linkages via phenolic crosslinks) b) polysaccharide-lignin (via ether- or ester-linkages of uronosyl-residues or via cinnamic acid bridges), c) lignin-protein and d) protein-polysaccharide (extensin).

In solution, polysaccharides can associate by non-covalent interactions to form a three-dimensional network. Highly hydrated regions, where association between two glycan chains is hindered either by ionic repulsion or by the presence of side chains, and "junction zones", linear, unsubstituted regions, where interactions by hydrogen-bonds or by ionic forces can occur (Rees 1982), determine the stability of this matrix. Well-known examples for non-covalent interactions are the association

of galacturonan-chains (egg-box model, Jarvis 1984) or intermolecular hydrogen-bonding of various polymers to cellulose, like xyloglucan (Hayashi *et al* 1987), xylans (Andrewartha *et al* 1979) and glucomannans (Chanzy 1982).

By covalent or non-covalent interaction the different cell wall polymers form matrices varying in rigidity, hydrophobicity and porosity (Bacic *et al* 1988). Both types of interactions are considered in the current cell wall models (Albersheim 1978, Lamport and Epstein 1983, Talbott and Ray 1992).

Compositional, morphological and taxonomic differences of cell walls

By variations in the types of polymers present and in their degree of interaction, distinct differences are created between cell wall layers (middle lamella, primary and secondary cell wall), between cell walls of different tissue types and between different taxonomic groups, like gymnosperms and angiosperms and, as subclasses of the latter, between dicotyledenous and monocotyledenous plants.

Primary cell walls, synthesised after formation of the middle lamella, can be regarded as a highly hydrated matrix in which exchange of solutes and small molecules is possible. Thus, cells surrounded by a primary cell wall are regarded as living cells at the end of their development. Cellulose, deposited in a rather irregular manner, reinforces the network of non-cellulosic 'matrix' polysaccharides. The latter are branched polysaccharides, like pectic compounds and xyloglucans (prevailing in dicot primary walls) or arabinoxylans and ß-1,3;1,4-linked glucans (prevailing in monocot primary walls), and structural proteins, extensins.

In contrast, secondary cell walls are deposited in some cell types when the growth of the primary wall has ceased. They are characterised by highly ordered, crystalline cellulose microfibrils and the presence of 4-*O*-methyl-glucuronoxylans (in dicots), little substituted (glucurono)arabinoxylans (in monocots) or glucomannans (in dicots and gymnosperms). An uncommon composition is encountered in secondary thickened cell walls of the endosperm or cotyledons of certain seeds. Mannans (in palms), xyloglucans (in tamarind) and galactans (in lupines) are deposited as reserve polysaccharides in the cell walls of these plants. Certain cell types undergo lignification in primary and/or secondary cell walls, thereby enhancing their mechanical stability and hydrophobicity (Bacic *et al* 1988).

Unthickened cells with primary walls are present in soft parenchyma tissue, like in fleshy fruits, cotyledons and legumes. Secondary thickened, lignified cells are typically found in support tissues (sclerenchyma), that give mechanical strength to the plant (fibers and sclereids) or conduct nutrients and water (xylem, with tracheids and vessel elements) (Brett and Waldron 1990).

Biodegradation of plant cell walls

For the utilisation of by-products, the complex plant cell wall structures have to be degraded. In nature, this process is mediated by microorganisms or, more specifically, by the enzymes produced by them. The following overview will focus on polysaccharide degrading enzymes only; lignolytic enzymes, contributing to natural decay processes also, are not considered here.

Non-starch polysaccharide degrading enzymes

The diversity of structures encountered amongst cell wall polysaccharides and their physical and chemical entanglement with each other or with other polymers requires the action of various enzyme activities for complete hydrolysis. The most important enzymes, grouped according to their target substrates, are briefly mentioned below, together with references of recent reviews.

Cellulolytic enzymes: These include endo-glucanases (E.C.3.2.1.4), exo-glucanases (cellobiohydrolase, E.C.3.2.1.91) and ß-glucosidases (E.C.3.2.1.21). A profound description of fungal cellulose degradation is given by Coughlan (1990).

Xylanolytic enzymes: Endo-xylanases (E.C.3.2.1.8) and ß-xylosidases (E.C.3.2.1.37) can attack the main chain of different xylans. In addition, the following activities are required for the removal of substituents: α-L-arabinofuranosidases (E.C.3.2.1.55), α-glucuronidases (E.C.3.2.1), acetyl esterases (E.C.3.1.1.6) and ferulic or coumaric acid esterases (Kormelink 1992).

Mannanolytic enzymes: Endo-mannanases (E.C.3.2.1.78) and ß-mannosidases (E.C.3.2.1.25) are activities which hydrolyse the backbone of mannans or glucomannans. The complete hydrolysis of highly substituted galacto-(gluco)mannans requires additionally the presence of α-galactosidases (E.C.3.2.1.22) (Matheson 1990).

Pectolytic enzymes: These include endo- (E.C. 3.2.1.15) and exo-polygalacturonases (E.C.3.2.1.67), endo- and exo-pectate-lyases (E.C.4.2.2.2 and E.C.4.2.2.9), all of which are active on non-methylesterified pectin, endo-pectin-lyases (E.C.4.2.2.10), which work on highly methylesterified pectins and pectin-methylesterases (E.C.3.1.1.11), which cleave the methyl ester (Rombouts and Pilnik 1980). Neutral sugar side-chains of pectins can be hydrolysed by endo- and exo-arabinanases (E.C.3.2.1.99 and E.C.3.2.1.55) and endo-ß-galactanases (E.C.3.2.1.89). These enzymes are not normally regarded as 'pectolytic enzymes'. An enzyme, cleaving specifically the linkage between galacturonic acid and rhamnose, rhamnogalacturonase, has recently been described by Schols *et al* (1990).

Limitations to enzymic plant cell wall degradation

Even though, according to our present knowledge, a suitable set of enzyme activities should theoretically degrade a given polysaccharide, the actual situation in intact tissues is far more complicated and the degradability of various plant materials differs widely. As outlined in the preceeding sections, heterogeneity of plant residues is not only manifested in non-starch polysaccharide composition, but also in botanical origin and consequently, in tissue types and cell wall types. The major part of lignocellulosic materials consists of secondary thickened, lignified cell walls. The effectivity of hydrolytic enzymes, however, decreases as complexity and hydrophobicity of the substrates increase. Numerous factors causing this behaviour, most of which are interrelated, have been discussed (Fan *et al* 1982): a) the crystallinity of the substrate, b) its moisture content, c) 'porosity' of the substrate and accessible surface area, d) lignification, e) (in)solubility, f) shielding by and association with other carbohydrate or non-carbohydrate polymers and g) product inhibition.

Some of these points will be considered in more detail. As outlined before, primary walls are regarded as highly hydrated matrices. The pore-size, as estimated in different primary walls, ranges from 3.5nm to 10nm in diameter (Carpita *et al* 1979, Tepfer and Taylor 1981, McCann 1990). Assumptions by the two former authors about the mass of a globular protein which would theoretically be able to penetrate such pores vary from 17kDa to 60kDa, respectively. Polysaccharidases generally have higher molecular masses than the lowest estimate and some are not globular (Shmuck *et al* 1986). If, like in secondary walls, pore size and hydration are even more unfavourable, enzyme action most likely remains surface-restricted. This is reflected in many studies, in which plant materials have been subjected to *in-vivo* or *in-vitro* digestion. Highly lignified structures, like sclerenchyma and xylem resist microbial degradation (Akin 1989, Grabber and Jung 1991). Taking into account the compositional differences within cell wall layers and cell types, it is not surprising that pectic polysaccharides, characteristically encountered in primary cell walls, are usually found to be most readily degraded to high extents, whereas the degradability of cellulose and (4-*O*-methyl)-glucuronoxylans, typical constituents of secondary walls, is lowest (Hatfield 1989, Wilson *et al* 1989).

The shielding of a substrate by association with other cell wall polymers represents another factor limiting the access of an enzyme to its substrate. An example is the interdependance of the hydrolysis of hemicellulose or cellulose with that of pectic polysaccharides (Voragen *et al* 1980, Renard *et al* 1991). The cooperative action of different polysaccharidases is equally important to overcome

this limitation and to proceed in a surface-oriented mode of attack.

Pretreatments

To enhance the efficacy of enzymic conversion of lignocellulosic materials, various pretreatments are applied. They can be grouped into the following classes or combinations of them: a) physical (e.g. particle size reduction, steam explosion (Toussaint *et al* 1991), b) chemical (e.g. acidic, alkaline or oxidative treatments (Barl *et al* 1991, Fox *et al* 1989, Morrison 1991, Rivers and Emert 1988, Thompson *et al* 1992), c) biological (Agosin *et al* 1986). These processes are aimed at an increase in available surface area, reduction of crystallinity of cellulose (or other non-starch polysaccharides), removal of lignin, cleavage or disruption of covalent and non-covalent bonds and consequent disintegration of the firm cell walls.

Outline of this thesis

Of the large amount of literature published on the enzymic conversion of plant biomass, the majority focusses especially on cellulose-degradation and cellulases. Considerably less information can be extracted in this context about other non-starch polysaccharides and their impact on the integral degradation of the residues. This information is, however, especially important for those materials which are characterised by a high proportion of non-cellulosic polysaccharides and for their use in different applications. At the same time, broad-range enzyme preparations are marketed for the use as supplement in animal diets. When we started our research, their implementation was mostly based on empirical findings and effects were rather unpredictable. Only recently, some working mechanisms have been revealed by fundamental studies (GrootWassink *et al* 1989, Bedford and Classen 1992).

The investigations described in this thesis were conducted to create necessary knowledge for the development of optimised, tailor-made enzyme-preparations directed to the degradation of plant cell walls in various agricultural by-products. Our studies focussed especially on the identification and characterisation of wall polysaccharides, the detection of structural barriers and on the identification of enzyme activities with potential to overcome these limitations. Particular attention was paid to the characterisation of reaction products formed, as these may explain *in-vivo* effects observed upon enzyme supplementation of animal feeds.

The two by-products investigated represent different taxonomic groups, sunflower meal belonging to the dicotyledenous and palm-kernel meal to the monocotyledenous plants and, as was evident from preliminary studies, varied widely in their non-starch polysaccharide composition.

A prerequesite for any investigation on enzymic degradation is the profound knowledge of the composition of the substrate. The detailed characterisation of the non-starch polysaccharides present in both raw materials is described in **Chapter 2** and **3**. Different types of polysaccharides have been identified in-situ and their chemical structure and composition were determined after extraction and partial purification. A quantification of all identifiable non-starch polysaccharides in the two meals was deduced from the results.

The enzymatic degradation of the cell wall materials was studied by three broad-range polysaccharidase preparations of different activity-spectra. General features governing and influencing the enzymic hydrolysis are described in **Chapter 4**. This enabled an evaluation of effectiveness and limitations of an enzymic treatment of the meals.

In **Chapter 5**, the reaction products solubilised upon treatment with the crude technical enzyme preparations and with their main fractions obtained from anion-exchange chromatography are described in detail with respect to their composition and size. Relations between type of enzyme activity and reaction products are shown.

In order to control and optimise the enzymatic process and product formation, the solubilising effect of individual components of the crude enzyme preparations was studied (**Chapter 6**). To this end, the preparations were fractionated by various chromatographic techniques and the most relevant enzyme activities were (partially) purified. Comparisons are made between the hydrolysis of an intact cell wall material and of its isolated major hemicellulose constituent, (4-*O*-methyl)-glucuronoxylan, in order to evaluate the impact of the primary structure of the polysaccharide and of cell wall architecture on enzymic action.

The approach and methodology used in this thesis and implications of the results with regard to other types of by-products and to various areas of application are discussed in **Chapter 7**.

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NON-STARCH POLYSACCHARIDES FROM SUNFLOWER (*HELIANTHUS ANNUUS*) MEAL AND PALM-KERNEL (*ELAEIS GUINEENSIS*) MEAL

I. PREPARATION OF CELL WALL MATERIAL AND EXTRACTION OF POLYSACCHARIDE FRACTIONS

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ABSTRACT

Two different chemical methods, sequential extraction with alkali and sodium chlorite and treatment with 4-methylmorpholine *N*-oxide (MMNO), were applied to the extraction of non-starch polysaccharides (NSP) from the enzymatically deproteinated, water-insoluble cell wall materials of sunflower (*Helianthus annuus* L.) meal and palm-kernel (*Elaeis guineensis* Jacq.) meal. The NSP content accounted for 55% (sunflower meal) and 75% (palm-kernel meal) of the cell wall materials. Neither of the treatments alone was capable of solubilising more than about half of the original NSP. Combined treatment using alkali/chlorite followed by MMNO completely dissolved cell wall material from palm-kernel meal, while a small residue (4% of original NSP) was left in sunflower meal. Loss of NSP occurred with both methods (total NSP-recovery ranging from 88% for alkali/chlorite extraction of sunflower to 64% for MMNO extraction of palm-kernel). Due to differences in solubility revealed upon acidification and/or dialysis, extracts became subdivided into precipitates and soluble fractions. The sugar composition of the resulting fractions enabled a tentative identification of the major non-starch polysaccharides: sunflower meal was found to contain cellulose, (acidic) xylans, polyuronide-containing fractions and xyloglucan; palm-kernel meal was found to contain mannans, cellulose and xylans, with the major part of the mannans originating from the endosperm and the xylans being almost exclusively located in the endocarp.

INTRODUCTION

Sunflower (*Helianthus annuus* L) meal and palm-kernel (*Elaeis guineensis* Jacq) meal, residues obtained after the extraction of oil, are two examples of lignocellulosic by-products that are produced in considerable quantities by the food and agricultural industries. World production in 1989 amounted to 9 million tonnes of sunflower meal and 1.6 million tonnes of palm-kernel meal (ISTA 1990).
Considerable effort has been directed towards the enzymic conversion of such materials, enabling their improved utilisation by the feed, chemical or energy producing industries (Coughlan 1989).

Detailed information about the substrate, i.e. plant cell walls and their constituent polysaccharides is a prerequisite for a better understanding of the enzymatic degradation of lignocellulosic materials.

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To date, only a general account of the non-starch polysaccharide composition of the two materials under investigation has been reported (Brillouet *et al* 1988, Thibault *et al* 1989). Specific polysaccharides have been investigated in related palm species (El Khadem and Sallam 1967; Saittagaroon *et al* 1983; Jarvis 1990). The aim of the present study was the extraction and identification of characteristic structural polysaccharides from the water-insoluble cell wall material of the two meals, using methods which extract as completely and non-destructively as possible.

Since lignified plant materials require severe chemical treatments to solubilise entirely their constituent polysaccharides, alkaline extraction and delignification have been applied sequentially (DuPont and Selvendran 1987, Selvendran and O'Neill 1987). As an alternative the cellulose solvent 4-methylmorpholine *N*-oxide (MMNO), previously described as a mild and non-degradative agent for solubilisation of primary (Joseleau *et al* 1981) and endosperm (Voragen *et al* 1987) cell walls, but being less effective on lignified tissues (Al Katrib *et al* 1988), was used as extractant.

The present study discusses the preparation of purified cell wall material and compares the two extraction procedures. Yields, chemical composition and monomeric sugar composition of cell wall materials and extracted fractions are presented.

MATERIALS and METHODS

Plant material

Sunflower (*Helianthus annuus*) meal and palm-kernel (*Elaeis guineensis*) meal were provided by Hendrix Nutrition B.V. (Boxmeer, The Netherlands). The two raw materials were ground in a laboratory mill (Model 100-AN, Peppink & Zn, Deventer, The Netherlands) to pass a 0.5-mm sieve. They were free of impurities as assessed by light microscopy.

Preparation of purified cell wall material (CWM)

The procedure described by Massiot *et al* (1988) was used with the following modifications: Meals were defatted by refluxing with hexane for 6h and air dried. Subsequently they were suspended in 0.1M phosphate buffer, pH 7.0 (100g litre^{-1}) and protease (Pronase Type XIV, Boehringer, Mannheim, Germany) was added (5mg g^{-1}CWM). The suspension was incubated 16h at 30°C with continuous

stirring. After centrifugation and washing, the treatment was repeated for 6h with a fresh amount of protease (2.5mg g⁻¹CWM). The supernatants were collected after centrifugation (16000 x *g*), concentrated *in vacuo* and freeze dried to give "water solubles" (WS). The residue (CWM) was washed with buffer and water. It was then resuspended in 80% ethanol, stirred overnight and finally air dried.

Separation of endocarp and endosperm fractions from palm-kernel CWM

Light microscopic observations confirmed the dark particles of the meal to be derived from endocarp (brownish coloured, thick walled cells) and the light ones from endosperm (Vaughan 1970). Sedimentation in water achieved a rough separation of particles with lower density (endosperm) from the ones with higher density (endocarp and endocarp/endosperm particles). From the latter fraction those particles solely consisting of dark (endocarp) material were selected manually after visual inspection.

Extraction of CWM's

The following extraction conditions have been applied either alone or in sequence: (20g CWM litre⁻¹ extractant; all alkaline extractions were carried out under nitrogen with 20mM $NaBH_4$ added to the extractant).

(1) 0.05M Na_2CO_3 (with 5mM EDTA for sunflower CWM) 15h, 4°C
(2) 1M KOH 3h, 20°C
(3) 4M KOH 3h, 20°C
(4) $NaClO_2$/HOAc (2% $NaClO_2$ (w/w), 0.5% HOAc (v/v)), 2h, 70°C
(5) 1M KOH as in (2)
(6) MMNO (24g g⁻¹CWM) 1h, 120°C, (2x)

Extracts were adjusted to pH 5.5 with acetic acid, dialysed and freeze dried. Precipitates formed upon acidification and/or dialysis were recovered separately by centrifugation (44300 x *g*), washed and freeze dried.

MMNO extraction (Voragen *et al* 1987) was applied to the CWM's directly (two cycles) or to the residue remaining after the extraction sequence 1 - 5. Extracts were dialysed and freeze dried, precipitates and residues were recovered by centrifugation, washed and freeze dried.

Analytical methods

All values were calculated on a dry weight basis. Moisture content was determined by Karl-Fischer Titration. The moisture content of freeze dried

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fractions was estimated to be 6.5%. The NSP content of the starting materials was determined according to Englyst and Cummings (1984). Neutral sugar composition of CWM's, residues and extracts was determined as alditol acetates with inositol as internal standard (hydrolysis and derivatisation according to Saeman *et al* (1954) and Englyst and Cummings (1984), respectively). Alditol acetates were separated on a 3m x 2mm i.d. glass column (packed with Chrom W AW 80 - 100 mesh, coated with 3% OV275) in a Carlo Erba GC operated at 200°C and equipped with an FID.

For the estimation of cellulose the samples were also subjected to direct hydrolysis with 1M H_2SO_4 (3 h, 100°C) and derivatised as above. The amount of cellulose was calculated from the difference in the percentage glucose obtained from measurement with and without the prehydrolysis step.

Uronic acids (AUA = anhydro-uronic acid) were estimated colorimetrically after Saeman hydrolysis with an automated 3-phenylphenol test (Thibault 1979) using concentrated sulfuric acid containing 0.0125M $Na_2B_4O_7$. Values were corrected for the interference of neutral sugars as determined by GLC.

Lignin was determined as Klason lignin (corrected for ash). Starch was determined enzymatically using the Boehringer test-kit. Ash was determined by incineration at 550°C overnight. Protein content was determined by a semi-automated micro-Kjeldahl method (N x 6.25).

Staining methods used for light microscopy of cell wall material were: Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany) for protein, acid phloroglucinol (1% (w/v) phloroglucine in HCl 15% (v/v)) for phenolic compounds, Calcofluor White (Polysciences Inc, Warrington, PA, USA) and Aniline Blue (water-soluble, BDH Chemicals, Poole, UK) for carbohydrates.

RESULTS and DISCUSSION

Preparation of CWM

Fractionation studies on non-starch polysaccharides require an adequate preparation of cell wall material, removing, as far as possible, protein, starch and other cytoplasmic constituents. The two meals were found to contain negligible amounts of starch (0.5% in sunflower meal and 0.1% in palm-kernel meal) but high amounts of protein (Table 1). In preliminary experiments the optimal incubation conditions for the enzymic digestion of protein were established. In contrast to the findings of Massiot *et al* (1988), the use of phosphate buffer at pH 7

Table 1

Yields and chemical composition of sunflower and palm-kernel meals and the
corresponding CWM's (% w/w)

	sunflower meal	CWM	palm-kernel meal	CWM
Yield from meal		59.0		72.6
Composition				
Protein	33.0	5.6	20.3	7.3
Lignin	14.3	20.8	12.0	17.5
Ash	n.dt	5.4	n.dt	5.0
Carbohydrates				
rha		0.6		0.1
fuc		0.2		0.0
ara		5.0		1.5
xyl		10.9		3.0
man		2.1		57.1
gal		1.4		2.4
glc (cellulosic)		24.6		8.1
(non-cell.)		2.0		0.9
aua		8.4		1.5
total NSP	31.2	55.2	50.0	74.6

led to maximal protein solubilisation without enhanced degradation or loss of pectic
or other polysaccharides compared with treatment with acetate buffer at pH 5.

The chemical composition of the raw materials and CWM's obtained is given
in Table 1. The efficiency of proteolysis (90% for sunflower meal and 74% for
palm-kernel meal) was similar to that reported by Massiot *et al* (1988) and
Brillouet *et al* (1988). The incomplete removal of protein was confirmed by light
microscopy. Although staining was markedly decreased after Pronase treatment,
both cell walls and cell contents still gave a positive reaction. This implies that
residual protein was either structurally bound in the cell wall or present as
inaccessible cytoplasmic material.

Along with the digestion of protein, small amounts of pectic substances were
dissolved (1.5% to 3% of neutral sugars, mainly arabinose and galactose, and, in
the case of sunflower meal, 5.5% of uronic acids) and recovered in the WS
fractions. However, the recovery of total NSP, calculated from Table 1, apparently
equals or even exceeds 100%. It appears that treatment with protease increased the
recovery of neutral sugars as analysed by GLC, indicating some "loosening effect"
on the cell wall structure. Intracellular non-starch polysaccharides present in palm-
kernel meal (see also next paragraph), were retained in the preparation and thus
contributed to the "cell wall material" of this meal.

Distribution of polysaccharides in different botanical tissues of palm-kernel meal

The meals under investigation contain different botanical tissues. In sunflower CWM, fractions of pericarp (hull) and seed (embryo, testa and endosperm) are present whereas, in palm-kernel CWM, endocarp (shell) and the large endosperm (with small embryo) can be distinguished (Vaughan 1970). The sugar composition of different tissues of sunflower meal has been reported by Sabir *et al* (1975) and Thibault *et al* (1989). The data from palm-kernel meal, given in Table 2, illustrate the highly uneven distribution of mannose and xylose in endosperm and endocarp tissues.

Table 2

NSP composition of endosperm and endocarp fractions
of palm-kernel meal (% w/w)

	endosperm	*endocarp*
rha/fuc	0.0	0.0
ara	1.3	1.0
xyl	0.6	19.7
man	71.8	3.7
gal	2.0	0.5
glc	8.8	6.5
aua	1.4	3.1
total NSP	85.9	34.5

The low NSP content of the latter fraction pointed to a preponderance of non-carbohydrate constituents. Light microscopic observations, after staining for phenolic compounds, gave positive reaction only in this endocarp fraction, indicating lignification of the tissue.

The content and the walls of endosperm cells reacted positively with Aniline Blue as well as Calcofluor White, both fluorescence stains for carbohydrates. Purified mannan reacted identically. After alkaline extraction, fluorescence intensity had decreased considerably in both cell content and walls. Combining these observations with the sugar composition determined (Table 2), we conclude that mannans are present both in the walls and the contents of the endosperm cells.

General comparison of extraction methods (Table 3)

For both materials, MMNO extraction achieved a higher solubilisation of NSP than ACA (alkali/chlorite/alkali). The total recovery of NSP, however, ranging from 64% to 88%, was slighlty better for the ACA extraction. Loss of NSP must

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have occurred during the extractions. Most likely reasons are degradation by alkaline conditions, extraction during delignification and losses on dialysis as well as inaccuracy in gravimetric measurements of small freeze dried fractions.

The solubilisation and recovery of individual sugars varied both with the extraction procedures and with the meals, indicating different attachment or linkage of certain sugar residues in the cell walls of the two meals. Recoveries exceeding 100% were determined in some instances for glucose and mannose. An increased recovery during the analysis of the extracted polysaccharides, in contrast to the insoluble starting material, might have caused this discrepancy. Saeman hydrolysis of lignified CWM never achieves complete solubilisation; instead, a small residue remains, known to contain essentially lignin and small amounts of ash, protein and carbohydrate. Underestimation of the sugar content in cell wall samples determined directly as alditol acetates has been observed previously by Lomax *et al* (1983).

By contrast, mannose recovery was strikingly low, when treating CWM of palm-kernel meal with MMNO. The non-degradative character of this chaotropic solvent is still a matter of discussion. Although MMNO does not degrade oat spelt xylan (Voragen *et al* 1987), it is reported to cause mid-chain cleavage of wheat bran arabinoxylans (Holloway *et al* 1985). Recent studies in our laboratory gave evidence of extensive breakdown of water-soluble arabinoxylans from wheat flour (unpublished results). The results obtained in the present study further confirm the assumption of a degradative solubilisation mechanism by MMNO and make it unsuitable as a mild extractant for certain polysaccharides.

Table 3

Solubilisation[a] and recovery[b] of NSP by ACA and MMNO extraction of CWM from sunflower meal and palm-kernel meal

| | solubilisation[a] | | | | recovery[b] | | | |
| | sunflower | | palm-kernel | | sunflower | | palm-kernel | |
	ACA	MMNO	ACA	MMNO	ACA	MMNO	ACA	MMNO
rha	85.1	80.0	68.5	100.0	45.7	73.0	71.6	32.0
fuc	100.0	100.0	-	-	22.5	29.3	-	-
ara	59.9	70.2	73.0	80.7	80.1	95.4	78.0	67.8
xyl	85.6	19.0	73.3	30.0	89.9	93.3	92.7	77.6
man	0.0	84.9	65.7	99.8	>100.0	>100.0	78.6	56.9
gal	73.9	78.8	54.2	95.9	77.3	99.5	80.0	76.4
glc	14.1	46.4	0.0	83.5	92.7	88.5	>100.0	97.8
aua	80.8	67.4	69.3	74.4	76.6	52.8	94.2	66.8
total NSP	44.6	49.2	57.8	94.1	88.3	85.6	83.2	63.9

[a] (Initial sugar (NSP) - sugar (NSP) in residue) per 100g initial sugar (NSP)
[b] (Sugar (NSP) in extract + sugar (NSP) in residue) per 100g initial sugar (NSP)

The main differences between the two extraction procedures are:

(1) The different amounts of glucose solubilised reflect the ability of MMNO to dissolve cellulose, while ACA only liberates non-cellulosic glucose.
(2) Solubilisation of arabinose, galactose and mannose residues was higher with MMNO.
(3) While ACA treatment released about 80% of xylose, only about 25% was solubilised by MMNO.

The selective insolubility of most of the xylose and the incomplete solubilisation of cellulose by MMNO treatment confirms that lignification is a limitation to MMNO extraction. Al Katrib *et al* (1988) observed an even stronger limitation in the case of wheat straw and demonstrated that, besides lignification, the physical structure and cohesion of the cell wall components contributes to this effect and may be overcome by pretreatment with alkali. This is in agreement with the enhanced efficacy of MMNO treatment of the residue of ACA observed in this study.

NSP fractions obtained by sequential extraction of sunflower CWM

Pectic substances, characterised by high galactose, arabinose and uronic acid contents, were extracted by the chelating agent EDTA. The carbohydrate content of the extracts was low. No attempts have been made to identify the non-carbohydrate part of these fractions.

Xylose-containing polymers with varying proportions of glucose, uronic acids and other neutral sugar residues were released by alkaline treatment. The most homogeneous xylan fractions with high NSP content became insoluble upon acidification or dialysis (1M KOHp[*], 1M KOHp[**], 4M KOHp[*], 4M KOHp[**], see Table 4). The water soluble fractions had a lower NSP content and a more heterogeneous sugar composition. This pointed to the presence of different populations of polysaccharides within these fractions. One of these (see fraction 4M KOHs) might be a xyloglucan, since all the characteristic constituent sugars, glucose, xylose, arabinose, galactose and fucose were encountered in this fraction (Stephen 1983). Taking into account the data reported by Thibault *et al* (1989) and Sabir *et al* (1975) on the NSP composition of flour and hull fractions of sunflower, we conclude, that all xylose-containing polymers originate from the hull.

Table 4

Yields, NSP and protein content and monomeric sugar composition of fractions resulting from extraction of sunflower CWM

Fraction	yield from CWM (%)	protein in fraction (%)	NSP in fraction (%)	rha	fuc	ara	xyl	man	gal	glc	aua
						(mol per 100 mol monosaccharides)					
ACA extraction											
CWM		5.6	55.3	1.2	0.4	10.5	23.0	3.6	2.5	45.6	13.2
Na₂CO₃s	4.6		6.1	1.2	0.0	22.4	9.8	7.8	16.5	14.6	27.6
Na₂CO₃p	4.7		23.7	0.7	0.0	3.6	0.4	0.0	2.5	0.2	92.5
1M KOHs*	4.5	5.9	51.9	0.3	0.0	3.0	72.1	1.6	0.1	8.9	11.2
1M KOHp*	1.9	3.6	85.5	0.3	0.0	0.3	80.6	0.1	0.1	9.6	9.1
1M KOHp**	1.0		78.9	0.5	0.0	0.5	85.4	0.0	0.0	1.4	12.2
4M KOHs*	3.4	4.0	72.0	0.3	1.8	4.5	42.2	9.4	7.4	25.9	8.5
4M KOHp	2.5		89.3	0.4	0.0	0.4	86.0	0.1	0.1	2.5	10.5
NaClO₃s	7.8	8.2	52.3	2.8	0.0	41.3	9.3	0.2	6.5	3.2	36.7
1M KOHCs**	7.3	3.4	38.3	1.1	0.0	10.4	50.4	0.3	3.4	13.6	20.8
1M KOHCp**	0.7		77.5	0.5	0.0	0.7	84.0	0.0	0.0	3.7	11.0
resACA	35.2	0.9	86.9	0.3	0.0	7.8	6.2	6.8	1.2	72.9	4.7
extraction of ACA residue with MMNO											
resACA		0.9	86.9	0.3	0.0	7.8	6.2	6.8	1.2	72.9	4.7
R-MMNOs	8.2		43.4	0.0	0.0	11.6	13.8	7.3	4.7	57.2	5.4
R-MMNOp*	45.6		97.5	0.2	0.0	0.2	1.9	6.5	0.0	89.8	1.4
resR-MMNO	16.7		37.4	0.8	0.0	42.9	1.9	4.1	1.7	46.4	2.2
direct extraction of CWM with MMNO											
CWM		5.6	55.3	1.2	0.4	10.5	23.0	3.6	2.5	45.6	13.2
MMNOsI	16.2	16.7	44.0	3.6	0.0	40.1	9.2	3.3	8.5	20.0	15.3
MMNOsII	3.3		48.9	5.0	0.0	27.4	10.8	6.6	7.9	27.7	14.6
MMNOpI	11.4		82.5	0.0	0.7	3.8	6.7	18.7	3.0	65.9	1.3
MMNOpII	1.3		83.9	1.2	0.0	9.1	9.5	9.8	2.9	67.5	0.0
resMMNO	58.3	1.8	48.2	0.5	0.0	6.0	35.9	1.1	1.0	47.2	8.3

* denotes precipitate upon acidification
** denotes precipitate upon dialysis
s, soluble

The high arabinose and protein contents and the low carbohydrate content determined in the fraction solubilised by chlorite ($NaClO_2s$) indicate the presence of glycoproteins, lignin-carbohydrate and lignin-protein complexes, and degradation products of lignin. The oxidative and acidic conditions of this treatment are known to split the linkages by which these compounds are held in the cell wall (Bacic *et al* 1988). A residue, mainly composed of cellulose, remained insoluble. Of its NSP content 20% comprised hemicellulosic constituents. As controlled by light microscopy, this material still contained minor amounts of phenolic compounds (probably lignin). MMNO was able to dissolve the major part of this material. A small residue, containing glucose and arabinose in equal amounts, resisted this final extraction. Direct MMNO extraction (Table 4) released hemicellulose and cellulose in one extraction step, the latter precipitating due to its insolubility in water.

NSP fractions obtained by sequential extraction of palm-kernel meal

Small amounts of pectic substances were released at low alkali concentrations (Na_2CO_3s, Na_2CO_3).

Mannans differing in solubility behaviour and extractability, were the major polymers released. In agreement with the investigations of El Khadem and Sallam (1967), Jarvis (1990) and Saittagaroon *et al* (1983) on other palm-species, the mannans extracted in this study had a very low degree of substitution as can be seen from the low galactose content of most of the fractions.

The major part of the xylose-containing polymers could only be extracted after treatment with chlorite and subsequent alkaline extraction. This provides evidence that most of the xylose-containing polymers in palm-kernel CWM were either bound to or shielded by lignin, preventing their extraction prior to delignification. From the carbohydrate distribution given in Table 2 it can be concluded that all xylose-containing polymers originate from the endocarp.

A residue, consisting of mannose and glucose, resisted the ACA extraction. It could be solubilised completely by MMNO. The close structural similarity between cellulose and mannans is indicated by these results.

Direct MMNO treatment yielded water-insoluble fractions containing mannan and cellulose and water-soluble material with high mannose and arabinose contents. Light microscopic examination revealed selective solubility of endospermic material and resistance of endocarp particles. This finding is consistent with the resistance of xylose to solubilisation (resMMNO) and its localisation in the endocarp fraction (Table 2).

Table 5

Yields, NSP and protein content and monomeric sugar composition of fractions resulting from extraction of palm-kernel CWM

Fraction	yield from CWM (%)	protein in fraction (%)	NSP in fraction (%)	rha	fuc	ara	xyl	man	gal	glc	aua
						(mol per 100 mol monosaccharides)					
ACA extraction											
CWM		7.3	74.6	0.2	0.0	2.5	4.8	75.6	3.2	11.9	1.8
Na$_2$CO$_3$s	1.2		36.4	0.0	0.0	6.3	1.2	77.4	5.7	1.6	7.8
Na$_2$CO$_3$p**	0.4		14.5	0.4	0.0	19.4	1.5	20.2	15.2	5.4	37.9
1M KOHs	9.7	4.6	91.0	0.2	0.0	1.8	1.2	91.7	2.8	1.3	1.1
1M KOHp*	0.3		34.9	1.1	0.0	3.5	21.4	18.4	1.6	49.6	4.5
1M KOHp**	1.4	1.6	94.1	0.0	0.0	0.6	0.5	96.8	0.4	1.2	0.5
4M KOHs	8.7		87.3	0.1	0.0	2.6	1.6	89.0	3.8	1.5	1.4
4M KOHp*	0.5	6.7	32.8	0.5	0.0	3.9	30.4	21.5	1.3	36.7	5.8
4M KOHp**	8.6		96.7	0.0	0.0	0.5	0.4	97.6	0.5	0.6	0.3
NaClO$_2$s	6.0	15.6	21.0	0.9	0.1	27.3	20.2	10.0	12.5	6.2	22.9
1M KOHCs	3.0	7.1	52.7	0.6	0.0	8.8	45.4	27.9	3.7	2.1	11.5
1M KOHCp**	1.0		90.3	0.0	0.0	0.4	90.8	0.1	0.0	1.6	7.1
resACA	31.5		100.0	0.1	0.0	1.6	3.1	61.7	3.5	28.7	1.3
extraction of ACA residue with MMNO											
resACA			100.0	0.1	0.0	1.6	3.1	61.7	3.5	28.7	1.3
R-MMNOs	10.7		79.0	0.5	0.0	7.9	2.3	69.7	9.8	4.8	5.0
R-MMNOp**	65.5		99.8	0.1	0.0	0.4	0.0	55.7	2.0	40.8	1.0
direct extraction of CWM with MMNO											
CWM		7.3	74.6	0.2	0.0	2.5	4.8	75.6	3.2	11.9	1.8
MMNOsI	21.4	21.8	53.6	0.3	0.0	6.4	0.7	78.2	6.5	5.2	2.7
MMNOsII	5.0		12.0	0.0	0.0	19.5	9.3	34.0	8.5	25.8	2.9
MMNOpI	32.7		93.1	0.0	0.0	0.9	0.4	74.4	3.1	20.7	0.6
MMNOpII	2.3		35.0	0.0	0.0	2.5	3.8	53.6	1.8	30.4	7.9
resMMNO	19.3	25.4	23.0	0.0	0.0	7.3	51.5	2.3	2.0	30.0	7.0

* denotes precipitate upon acidification
** denotes precipitate upon dialysis
s, soluble

CONCLUSIONS

Major polysaccharide fractions of sunflower meal and palm-kernel meal could be extracted with differing degrees of "purity", thereby subdividing two classes of polysaccharides according to their solubility or insolubility in water. Extraction with MMNO was limited, probably due to lignification and strong physical associations between the polymers in the cell walls. Unless combined with other treatments such as delignification and alkaline extraction, incomplete solubilisation of hemicellulose and cellulose was observed. With both procedures degradation of NSP occurred. The results of the two chemical approaches provide evidence for different attachment of some of the polymers in the two materials. The NSP composition of the extracts allows a tentative interpretation of some major polysaccharides, as polyuronide-containing fractions, (acidic) xylans, xyloglucan and cellulose in sunflower meal and mannans, cellulose and xylans in palm-kernel meal. Further purification and linkage analysis of extracts is necessary to identify clearly the polymers involved.

ACKNOWLEDGEMENTS

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NON-STARCH POLYSACCHARIDES FROM SUNFLOWER (*HELIANTHUS ANNUUS*) MEAL AND PALM-KERNEL (*ELAEIS GUINEENSIS*) MEAL

II. INVESTIGATION OF THE STRUCTURE OF MAJOR POLYSACCHARIDES

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ABSTRACT

For the identification and characterisation of major non-starch polysaccharides from sunflower (*Helianthus annuus* L) meal and palm-kernel (*Elaeis guineensis* Jacq) meal, extracts obtained by sequential alkaline extraction and delignification of the corresponding cell wall materials were subfractionated by graded ethanol precipitation and size-exclusion and adsorption chromatography. Determination of the sugar and glycosidic linkage composition of extracts and subfractions and intact cell wall materials allowed the identification and quantification of a variety of polysaccharides. In sunflower meal, cellulose (42% of total non-starch polysaccharides), pectic polysaccharides (24%), (4-*O*-methyl)-glucuronoxylans with 8-11% uronic acid substitution (24%), (gluco)mannans (5%) and fucoxyloglucans (4.5%) were encountered. Major polysaccharides in palm-kernel meal were linear mannans with very low galactose substitution (78% of total non-starch polysaccharides), followed by cellulose (12%) and small amounts of (4-*O*-methyl)-glucuronoxylans and arabinoxylans (3% each).

INTRODUCTION

Sunflower (*Helianthus annuus* L) meal and palm-kernel (*Elaeis guineensis* Jacq) meal are residues obtained after the extraction of edible oils. Due to their high contents of protein and carbohydrate, the meals represent potentially valuable raw materials for the feed industry (Onwudike 1986, 1988, Musharaf 1991). However, as with other lignocellulosic by-products, their utilisation is limited owing to their low digestibility. Enzymic treatment of such materials could enhance their agro-industrial utilisation and consequently much effort has been devoted in the study of the degradation mechanisms (Coughlan 1989). Knowledge about the structural characteristics of the subtrates, i.e. the cell walls and, in particular, their constituent polysaccharides, is a prerequesite to the understanding of these mechanisms.

General accounts of the non-starch polysaccharide (NSP) composition of the meals were reported by Brillouet *et al* (1988) and Thibault *et al* (1989). In related palm-species the chemical structure of mannans was investigated and close similarities with cellulose were found (El Khadem and Sallam 1967, Saittagaroon *et al* 1983, Jarvis 1990). Major non-cellulosic polysaccharides from both meals were extracted by different chemical methods and tentatively identified on the basis of

the sugar composition of extracts and residues (Düsterhöft *et al* 1991).

The present study was undertaken to obtain detailed information on the structure of these polymers. After subfractionation of heterogeneous extracts, the glycosidic linkage composition and, if appropriate, the molecular size distribution of characteristic fractions was analysed. Results obtained from the extracted polymers were compared with those from the glycosyl linkage analysis of the intact cell wall materials (Lomax *et al* 1983). This allowed a calculation of the quantitative distribution of constituent polysaccharides in the two meals.

MATERIALS AND METHODS

Plant material

Sunflower meal and palm-kernel meal were defatted and treated with protease to give "cell wall materials" (CWM) (Düsterhöft *et al* 1991).

Extraction and fractionation of polysaccharides

Polysaccharides were extracted sequentially with alkali of increasing strength (1M and 4M KOH), followed by delignification with chlorite and further alkaline extraction (1M KOH) (Düsterhöft *et al* 1991). Precipitates formed upon acidification of alkaline extracts to pH 5.5 or upon dialysis of acidified extracts were separated by centrifugation (44,300 x *g*), washed and freeze dried.

After dialysis and concentration under reduced pressure to a final concentration of about 5g litre^{-1}, water-soluble fractions were subfractionated by adding 96% ethanol, while stirring on ice. The ethanol concentration was increased in steps of 10% (v/v). Whenever a precipitate formed, the extract was kept overnight at 4°C and then separated as described above.

Size-exclusion chromatography

Samples were dissolved (10mg ml^{-1}) in sodium acetate buffer (0.1M, pH 5.0, containing 0.1g thiomersal litre^{-1}) and chromatographed in the same buffer on columns of Sephacryl S1000 or S500 (Pharmacia LKB, Uppsala, Sweden). The column flow rates were 9ml h^{-1} for S1000 (115cm x 1,4cm) and 13ml h^{-1} for S500 (115cm x 1,1cm). Fractions (2ml and 2.7ml respectively) were assayed colorimetrically for total neutral sugar and uronic acid content (see 'General analytical methods'). The void (45ml) and total bed volume (120ml) of S1000 were determined using *Escherichia coli* cells (ATCC 11229, turbidimetric estimation)

and glucose, respectively; void (84 ml) and total bed volume (190 ml) of S500 by amylopectin (Avebe, Veendam, Netherlands) and glucose.

Ion-exchange chromatography of uronic acid residues

For the identification of galacturonic, glucuronic and 4-*O*-methyl-glucuronic acids, high-performance anion-exchange chromatography (HPAEC) was performed on a Dionex (Sunnyvale CA, USA) system, composed of an Eluant Degas Module, a Gradient Pump Module (GMP2) and an Electrochemical Detector (PED). Samples were hydrolysed (Saeman 1954) and the hydrolysates were analysed on a CarboPac PA1 column (250mm x 4mm) and a CarboPac PA guard column (25mm x 3mm) at a flow rate of 1ml min^{-1}, running a linear gradient of water and 1M sodium acetate/100mM NaOH. Elution was monitored by triple-pulsed amperometric detection (PAD) using a gold electrode.

Adsorption chromatography of xyloglucan-containing fractions on cellulose

The sample (30 mg) was dissolved in 0.025M sodium acetate buffer, pH 5 (5ml), and applied to a column (16cm x 0.7cm) of microcrystalline cellulose (ca. 0.019mm, Serva, Heidelberg, Germany). Elution of the unbound fraction took place in the same buffer (36ml). Bound fractions were eluted by 7M urea (25ml), followed by 0.5M NaOH (35ml) (Aspinall and Molloy 1969). The latter fraction was neutralised using 1M HCl; all fractions were dialysed against distilled water and freeze dried.

Methylation analysis

Cell wall materials were milled to a fine powder using a freezer mill cooled by liquid nitrogen (SPEX Industries Inc, Edison, USA) (Lomax *et al* 1983). The particle size, determined microscopically, varied from 20 μm - 200 μm. In addition, cell wall material of palm-kernel meal was partially delignified by sodium chlorite (Düsterhöft *et al* 1991) prior to milling.

Acidic fractions were carboxyl reduced according to Taylor and Conrad (1972). Their linkage composition was analysed before (data not shown) and after reduction. Upon reduction, glucuronic and galacturonic acid units were converted to their corresponding glucose and galactose units, which, after derivatisation are represented in the Tables 2 and 4 by the values underlined. Samples were methylated by a modification of the Hakomori method (Sandford and Conrad 1966) and subsequently dialysed and dried in a stream of air. Solubility in CHCl$_3$/MeOH (1:1 v/v) was used to check for the completeness of methylation. If any material remained insoluble in this solvent, the procedure was repeated. For the formation

of glycitol acetates, methylated CWM was hydrolysed by formic acid (90% v/v), 100°C, 6h) followed by 0.25M sulphuric acid (100°C, 16h), whereas extracted polysaccharides were hydrolysed using 2M TFA (121°C, 1h). Reduction was performed using sodium borodeuteride. The partially methylated glycitol acetates were separated and quantified on a fused silica capillary column (30m x 0.32mm; wall coated with DB1701; 0.25μm) in a Carlo Erba Fractovap 4160 GC equipped with a FID. The sample composition was calculated using effective carbon response (ECR) factors (Sweet *et al* 1975). Identification of the compounds was confirmed by GC-MS (Hewlett Packard, MSD 5970-B coupled to a HP 5890 equipped with a fused silica column (CPSIL 19CB, 26m x 0.22mm; 0.18μm)). The 3,4- and 2,3-*O*-methylated and, separately, the 2-and 3-*O*-methylated xylitol acetates co-eluted and their relative amounts were calculated from the relative abundance of the ions at m/z 117 and m/z 118, and m/z 118 and m/z 129+130, respectively.

General analytical methods

Monosaccharide composition of samples was determined as glycitol acetates by GLC (column 3m x 2mm, packed with Chrom W AW 80-100 mesh, coated with 3% OV275). Myo-inositol was used as internal standard. Hydrolysis of (unmethylated) CWM and mannan-containing fractions was performed by Saeman hydrolysis (Saeman *et al* 1954), of all other fractions by 2M TFA (121°C, 1h). Derivatisation was conducted according to Englyst and Cummings (1984). On GLC, 4-*O*-methyl-glucose, present in carboxyl reduced samples, eluted as a distinct peak between galactose and glucose and was quantified accordingly.

Uronic acids (AUA=anhydro-uronic acid), in water-soluble fractions from size-exclusion chromatography or in acid hydrolysates, were estimated colorimetrically by an automated 3-phenylphenol test (Thibault 1979). The sulfuric acid used contained 0.0125M sodium tetraborate. Values were corrected for the interference of neutral sugars as determined by GLC.

Total neutral sugar content of fractions from size-exclusion chromatography was determined by an automated orcinol-sulfuric acid method (Tollier and Robin 1979).

RESULTS AND DISCUSSION

General lay-out of experiments

In contrast to investigations conducted on single, prominent polysaccharides isolated from certain plant species, the study of all cell wall polysaccharides present

in a raw material encounters many difficulties. Chemical extractions, especially in lignified samples, are the only means to solubilise effectively all constituent polysaccharides of plant cell walls. However, complete dissolution is rarely achieved. A significant drawback of these methods is the risk of formation of artefacts, resulting from scission of alkali- or acid-labile linkages. Although some polysaccharides may be obtained in reasonably pure form, most are co-extracted with a number of different polymers, and further subfractionation is needed to identify the multiple components.

Table 1

(Sub)fractions investigated from sunflower and palm-kernel meal, their origin and yield

designation[a]	origin[b]	sub-fractionation	yield[c]
sunflower			
CWM	-	-	100.0
S-X	1M KOH extract(p)	-	2.9
S-X,XG	4M KOH extract(s)	EtOH (20%)	1.5
S-X,PC	delignif.,1M KOH extr.(s)	EtOH (40%)	1.6
S-XG	S-X,XG	adsorption-chr.	0.5
palm-kernel			
CWM	-	-	100.0
P-M	1M KOH extract(s)	EtOH (10%)	8.9
P-X	delignif.,1M KOH extr.(p)	-	1.2
P-X,AX	delignif.,1M KOH extr.(s)	EtOH (50%)	0.5

[a] abbreviations used: S=sunflower, X=xylan, XG=xyloglucan, PC=pectic compounds
  P=palmkernel, M=mannan, AX=arabinoxylan
[b] for origin of extracts see Düsterhöft *et al* 1991, Tables 4 and 5
  abbreviations used: (s)=soluble, (p)=precipitate upon dialysis/acidification of extract
[c] proportion of total NSP in CWM recovered in (sub)fraction (%)

Thus, for the investigation of constituent polysaccharides from sunflower meal and palm-kernel meal, characteristic fractions obtained from sequential chemical extraction of the meals (Düsterhöft *et al* 1991) were selected and, if necessary, subfractionated by graded precipitation with ethanol and/or adsorption chromatography. A survey of all (sub)fractions dealt with in detail in the present study is given in Table 1. These (sub)fractions cover all types of polysaccharides that were anticipated after determination of the sugar and glycosidic linkage composition of numerous extracts and (sub)fractions. They contain either pure polysaccharides or are enriched in the types of polysaccharides designated.

Regarding the limitations outlined above, a second approach, the *'in-situ'*

determination of the glycosyl-linkage composition in intact cell wall materials, was taken to validate the results.

Identification and structure of polysaccharides from sunflower meal

Xylans. Alkaline extraction (1M and 4M KOH) predominantly released xylose-containing polymers from the cell wall material. The majority, accounting for more than 63% of extracted xylose, occurred as water-insoluble fractions and was accompanied by variable amounts of uronic acids (9 - 12 mol-%) and glucose (1.4 - 9.6 mol-%). Similar polysaccharides were present in heterogeneous water-soluble fractions (Düsterhöft *et al* 1991). Examination of a number of these fractions revealed structural features similar to those reported below.

The glycosidic linkages determined (Table 2) show that fraction 'S-X' contains a (1→4)linked xylan with about 10% glucuronic acid substitution at the O-2 position. Terminal glucose residues were exclusively encountered in the reduced sample, but absent in the unreduced sample (data not shown). The comparison of the proportion of terminal glucose determined after methylation of the reduced sample with the proportion of 4-O-methyl glucose determined in the reduced, but unmethylated sample (data in parentheses, italicised) shows that about 80% of the glucuronic acid residues are methyl-etherified at position O-4. The presence of 4-O-methyl-glucuronic acid and minor amounts of glucuronic acid was confirmed by HPAEC. Substitution with (4-O-methyl)-glucuronic acid in other xylan fractions (data not shown) varied between 8-11%. These features are typical of xylans encountered in secondary cell walls of dicotyledenous plants. In their native state, these polysaccharides are reported to be O-acetylated (Stephen 1983). During the alkaline extraction applied in this study these ester-linkages will be saponified, thereby changing the solubility of the polymers.

The reason for the different extractability and solubility of the various xylans obtained in the present study can be due to either differences in molecular size and/or uronic acid substitution or to difference in "location" in the cell wall and interlinkage to other cell wall compounds.

In order to check the homogeneity and molecular size distribution of fraction 'S-X', size-exclusion chromatography (Sephacryl S1000) was performed (Fig.1). The fraction was only partially soluble in the buffer used for chromatography. The broad, asymmetric shape of the resulting peak and the sugar composition measured at different elution volumes indicate the presence of several populations of acidic

Table 2

Partially methylated alditol acetates from sunflower CWM and polysaccharide fractions derived from it [mol per 100 mol]

O-methyl derivative	CWM	S-X[a]	S-X,XG[a]	S-X,PC[a]	S-XG
235 ara*	2.7		0.5	3.0	0.7
23 ara	3.1		0.7	2.2	
35 ara	0.2				
3 ara	0.7				
2 ara	3.1		0.5	2.7	
ara	1.5				0.3
total	11.3(12.1)[b]	(0.6)	1.7 (1.9)	7.9 (7.8)	1.0 (0.7)
234 xyl	0.5	0.2	8.0	2.0	9.3
23 xyl	20.6	71.7	27.4	56.2	17.4
34 xyl	1.0	3.3	4.8	4.5	6.0
3 xyl	1.3	6.6	2.5	4.8	0.9
2 xyl	0.5	0.3	0.1	0.1	
xyl	0.9				0.3
total	24.8 (26.5)	82.1(84.5)	42.8(44.7)	67.6(65.1)	33.9(31.9)
234 fuc	0.1		2.2		3.3
3 fuc			0.2		
total	0.1 (0.5)		2.4 (2.4)		3.3 (2.7)
234 rha	0.2				
34 rha	0.2			0.8	
3 rha				1.1	
total	0.4 (1.4)	(0.6)		1.9 (1.7)	
2346 glc	1.3	8.7[c]	6.3	9.0	2.2
236 glc	52.0	7.0	18.5	2.8	31.5
246 glc	0.9		1.4	0.6	0.3
23 glc	2.7		13.8	1.4	15.3
glc	0.9		0.5	2.7	1.4
total	57.8 (52.5)	15.7 (4.6)	40.5(31.6)	16.5 (6.6)	50.7(53.6)
		(6.9)[d]	(3.8)	(10.7)	
2346 gal	0.7		3.8	2.3	3.0
236 gal	0.4	1.5	0.7	2.6	0.2
total	1.1 (2.9)	1.5 (2.7)	4.5 (7.5)	4.9 (8.1)	3.2 (5.6)
236 man	3.8	0.4	8.1	1.1	7.1
23 man					0.4
man	0.6	0.3			0.3
total	4.4 (4.1)	0.7 (0.0)	8.1 (8.0)	1.1 (0.0)	7.8 (5.5)

Abbreviations used: S=sunflower meal, X=xylan, XG=xyloglucan, PC=pectic compounds
* 235 ara denotes 1,4-di-O-acetyl-2,3,5-tri-O-methylarabinitol etc.
[a] carboxyl reduced
[b] values in parentheses: amount of parent sugar determined in unmethylated sample
[c] values underlined derive from (4-O-methyl)glucuronic or from galacturonic acid
[d] values italicised: 4-O-methyl-glucose (determined in unmethylated sample)
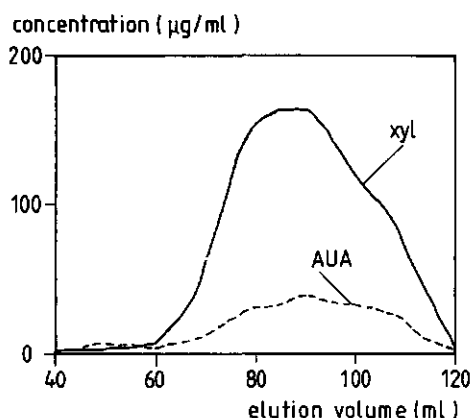
concentration (μg/ml)

Figure 1: Size-exclusion chromatography of (4-*O*-methyl)-glucuronoxylan. Elution profile of constituent sugars as determined by GLC and of uronic acids (AUA)

xylans with varying amounts of (4-*O*-methyl)-glucuronic acid substitution. Glucose was exclusively found in the insoluble residue, which accounted for 13% (w/w) of the original fraction. This implies that the glucose moiety derives from separate polymers which are co-extracted/co-precipitated with the xylans. Similarly, minor amounts of pectic compounds probably were co-extracted, as indicated by galacturonic acid detected by HPAEC and 2,3,6-*O*-methyl galactose residues formed upon methylation of the reduced sample (Table 2).

Xyloglucans. Xyloglucans were extracted by 4M KOH and recovered after subfractionation of heterogeneous, water-soluble fractions (Table 1, 'S-X,XG', 'S-XG'). The presence of xyloglucans was indicated by (1→4)linked glucose residues forming the XG-backbone, about 45% of which were branched at O-6. Terminal xylose, galactose and fucose and (1→2)linked xylose residues may derive from different side chains (Table 2, 'S-X,XG').

The molecular size distribution of fraction 'S-X,XG' and its sugar composition at different elution volumes (Fig.2) reveals that this fraction, even after subfractionation, contains various types of polysaccharides. Xyloglucans of different molecular size eluted near the void volume and in the main fraction (elution volume 70-100 ml). The latter co-eluted with a (4-*O*-methyl)-glucuronoxylan. Furthermore, (1→4)linked mannose residues indicate the presence of (gluco)mannans, eluting both near the void and included volume. Since the differences in molecular size were too small to achieve a good resolution between

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the various polymers, adsorption chromatography on a cellulose column was applied to bind specifically the XG moiety of the fraction (Hayashi *et al* 1987). However, xyloglucans, xylans as well as mannans partly bound to the column. By stepwise elution with buffer, 7M urea and 0.5M NaOH, fractions enriched in xylan or xyloglucan were obtained (Table 3).

Table 3

Yield and non-starch polysaccharide (NSP) composition of subfractions obtained by adsorption chromatography of xyloglucan-containing fraction 'S-X,XG'

fraction	yield[a]	NSP[b]	rha	fuc	ara	xyl	man	gal	glc	aua
						[mol per 100 mol]				
not bound	31	73.5	0.2	0.4	4.1	48.0	5.7	3.6	27.4	10.5
7M urea	18	81.7	0.1	0.5	1.3	64.6	1.4	2.6	18.2	11.2
0.5M NaOH	38	96.4	-	2.6	0.7	30.8	5.3	5.4	51.8	3.4

[a] g per 100g of starting material
[b] proportion of NSP from total weight of fraction (g NSP per 100g fraction)

The xyloglucan fraction released by the latter eluant was still accompanied by a mannose-containing polymer and some uronic acid containing material. Structural details from this fraction can be deduced from Table 2 ('S-XG'). Only about 30% of the glucosyl residues forming the backbone carried side chains at O-6. Terminal xylopyranosyl-, galactopyranosyl- or fucopyranosyl-groups may either be attached as single unit side chains or, to a lesser extent, via a xylose residue, thus representing a two-membered side chain. The chromatographic behaviour of the crude fraction implies that the polymers present occur in multiple forms and probably interact strongly with each other *in situ* and/or after extraction. The presence of (gluco)mannans in XG preparations has been ascribed to the strong attachment of both polymers to cellulose (Selvendran and O'Neill 1987).

Pectic polysaccharides. Galacturonans were primarily extracted by the chelating agent EDTA. Delignification with chlorite and subsequent alkaline extraction released pectic components containing considerable amounts of neutral sugar residues (Düsterhöft *et al* 1991). A subfraction from this extract (Table 1, 'S-X,PC') was investigated further. Its glycosidic linkage composition (Table 2, 'S-X,PC') revealed the presence of (1→5)linked arabinose residues, about 50% of which were branched at O-3, terminal arabinose and differently linked rhamnose residues.
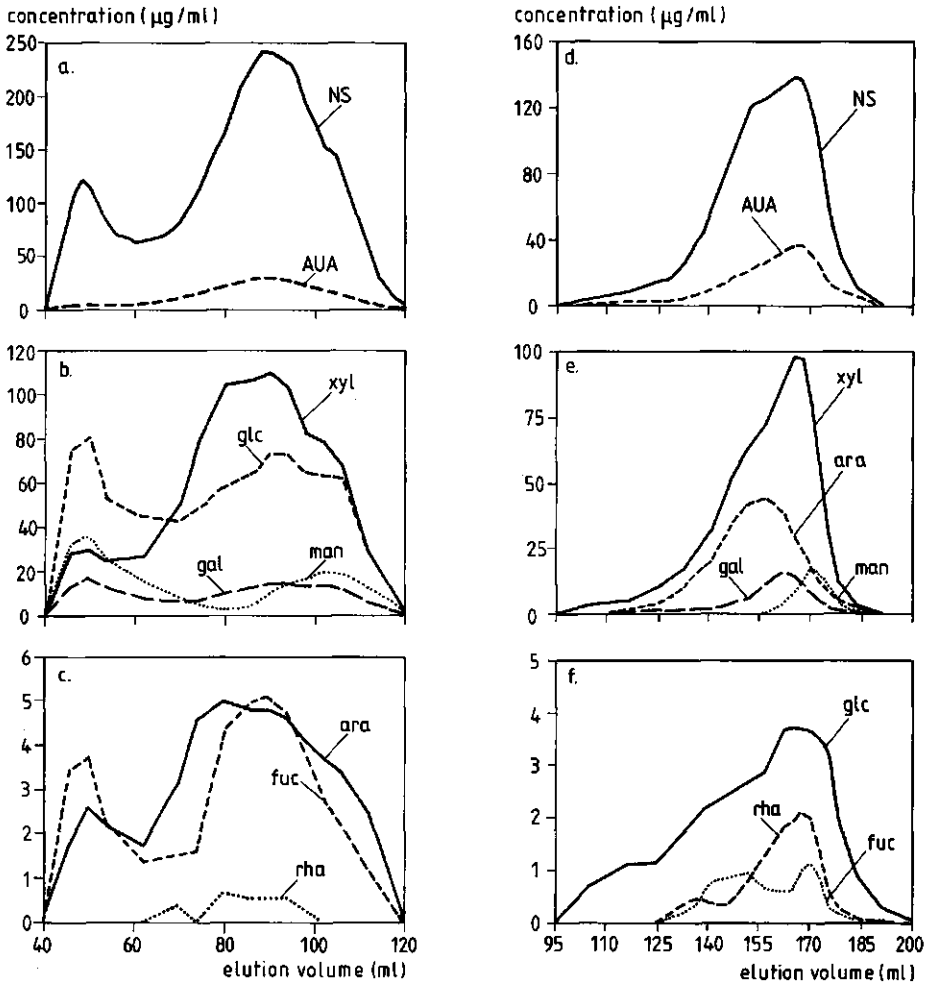
Figure 2: Size-exclusion chromatography of fractions 'S-X,'XG (left) and 'P-X,AX' (right).
(a),(d): elution profile of total neutral sugars (NS) and uronic acids (AUA);
(b),(c),(e),(f): Elution profile of constituent sugars as determined by GLC

These derivatives may be ascribed to rhamnogalacturonan structures of pectic origin. They were tightly bound in the cell wall and only released after delignification. As delignification and alkaline conditions are rather degradative especially to pectic and arabinose-containing polysaccharides (Ford 1986), no attempts were undertaken to study the pectic material in more detail. An acidic xylan was major constituent of this fraction.

Identification and structure of polysaccharides from palm-kernel meal

Mannans. Mannose is the major constituent monosaccharide in the cell wall material of palm-kernel meal (75% of NSP). Around 66% of it could be solubilised by sequential extraction with alkali and sodium chlorite and both water-soluble as well as water-insoluble polymers containing minor amounts of other hemicelluloses (1-3% arabinose, 1.5% xylose, 0.5-4% galactose, 1.5% glucose and 1.5% uronic acid (Düsterhöft *et al* 1991)) were obtained. The glycosidic linkage composition of a representative subfraction (Table 4, 'P-M') shows that the mannose residues are (1→4)linked, forming linear mannans with a very low degree of galactose substitution. Daud and Jarvis (1992) recently reported similar results from ^{13}C NMR studies of palm-kernel mannans. From the ratio of terminal to (1→4)bound residues a degree of polymerisation of 12 can be calculated. Similar structural features have been reported in other palm species (Aspinall *et al* 1953, El Khadem and Sallam 1967).

Xylans. By sequential chemical extraction, 73% of the xylose present in palm-kernel meal could be solubilised. The major part (85%) could only be released after a delignification step (Düsterhöft *et al* 1991). In subfractions obtained from this treatment different types of xylans were encountered. The water-insoluble fraction 'P-X' (Table 4,'P-X') contains a 4-*O*-methyl-glucuronoxylan with about 6% glucuronosyl-substitution at O-2 or, less frequently, at O-3. 4-*O*-methyl-glucuronic acid was the only acidic residue detected by HPAEC. From heterogeneous, water-soluble fractions, subfractions enriched in arabinose could be precipitated by moderate to high ethanol concentrations (>50%). Their carbohydrate content was low (< 50%). Detailed examination of fraction 'P-X,AX' by size-exclusion chromatography (Fig.2d-f) and glycosyl-linkage analysis (Table 4, 'P-X,AX') revealed the presence of two major polymers:
(1) an arabinoxylan, with xylose residues carrying single unit side chains of arabinose at O-2 and/or O-3 (elution volume 150 ml), (2) a (4-*O*-methyl)-glucuronoxylan, eluting near the included volume. If we assume that around one third of the xylose in this fraction derives from the arabinoxylan, the residual glucuronoxylan has a degree of substitution of at least 20%, which is significantly higher than for the water-insoluble xylan described above. Terminal galactopyranosyl groups have been reported to be present in xylan side chains from lignified tissues of monocotyledenous plants (Aspinall 1980). In xylans from soft- and hardwoods, sequences of ß-D-xyl*p*(1→3)-α-L-rha*p*(1→2)-α-D-gal*p*A(1→4)-xyl were determined at the reducing terminus (Anderson and Samuelsson 1983).

Table 4

Partially methylated alditol acetates from palm-kernel CWM and polysaccharide
fractions derived from it [mol per 100 mol]

O-methyl derivative	CWM	P-M	P-X^a	P-X,AX^a
235 ara*	0.8	0.9		13.4
23 ara	0.4			2.8
25 ara				0.7
2 ara	0.2			1.5
ara	0.3			0.8
total	1.7 (2.5)	0.9 (0.8)^b		19.2(19.7)
234 xyl	0.2			3.7
23 xyl	2.8	1.5	78.2	33.2
34 xyl	0.1		3.9	2.0
2 xyl	0.4		0.8	6.6
3 xyl	0.4		4.7	7.6
xyl	0.5			3.2
total	4.4 (4.9)	1.5 (0.7)	87.6 (92.5)	56.3(43.2)
34 rha	0.2			
total	0.2 (0.2)			(0.9)
2346 glc			7.5^c	8.2
236 glc	15.0	1.0	3.9	5.2
23 glc	0.2		0.3	
glc	0.6			
total	15.8(12.1)	1.0 (0.7)	11.7 (2.7) (4.8)^d	13.4(11.5) (8.3)
2346 gal	1.4	0.6	0.2	1.7
346 gal				2.3
236 gal	0.6			0.7
gal				1.9
total	2.0 (3.2)	0.6 (0.6)	0.2 (0.0)	6.6(11.5)
2346 man	3.8	7.2		
236 man	64.3	87.3	0.4	4.5
23 man	2.1	1.1		
man	5.8	0.3		
total	76.0(77.0)	95.9(97.2)	0.4 (0.0)	4.5 (4.8)

Abbreviations used: P=palm-kernel meal, M=mannan, X=xylan, AX= arabinoxylan
* 235 ara denotes 1,4-di-O-acetyl-2,3,5-tri-O-methylarabinitol etc.
^{a} carboxyl reduced
^{b} values in parentheses: amount of parent sugar determined in unmethylated sample
^{c} values underlined derive from (4-O-methyl)-glucuronic or from galacturonic acid
^{d} values italicised:4-O-methyl-glucose (determined in unmethylated sample)

Either of these structures might explain the small proportions of terminal and
(1→2)linked galactose residues (the latter deriving from galacturonic acid)
encountered in this fraction.

That (arabino)xylans are involved in the linkage between lignin and

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carbohydrate has been shown by various studies, for example on grasses or jute fibre (Das *et al* 1981, Neilson and Richards 1982, Ford 1986). In palm-kernel meal, we found that xylose was exclusively present in the highly lignified endocarp part of the meal (Düsterhöft *et al* 1991). This strengthens the assumption that the arabinoxylans and/or acidic xylans encountered in this extract may actually be involved in such cross-linking structures.

Glycosyl-linkage composition of intact CWM's and comparison with structural features obtained from extracted polysaccharides

Due to the insolubility of intact cell wall material in DMSO, special precautions had to be taken to enhance the completeness of methylation. The finely milled samples were methylated twice. Additionally, the reaction mixture was worked up by dialysis. In contrast to the frequently used method of extraction into an organic layer, the dialysis step ensures the complete recovery of a sample, whether it be soluble or insoluble, methylated or not. Losses might only occur by transferring the sample solution or by passage of low molecular weight compounds through the dialysis bag.

While this procedure leads to satisfying results for sunflower meal (Table 2,'CWM'), the cell wall material of the palm-kernel remained severely undermethylated (67% of the mannose residues were recovered in unmethylated form, data not shown). After partial delignification of the CWM, removing 70% of the lignin and concomitantly, 15% of NSP, the completeness of the reaction could be greatly improved (Table 4, 'CWM'). At present, we do not know how chlorite treatment renders this CWM more susceptible to methylation. Microscopic observations (Düsterhöft *et al* 1991) confirmed the lignin to be concentrated in the endocarp fraction of the CWM. No indication for uniform distribution throughout the endosperm was found. It is therefore questionable whether delignification itself or secondary effects of this treatment are responsible for the observed effect.

However, unmethylated residues of all parent sugars were still determined in both CWM's in too high proportions to be explained as double or triple substituted residues. The present results support the view that the majority of these residues derives from residual insoluble material which was left in the reaction vials. This material in consequence is not reached by the methylating reagents and remains completely unmethylated (Lomax *et al* 1983).

Despite these limiting observations, essentially all linkage-types encountered in extracted polysaccharides and discussed above were confirmed by the results

obtained from the intact cell wall material (Tables 2 and 4). This supports the validity of the structural data deduced from the study.

Relating the proportions of different derivatives determined in intact CWM with the structural features of the polysaccharides identified, an approximate estimation of the distribution of the major non-starch polysaccharides in the two meals was calculated. In sunflower meal, cellulose comprises 42% of the non-starch polysaccharides, pectic compounds (galacturonans, arabinogalactans, rhamnogalacturonans) and (4-*O*-methyl)-glucuronoxylans about 24% each. Xyloglucans and (gluco)mannans are minor constituents (4.5% and 5% of NSP respectively). In palm-kernel meal, mannans are the prevailing constituent (78% of NSP). Cellulose accounts for only 12%. (4-*O*-methyl)-glucuronoxylans and arabinoxylans represent about 3% of NSP each.

CONCLUSIONS

From the parallel study of complete cell wall materials and chemically extracted fractions the main non-starch polysaccharides of the two meals could be identified, their structure investigated and an estimation of their contents deduced. In decreasing order these are, for sunflower meal: cellulose > (4-*O*-methyl)-glucuronoxylans, pectic compounds > (gluco)mannans > fucoxyloglucans; and, for palm-kernel meal: mannans > cellulose > (4-*O*-methyl)-glucuronoxylans, arabinoxylans.

As minor constituents were not obtained in pure form, their chemical structure could not be determined unambiguously. The results presented here do not allow conclusions to be drawn on the structure of the pectic polysaccharides encountered in the raw materials. These compounds, representing a significant part of the NSP of sunflower meal, would need a less degradative approach to extraction if more detailed information about their chemical structure were to be obtained.

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PARAMETERS AFFECTING THE ENZYMIC HYDROLYSIS OF OIL-SEED MEALS, LIGNOCELLULOSIC BY-PRODUCTS OF THE FOOD INDUSTRY

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ABSTRACT

The enzymic hydrolysis of cell wall materials (CWM) from sunflower and palm-kernel meals was studied using crude enzyme preparations containing different cell wall polysaccharide degrading activities. At high enzyme concentrations and very small particle size a maximum of 30% and 53% of the non-starch polysaccharides present in sunflower and palm-kernel CWM, respectively, was solubilised after 72h. Partial delignification of the CWM's gave residues with decreased lignin content but lower or slightly enhanced degradability using sodium chlorite treatment and alkaline peroxide treatment, respectively. Although the composition of enzyme preparations was found to influence the extent of solubilisation, the type of sugars released and the site of enzymic attack within the tissues, the results of the study indicate that of all parameters investigated, accessibility of the substrates was the overriding factor limiting further hydrolysis.

INTRODUCTION

Because of their high carbohydrate content, by-products of the food and agricultural industries may be utilised as chemical feedstock, for the production of fermentable sugars or as feed compounds. Conversion or modification of such lignocellulosic substrates may be achieved by enzymatic processes (Vallander and Eriksson 1990; Fan *et al* 1982); however, unless chemical or physical pretreatments are used, saccharification yields remain uneconomically low. Conflicting evidence about the efficacy of pretreatments indicates that raw materials differ widely in susceptibility and thus results may not be generalised. The majority of numerous reports on the enzymic saccharification of lignocellulosic substrates, before and after various pretreatments, focus on cellulose hydrolysis and cellulase systems (Rivers and Emert 1988; Vallander and Eriksson 1985; Gould 1984, 1985; Fox *et al* 1989). If hemicelluloses constitute a significant part of the total carbohydrate content, as, for example, in soy-bean meal, sugar beet pulp, palm-kernel meal, bagasse (Brillouet *et al* 1988) or spent grain (Beldman *et al* 1987), polysaccharidases other than cellulases are additionally required for efficient saccharification (Beldman *et al* 1984; Coughlan *et al* 1985).

Sunflower (*Helianthus annuus* L) meal and palm-kernel (*Elaeis guineensis* Jacq) meal, which are residues obtained after the extraction of edible oils, are two examples of such by-products. They contain relatively high amounts of protein (33% in sunflower meal and 20% in palm-kernel meal) and carbohydrates (31%

non-starch polysaccharides (NSP) in sunflower and 50% in palm-kernel meal) and are highly lignified (Düsterhöft *et al* 1991). Their use as feed compound, however, is limited, because of low digestibility and palatability (Babatunde *et al* 1975; Musharaf 1991). A profound characterisation of the substrates, especially with respect to their non-starch polysaccharide composition has been reported earlier. Major polysaccharides in cell wall material from sunflower meal were cellulose, 4-*O*-methyl-glucuronoxylans and pectic compounds; in cell wall material from palm-kernel meal, mannans and cellulose were predominant (Düsterhöft *et al* 1992).

The present investigation was made to assess the efficacy of enzymic treatment of these two by-products and to elucidate major factors limiting their biodegradation. Almost complete saccharification of sunflower hulls, a constituent of the meal used in this study, was reported after autohydrolysis-explosion pretreatment and cellulase digestion (Dekker and Wallis, 1983). The pretreatment alone solubilised more than 80% of the hemicellulose and pectic compounds of the hulls. In the present study, particle-size reduction and delignification, pretreatments which were expected to improve the enzymic degradation without solubilising major parts of the substrates by themselves, were tested. The susceptibility of original and pretreated samples to enzymic hydrolysis was investigated with crude enzyme preparations containing different combinations of cellulolytic, hemicellulolytic and pectolytic activities. The data from chemical analyses were supplemented by direct visualisation of the site of enzymic attack in the tissues by transmission electron microscopy.

MATERIALS AND METHODS

Substrates

Sunflower meal and palm-kernel meal, supplied by Hendrix Nutrition Nederland B.V. (Boxmeer, The Netherlands), were ground in a laboratory mill to pass through a 0.5mm sieve (in the following referred to as 'coarse'). Cell wall materials (CWM's) were prepared by extraction with hexane and treatment with protease (Düsterhöft *et al* 1991).

Pretreatments

particle-size reduction. The CWM's were milled (3 min) to a fine powder (particle-size distribution, as assessed microscopically: $20\mu m$ - $200\mu m$) using a freezer mill, cooled by liquid nitrogen (Spex Industries Inc, Edison, USA).

delignification with sodium chlorite. CWM's (particle size < 0.5mm) were treated with $NaClO_2$/HOAc ($NaClO_2$, 2%; HOAc, 0.5%) at 70°C for 2h. The insoluble residue was recovered by filtration, washed extensively with distilled water, suspended in 80% ethanol and finally air-dried.

delignification with alkaline H_2O_2. CWM's (particle size <0.5mm) were treated with alkaline hydrogen peroxide (3% H_2O_2 (w/v), pH 11.5, room temperature) for 7h. The pH was kept constant by addition of HCl (Gould 1985). The insoluble residue was recovered as described above.

Enzymes and determination of their glycanase (glycosidase) activities

Driselase ('D', from *Irpex lacteus*, Sigma, St.Louis, MO, USA) and an experimental preparation of fungal origin ('E', Novo Industries, Bagsvaerd, Denmark) were used for the hydrolysis of sunflower CWM. Additionally Gamanase ('G', from *Aspergillus niger*, Novo Industries) was used for incubations of palm-kernel CWM. The preparations were desalted on BioGel P10 (BioRad, Richmond, CA, USA) prior to use. They were tested for activity towards CM cellulose (Type AF 0305, Enka B.V., Arnhem, The Netherlands), Avicel (Serva, Heidelberg, Germany), xylan (ex oat spelt) and polygalacturonic acid (both from Sigma), mannan (alkali-extracted from palm-kernel meal (Düsterhöft *et al* 1991) and debranched, dyed arabinan (Megazyme, North Rocks, N.S.W., Australia). Glycosidase activities were measured using the *p*-nitrophenyl derivatives of ß-D-glucopyranose, α/ß-D-galactopyranose, ß-D-xylopyranose, α-L-fucopyranose, ß-D-mannopyranose and α-L-arabinofuranose (Sigma) as substrates. For the determination of arabinanase activity, 1% substrate (w/v) was incubated with enzyme in 50mM sodium acetate buffer, pH 5.0 at 30°C (1h). The reaction was stopped by addition of ethanol. From the absorption of the supernatant at 520nm the activity was calculated by reference to a standard curve. For other glycanase assays, 0.1 % (w/v) and for glycosidase assays 0.02% (w/v) of substrate was incubated with enzyme in 50mM sodium acetate buffer, pH 5 at 30°C (1h). Enzyme activities were calculated from the increase in reducing endgroups as measured by the Nelson-Somogyi (Somogyi 1952) assay, or from the release of *p*-nitrophenol from the corresponding glycoside, measured at 405nm after raising the pH to 9.0. One Unit of activity is defined as the amount of enzyme that catalyses the release of 1μmol of reducing sugar or *p*-nitrophenol per minute.

Enzymatic degradation of CWM's

Generally, an amount of CWM (cryo-milled) corresponding to 10mg NSP

(non-starch polysaccharides) was weighed into Eppendorf cups (volume 1.5 ml, with screw caps). The incubation volume in sodium acetate buffer (0.05M, pH 5.0, containing 0.01% NaN$_3$) was 0.5ml. Enzyme addition was standardised to 50μg protein per sample. The cups were rotated 'head over tail' at 38°C for a period of 72h. To stop the reaction, they were placed in a boiling water bath for 10 minutes, then centrifuged at 18,000 x g (7min). The supernatant was removed and appropriately diluted for subsequent analyses.

Delignified CWM's were incubated at coarse particle-size (<0.5mm) and compared to the original substrate of the same particle-size.

In a separate experiment, CWM's were incubated with enzymes as above, but in 24h-intervals the supernatants were removed by centrifugation and replaced by equal amounts of buffer containing fresh enzymes. The supernatants obtained after 24h, 48h and 72h were separately treated and analysed as described above. Mean values of triplicate experiments are reported. The standard deviation of experiments conducted on different data was found to be 5% of the mean value.

Microscopic investigation of enzyme action

The ETAg-staining (enzyme-thiocarbohydrazide-silver-proteinate) was used to directly localise the site of enzymic attack in the CWM's. The procedure of Joseleau and Ruel (1985) was modified to allow for the investigation of enzyme action on whole plant particles rather than on thin-sections (Engels and Schalk, 1992). To this end, cryo-milled CWM's were first incubated with crude enzyme preparations as described above and then stained with thiocarbohydrazide and silver-proteinate. Samples then were embedded in an epoxy-resin and ultra-thin sectioned (50-70nm). Stained sections were examined using a Philips EM301 electron microscope.

General analytical methods

The neutral sugar composition of the cell wall materials (before and after pretreatments) and of digests was measured by GLC as alditol acetates. Insoluble samples were hydrolysed by 72% (w/w) sulphuric acid (1h, 30°C) followed by dilution to 1M concentration (3h, 100°C); digests were hydrolysed by 2M TFA (121°C, 1h). Derivatisation to alditol acetates was carried out according to Englyst and Cummings (1984).

The total amount of neutral sugars in digests was measured by an automated orcinol-sulfuric acid test (Tollier and Robin 1979). A reference standard solution containing arabinose and glucose in equal amounts was used for calibration in sunflower incubations and the values were corrected for uronic acid interference. In

palm-kernel incubations, mannose was used as standard.

Uronic acids in acid or enzymic hydrolysates were assayed by an automated 3-phenylphenol-test (Thibault *et al* 1979); the sulphuric acid used containing 0.0125M sodium tetraborate. Galacturonic acid was used as standard and values were corrected for any interference by neutral sugars.

The percentage solubilisation of NSP was calculated according to formula (1)

$$\text{(1)} \quad \frac{(\mu g \text{ neutral sugars} + \mu g \text{ uronic acids released}) \times 0.89\ (0.90)^{a}}{(\mu g \text{ non-starch polysaccharides in substrate})} \quad \text{X } 100$$

[a] a factor of 0.89 and 0.90 was used for sunflower meal and palm-kernel meal, respectively, to correct for uptake of water during hydrolysis of pentose and/or hexose based polysaccharides.

As the content of uronic acids in palm-kernel CWM and its digests was extremely low compared with the amounts of neutral sugars present, the standard error of the uronic acid measurements was inacceptably high. Therefore (and because of their minor importance for this raw material), uronic acids were not determined in experiments with palm-kernel CWM.

The amount of reducing sugars released during incubations of the CWM's was determined according to the method of Nelson-Somogyi. Based on the monosaccharide composition of digests from sunflower incubations a theoretical mean molecular weight of 170 was used to express the data on a weight basis.

The protein content of enzyme preparations was determined by a modification of the Lowry-method (Bensadoun and Winterstein 1976). Bovine serum albumine was used as standard.

The moisture content of substrates before and after pretreatment was measured by Karl-Fischer titration.

Lignin ('Klason' lignin) was determined gravimetrically as the residue of hydrolysis with sulphuric acid (72%, 1h, 30°C, followed by dilution to 1M, 3h, 100°C), corrected for ash. Ash was determined by incineration at 550°C, overnight.

RESULTS

Characterisation of enzyme preparations

In Table 1 the specific activities of the preparations 'E', 'D', and 'G' towards the most relevant substrates for the degradation of the CWM's are listed. 'E' and

'D' both contain a broad spectrum of cellulolytic and hemicellulolytic activities. Major difference is the level of pectolytic activity which dominates in 'E'. Preparation 'G' almost exclusively contains mannan-hydrolysing activities with very low amounts of xylanase and CMCase as side activities.

Table 1

Specific activities of enzyme preparations 'E', 'D' and 'G' [U/mg protein]

substrate	'E'	'D'	'G'
CM cellulose	0.29	1.37	0.05
Avicel	0.02	0.05	0.00
mannan	1.45	0.92	25.00
xylan	1.25	2.00	0.17
polygalacturonic acid	21.55	0.92	0.00
arabinan	0.25	0.81	0.00
pnp-ß-D-glc$p$	0.10	0.07	0.02
pnp-ß-D-gal$p$	0.17	0.05	0.01
pnp-α-D-gal$p$	0.27	0.05	0.02
pnp-ß-D-xyl$p$	0.02	0.02	0.00
pnp-α-L-fuc$p$	0.00	0.01	0.00
pnp-ß-D-man$p$	0.00	0.00	0.01
pnp-α-L-ara$f$	0.20	0.10	n.dt

Time course of incubations

The enzymatic degradation of the CWM's, measured as the solubilisation of neutral sugars and, (in sunflower CWM), uronic acids, was followed over a period of 0 - 72h. Fig.1 shows the percentage solubilisation as a function of incubation time. For both raw materials and all tested enzyme preparations curves of similar shape were obtained, with highest reaction velocity in the first 8 hours. At that time, in sunflower incubations almost 80% of the maximally obtainable solubilisation was achieved and the reaction rate levelled off. On the contrary, the saccharification of palm-kernel CWM continued with a constant, though reduced rate after the initial phase of the reaction. As the release of reducing sugars followed similar curves (data not shown), the degree of polymerisation (DP) of reaction products which can be calculated from the ratio total sugar to reducing sugar, remained fairly constant during the course of hydrolysis. Maximum values at the beginning of the reaction of DP 2-3 and minimum values of about 1.8 - 1.3 were found with different enzyme-substrate combinations.
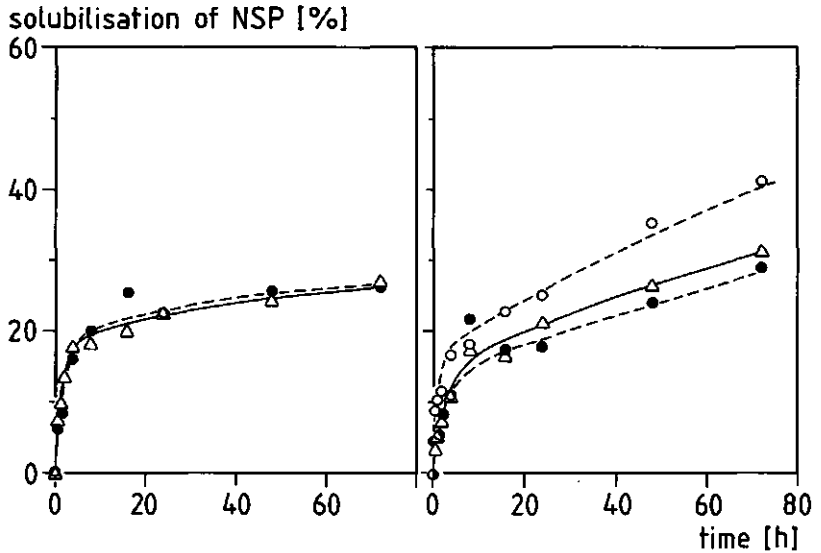
solubilisation of NSP [%]

Figure 1: Time course of enzymic degradation of sunflower CWM (left) and palm-kernel CWM (right) by different enzyme preparations. △ = prep. 'D', ● = prep. 'E', O = prep. 'G'

Effect of enzyme concentration

To study the effect of enzyme concentration on the extent of solubilisation of NSP, incubations (72h) were carried out varying the amount of added protein from 10μg to 150 μg, i.e. from 0.1% to 1.5% (w/w) on basis of NSP. Fig.2 shows that, with increasing enzyme concentration, the solubilisation could be enhanced. However, a ten-fold increase in enzyme concentration only resulted in a two-fold increase in solubilisation. The difference in the level of solubilisation achieved by the preparations under standard conditions (50 μg protein) was consolidated at higher concentrations.

Enzyme inactivation

Compared to uninterrupted incubation for 72 hours, repetitive removal of reaction products and addition of fresh buffer and enzymes in 24h-intervals did not change the total extent of solubilisation from sunflower CWM or from palm-kernel CWM treated with 'G'. Slight increases were obtained for palm-kernel CWM after incubation with 'E' and 'D', but these were not significant, taking into account the mean deviation of 5% (Fig.3).

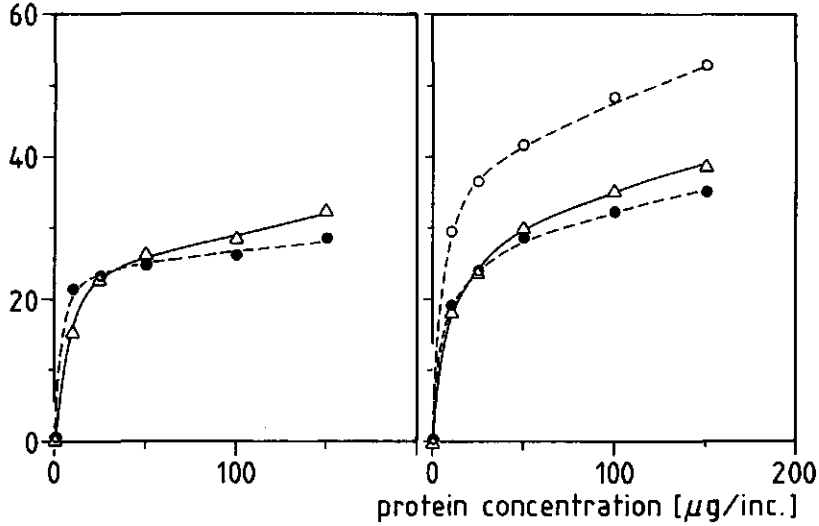**Figure 2**: Release of reaction products during enzymic hydrolysis of the CWM's as a function of enzyme concentration. Left: sunflower CWM, right: palm-kernel CWM. $\triangle$ = prep. 'D', $\bullet$ = prep. 'E', $\bigcirc$ = prep. 'G'
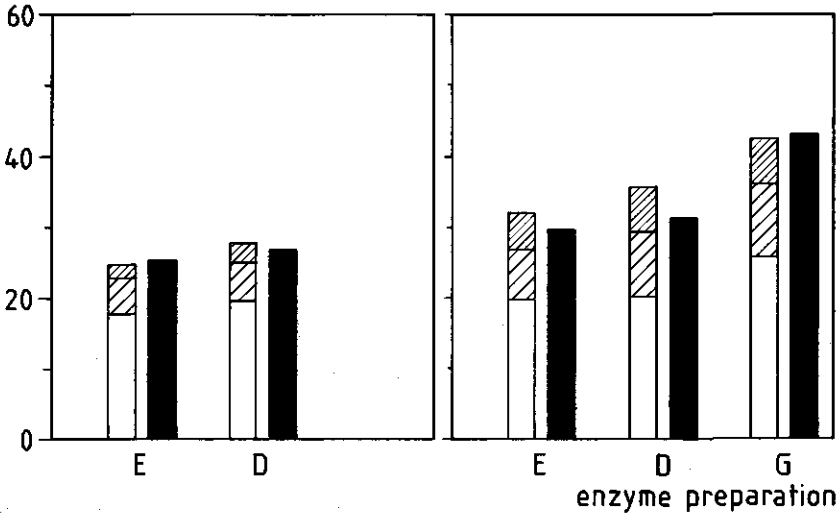

Figure 3: Comparison of direct 72h-incubation with three 24h-incubation intervals. Left: sunflower CWM, right: palm-kernel CWM. ▬ = direct 72h, ☐ = 0-24h, ▨ = 24-48h, ▨ = 48-72h

Effect of different enzyme activities on the composition of reaction products

Crude enzyme preparations with different activity spectra (Table 1) were used to evaluate the relative importance of enzyme composition on the degradation of the CWM's. The composition of reaction products released from a substrate by different preparations (Table 2) was found to be similar, with arabinose, glucose and uronic acids as the major constituents of reaction products from sunflower CWM and mannose from palm-kernel CWM. Distinct differences in the proportion of some components, however, were detected. Thus, for sunflower incubations, the release of uronic acids and glucose differed for preparations 'E' and 'D', possibly reflecting the high amounts of pectolytic activity in 'E' and cellulolytic activity in 'D'. When palm-kernel CWM was used as substrate, 'E' and 'D', in contrast to 'G' containing cellulolytic activity, released larger proportions of glucose.

Table 2

Monomeric sugar composition (rel. mol-%) of sunflower and palm-kernel CWM and of digests obtained from incubations with the crude enzyme preparations 'E', 'D' and 'G'

monosaccharide	sunflower			palm-kernel			
	CWM	'E'	'D'	CWM	'E'	'D'	' G '
rha	0.8	4.4	2.9	0.0	0.0	0.3	0.0
fuc	0.1	tr.	tr.	0.0	0.0	0.0	0.0
ara	11.5	23.2	21.9	2.8	1.7	1.7	1.0
xyl	23.5	7.9	8.7	5.9	0.5	0.6	0.9
man	3.8	7.5	8.9	73.4	83.4	81.7	91.0
gal	2.8	5.1	4.7	3.2	3.0	4.0	3.6
glc	43.2	26.3	32.0	13.2	9.2	9.4	1.8
aua	14.2	25.7	20.8	1.5	2.2	2.3	1.6
percentage solubilisation[a]		26.2	27.2		29.0	31.5	41.4

[a] obtained under standard incubation conditions (cryo-milled, $50\mu g$ protein added); determined spectrophotometrically and calculated according to formula (1)

Influence of particle size

Compared with the standard (cryo-milled) samples, markedly less NSP was solubilised when the CWM's were incubated as coarse particles (<0.5mm). The effect was more pronounced in palm-kernel meal (Table 3, about 50% less solubilisation) than in sunflower meal (20% to 40% less solubilisation). Prolonged cryo-milling (10 min) however, only achieved marginal further improvements.

Influence of lignification

Different methods for partial removal of lignin were tested and the susceptibility of the pretreated materials to enzymic degradation was re-evaluated. Chlorite treatment removed about 40% of lignin from sunflower CWM and 70% from palm-kernel CWM (Table 4). It was more effective than alkaline hydrogen peroxide, which removed 30% and 45% from sunflower and palm-kernel CWM's, respectively. Regardless of the treatment used, palm-kernel meal was more susceptible to delignification than sunflower meal. Although the methods chosen were designed to remove as little as possible non-starch polysaccharides by themselves, 15 - 20% NSP were solubilised by the treatments. Since the sugar composition of original and pretreated substrates was found to be very similar, it was concluded that, except some uronide-containing material from sunflower CWM, no other polysaccharides were selectively removed.

Table 3

Influence of particle-size, enzyme concentration and partial delignification on the enzymic hydrolysis of sunflower and palm-kernel CWM's (expressed as percentage of solubilisation at standard incubation conditions[a]) with enzyme preparations 'E', 'D' and 'G'

| treatment | sunflower | | palm-kernel | | |
	'E'	'D'	'E'	'D'	'G'
standard[a]	100	100	100	100	100
particle size <0.5mm	81	62	49	51	54
enzyme concentration					
x 0.5[b]	92	86	83	79	88
x 2	106	109	113	117	116
x 3	115	123	124	129	127
delignification					
a) chlorite	89 (136)[c]	105 (164)	72 (164)	84 (146)	58 (113)
b) alk.H$_2$O$_2$	95 (151)	168 (225)	117 (244)	114 (200)	96 (170)

[a] standard incubation conditions: cryo-milled, 50µg protein added
[b] 'x 0.5' means: half the amount of enzyme at standard conditions (= 25µg), etc.,
[c] data in parentheses: amount of neutral sugars released by the pretreatment itself added to the value obtained from enzymatic digestion of pretreated residue

The chlorite-pretreated substrates were less susceptible to enzymic hydrolysis than the original substrates, except for the combination sunflower CWM/'D' (Table 3). Although less lignin (and more NSP) was removed by treatment with alkaline peroxide, the residues exhibited equal or slightly enhanced degradability, the amounts being dependent on the enzyme-substrate combination used. If it is assumed that the part of NSP solubilised by the pretreatment itself would be enzyme-degradable, correction for this amount would result in relative improvements of solubilisation of 13% to 144%, again depending on the substrate-enzyme combination (Table 3, data in parentheses).

Table 4

Chemical composition and yield of cell wall materials from
sunflower and palm-kernel meals, before and after
different delignification treatments

substrate	NSP %	lignin %	yield %
sunflower			
CWM	62.9	23.4	100.0
CWM_c^a	70.6	16.9	79.9
CWM_{H2O2}^b	63.0	19.4	81.5
palm-kernel			
CWM	80.3	18.6	100.0
CWM_c	91.8	6.8	72.4
CWM_{H2O2}	85.0	13.9	74.0

[a] C denotes: delignified by $NaClO_2/HOAc$
[b] H2O2 denotes: delignified by alkaline peroxide

Microscopic observation of enzyme action

Specific staining of the reducing end-groups created by enzymic hydrolysis of glycosidic linkages allows the visualisation of the site of action in an insoluble substrate. Control incubations were carried out with denaturated enzyme and the thin-sections examined were almost devoid of staining. As observed at low magnification (cellular level), staining in seed-hull tissue from sunflower CWM was essentially restricted to 1 - 2 cell layers at the surface of small particles (Fig. 4). In palm-kernel CWM, the cell content (C) of peripherically located endosperm cells was generally extensively stained (e.g. Fig.5(c)). At higher magnification (cell

Figure 4: Sunflower CWM treated with preparation 'E' (ETAg-staining). Seed hull tissue showing intensive staining in cell walls (CW) of cells located at the outer border of the particle and neighbouring cells almost completely unstained (Engels and Schalk 1992, with permission)

wall level), the effect of varying enzyme composition could be demonstrated with palm-kernel incubations. After treatment with 'G' (Fig. 5(a)), enzymic action was restricted to the cell content and the border area between the cell contents and secondary cell wall (SW). After treatment with 'E' and 'D', hydrolytic action was also indicated in different layers of the primary and secondary cell walls. The primary wall- and middle lamella-regions (PM) were mainly stained in endosperm cell walls treated with 'E' (Fig. 5(b)); additional staining was observed in the secondary wall after treatment with 'D' (Fig. 5(c)).

DISCUSSION

The efficacy of the enzymatic saccharification of lignocellulosic raw materials depends on a variety of factors. The physical structure and chemical composition of the substrate, the composition of enzyme preparations used for treatment and the process conditions applied influence the yield of sugars obtained. In the present investigation a number of these parameters were studied for the treatment of two
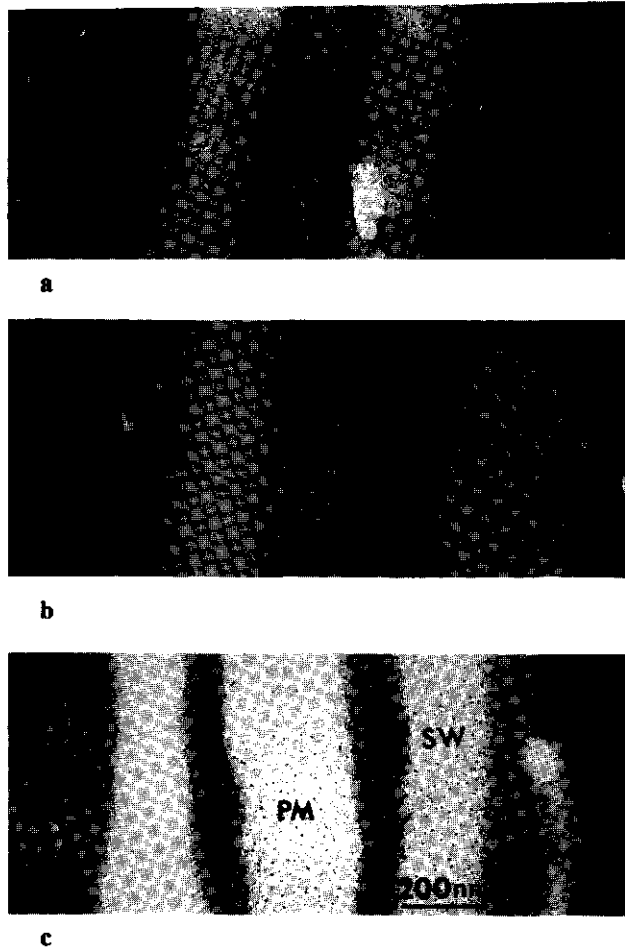
Figure 5: Endosperm tissue of palm-kernel CWM treated with different enzyme preparations (ETAg-staining).
(a) After treatment with preparation 'G', silver grains are located at the border of the cell content (C) and the secondary wall (SW). Some silver grains are also perceivable in the cell contents (→).
(b) Treatment with preparation 'E'. Silver deposition is found in the primary wall/middle lamella regions (PM).
(c) Treatment with preparation 'D'. Silver staining is present in cell content (C), secondary wall (SW) and primary wall/middle lamella (PM). The intensity of staining in the neighbouring cell/cell wall (left side) is much lower.

by-products, which, in addition to cellulose and lignin, contain considerable amounts of hemicellulose.

Raw materials from mono- and dicotyledenous plants were chosen as

substrates, since the two groups are known to have different cell wall composition and may therefore respond differently to enzymic hydrolysis or pretreatments. The preparation of cell wall materials (CWM's) removed protein and small amounts of water-soluble compounds of expected high enzymic degradability and left a residue of structural polysaccharides and lignin with increased resistance.

Under standard conditions (cryo-milled substrate, $50\mu g$ protein) solubilisation of sunflower CWM was restricted to 20-30% (on basis of NSP). In palm-kernel meal, 30-40% was solubilised during 72 hours. The time-course study (Fig.1) indicated that further solubilisation of the palm-kernel substrate could be achieved with prolonged incubation. Additional yield could also be obtained by increasing the enzyme concentration (Fig. 2 and Table 3); but the effect was not proportional to the amount of enzyme applied.

These degradation features may be caused by numerous factors. Provided that all enzyme activities needed for the breakdown of a substrate are present, the low additional effect obtained with increasing enzyme concentrations may indicate that the accessibility of the substrate is limiting hydrolysis. Reduction of particle-size, thereby increasing the surface area available for enzymes to attack, did enhance solubilisation which is consistent with improving accessibility. Milling to a fine particle-size as in the present study, however, is uneconomical on a technical scale and would cause difficulties for animal feeding purposes.

It is generally accepted that lignin represents a major constraint to enzymic degradation (Ford 1978; Morrison 1988, 1991). As both meals investigated in this study are highly lignified, their accessibility might be hindered by lignin and low molecular-weight phenolic compounds forming covalent linkages with sugar residues. With proceeding enzymic degradation of the cell walls, these structures might exert a 'shielding' effect and finally prevent further hydrolysis (Chesson 1993). Lignin may also adversely affect enzymatic hydrolysis by non-specific adsorption of enzymes. This effect has been studied with cellulases (Chernoglazov et al 1988; Ooshima et al 1990) and xylanases (Senior et al 1991). Studies on different raw materials unanimously showed that partial removal (about 50%) of lignin was sufficient to enhance enzymic hydrolysis (Gould 1984; Tanaka et al 1985).

In the present study, up to 40% - 70% of the lignin could be removed from sunflower and palm-kernel meal, but the susceptibility to enzymic hydrolysis decreased for chlorite-treated residues; equal or slightly improved results were obtained for alkaline peroxide-treated residues. Gould (1985) has reported that the efficacy of alkaline peroxide treatment was largely dependent on the type of raw material; dicotyledenous materials being less susceptible than monocotyledenous,

especially grasses. This tendency was not confirmed in the present study and the adverse effect of chlorite treatment is in contrast to numerous reports of its effect on other raw materials. As it is questionable, whether the part of NSP solubilised by the pretreatment itself is enzyme-degradable, -if so, it could be added to the value obtained from enzymic hydrolysis-, the results are interpreted to indicate that the lignification of the raw materials tested in the present study is not directly limiting enzymic action. Al Katrib *et al* (1988), studying the enzymic saccharification of wheat straw before and after various pretreatments, drew similar conclusions. Secondary effects, like increased hydration of the substrate (Gould 1985) might bring about the positive effects of alkaline peroxide treatment.

The microscopic observation of a surface-restricted attack in the (relatively) largest particles (0.2mm ϕ) of the cryo-milled samples supports the chemical results pointing to a limited substrate accessibility. However, as the microscopic technique applied only reflects the situation in the residual material, no information could be obtained about the solubilised fraction. Micrographs taken at earlier stages of incubation would need to be compared for confirmation of the results.

In addition to the above mentioned substrate-dependent factors, a number of enzyme-dependent factors may contribute to the limited degradation. Enzymes may become inactivated upon prolonged exposure to elevated temperatures or inhibition by reaction products may occur. Thus, removal of reaction products and addition of fresh amounts of enzymes at intervals has been reported to enhance the saccharification of wheat straw (Vallander and Eriksson 1985). Although the similar experiments conducted in the present study cannot unambiguously exclude these effects, the results (Fig.3) indicate that, if present at all, they are superimposed by other, dominating factors. Although actually thrice the standard amount of enzyme was added by exchange of buffer/enzyme in 24 hour intervals, addition of 150 μg protein directly at the beginning of the reaction led to higher solubilisation (compare Fig.s 2 and 3). The initial adsorption of enzymes to the substrates therefore seems to determine the overall extent of solubilisation.

The composition of enzyme preparations influenced the extent of solubilisation, the type of reaction products released (Table 2) and, as observed microscopically in palm-kernel CWM, the site of enzymic action (Fig.5). Absence of staining may not only indicate the absence of a substrate in the unstained part, but also the lack of activities able to attack the substrate or its inaccessibility in this region. Previous studies indicated that mannans in palm-kernel CWM occurred both in cell walls and cell content (Düsterhöft *et al* 1991) and in related palm species they were located in secondary thickened cell walls (Matheson 1990). Enzymes present in 'G' obviously are unable to attack any substrates in the cell

walls, while those deriving from 'E' and 'D' can. As the major difference in composition of the solubilised material from palm-kernel CWM was the proportion of glucose, and as cellulose is the only glucose-containing polysaccharide detected in significant amounts in this meal (Düsterhöft *et al* 1992), staining within the cell walls in samples treated with 'E' and 'D' is likely to be caused by the cellulolytic activities of these two preparations. Variations of cellulose microstructure in different cell wall regions and differences in the cellulolytic enzymes of 'E' and 'D' might cause these observations. While this emphasizes the importance of enzyme composition for an optimisation of the treatment, it becomes obvious that fractionation of the preparations or supplementation with purified enzymes would be necessary in order to determine the individual effect of different activities.

A summary of the experiments shows that the results vary considerably according to the substrates tested. Clearly, the two raw materials in the present investigation respond differently to the pretreatments applied and to enzymic degradation in general, sunflower CWM exhibiting highest resistance. The fact that these oil seed meals have already undergone severe chemical and physical treatments during their processing (solvent extraction, pelleting, drying) possibly contributes to their resistance compared with other by-products described in the literature. The results suggest that the accessibility of the substrates, influenced by parameters other than lignification, is the major limiting factor in the enzymic degradation of the CWM's. Further research, directed towards an understanding of the role of different enzyme activities, may enable the optimisation of enzyme composition, in order to influence the extent and type of reaction products released.

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CHAPTER 5

SOLUBILISATION OF NON-STARCH POLYSACCHARIDES FROM OIL-SEED MEALS BY POLYSACCHARIDE-DEGRADING ENZYMES

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ABSTRACT

The solubilisation of non-starch polysaccharides from cell wall materials of palm-kernel and sunflower meals was studied using multi-component polysaccharidase preparations or fractions thereof. In sunflower CWM, the degradability of pectic compounds and mannose-containing polysaccharides was highest, as estimated from 40% - 70% solubilisation of their constituent monosaccharides arabinose, galactose, galacturonic acid, rhamnose and mannose. Dimers and monomers were produced as major neutral and acidic end products. Mannans in palm-kernel CWM were hydrolysed to about 20-50%, depending on enzyme composition, with the monomer and dimer of mannose as major end products. In both materials, xylans or other xylose-containing polymers were most resistant to enzymic hydrolysis. The results indicate the preferential solubilisation of parenchyma tissues from the seed in sunflower meal and the endosperm in palm-kernel meal, and the resistance of sunflower hull and palm-kernel endocarp to enzymic attack. The concentrations of notably cellulolytic, mannanolytic and arabinolytic activities clearly determined the extent to which the corresponding constituents were solubilised.

INTRODUCTION

The (partial) hydrolysis of structural polysaccharides from plant cell walls by polysaccharidases is a target in various areas of food, feed or chemical industries: For the production of fermentable sugars, which in turn can be used as energy source or chemical feedstock (Magee and Kosaric 1985; Vallander and Eriksson 1990), to enhance the utilisation of raw materials as animal feed compounds in monogastric diets (Chesson 1987; Petterson et al 1991), to modify the chemical structure of polysaccharides and influence their functional properties (McCleary 1986) or to improve the processing of foods, like the extractability of oils (Sosulski et al 1988), the pressability of fruits, filterability of beverages (Wucherpfennig et al 1991, Oksanen et al 1985) and the maceration of tissues (Chesson 1980).

To our knowledge, in only few investigations commercial multi-component polysaccharidase preparations have been used to study the hydrolysis of various plant materials of nutritional importance (Massiot et al 1989, Sinoquet 1989, Thibault and Rouau 1990, Mulder et al 1991). In all these studies the substrates derived from low- or non-lignified parenchyma tissues.

By-products from the food and agricultural industries often contain

considerable amounts of non-starch polysaccharides, the utilisation of which may likewise be enhanced by treatment with polysaccharidases. Two such by-products, sunflower (*Helianthus annuus* L) meal and palm-kernel (*Elaeis guineensis* Jacq) meal, used to limiting extents in animal diets, have been subject of previous investigations concerning their non-starch polysaccharide composition (Düsterhöft *et al* 1992) and their degradability by cellulolytic/hemicellulolytic enzyme preparations (Düsterhöft *et al* 1993a). While it was found that the solubilisation of the lignified cell wall materials of these meals, consisting primarily of cellulose, glucuronoxylans, pectic compounds (sunflower) and mannan (palm-kernel), was mainly governed by their limited accessibility to the enzymes, the results also indicated, that the composition of enzyme activities did influence the extent and the site of hydrolysis, as well as the composition of solubilised reaction products.

The present study was undertaken to characterise these products in detail and to evaluate the effect different enzyme activities had on their formation and composition. To this end, multi-component enzyme preparations were fractionated and the products obtained from hydrolysis with these fractions were isolated and partially characterised. The results will be discussed with regard to substrate morphology, energy gain and physiological implications.

MATERIALS AND METHODS

Substrates

Cell wall materials (CWM's) were prepared from sunflower (*Helianthus annuus* L) and palm-kernel (*Elaeis guineensis* Jacq) meals as described previously (Düsterhöft *et al* 1991). Their non-starch polysaccharide (NSP) composition (given as relative mol-%) was rhamnose 1% (0%), fucose 0.4% (0%), arabinose 10.5% (2.5%), xylose 23.0% (4.8%), mannose 3.6% (75.6%), galactose 2.5% (3.2%), glucose 45.6% (11.9%) and uronic acids 13.2% (1.8%) for sunflower and palmkernel (data in parentheses) CWM. The CWM's were milled to a fine powder (particle size distribution $20\mu m$ - $200\mu m$) using a freezer mill (Spex Industries Inc, Edison, USA).

Enzymes

Driselase ('D', from *Irpex lacteus*) was purchased from Sigma (St.Louis, MO, USA); an experimental preparation from fungal origin, designated 'E', was kindly provided by NOVO Nordisk (Bagsvaerd, Denmark), Gamanase ('G', from *Aspergillus niger*) was purchased from the latter manufacturer. For experiments on

sunflower CWM, 'D' and 'E', on palm-kernel CWM additionally 'G', or fractions of these preparations were used.

Fractionation of enzyme preparations

Preparations obtained in powder form were dissolved/suspended in sodium acetate buffer (10mM, pH 5.0), remaining solids were removed by centrifugation (10min, 10,000 x g); liquid preparations were diluted if their viscosity was very high or otherwise applied directly on a BioGel P10 column (BioRad Laboratories, Richmond, LA, USA, column 85cm x 2.8cm) to remove low molecular weight additives. In the following these 'desalted' preparations are referred to as 'crude'. Subsequently they were fractionated by anion-exchange chromatography on DEAE BioGel A (BioRad, column 15cm x 3cm, equilibrated with 10mM sodium acetate buffer, pH 5.0). Non-bound fractions eluted with the starting buffer, bound fractions were eluted with a peak-controlled NaCl gradient (10mM sodium acetate buffer, pH 5.0 to 50mM sodium acetate buffer, pH 5.0, containing 0.5M NaCl). Eluted fractions were assayed for different glycanase activities and pooled according to the elution pattern of activities. This resulted in fractions enriched in certain activities. Those fractions which, after incubation with the CWM's, achieved the highest solubilisation of NSP, were selected and used for further experiments in the present study ('E1', 'E2', 'D1', 'D3', 'G3').

Measurement of glycanase and glycosidase activity

Crude preparations and fractions were tested for activity towards CM cellulose (Type AF 0305, Enka B.V., Arnhem, The Netherlands), Avicel (Serva, Heidelberg, FRG), xylan (ex oat spelt) and polygalacturonic acid (both from Sigma), mannan (alkali-extracted from palm-kernel meal (Düsterhöft *et al* 1991) and linear, dyed arabinan (Megazyme, North Rocks, N.S.W., Australia) and towards *p*-nitrophenyl derivatives of ß-D-glucopyranose, α/ß-D-galactopyranose, ß-D-xylopyranose, α-L-fucopyranose, ß-D-mannopyranose and α-L-arabinofuranose (Sigma). For the determination of arabinanase activity, substrate (10g litre^{-1}) was incubated with enzyme in 150mM sodium acetate buffer, pH 5.0 at 30°C (1h). The reaction was stopped by addition of ethanol. From the absorption of the supernatant at 520nm, the activity was calculated by reference to a standard curve. For other glycanase assays, 1g litre^{-1} and for glycosidase assays 0.2g litre^{-1} substrate was incubated with enzyme in 50mM sodium acetate buffer, pH 5.0 at 30°C (1h). Enzyme activities were calculated from the increase in reducing end-groups as measured by the Nelson-Somogyi (Somogyi 1952) assay, or from the release of *p*-nitrophenol from the corresponding glycoside, measured at 405nm. One Unit of

activity is defined as the amount of enzyme that catalyses the release of 1μmol of reducing sugar or p-nitrophenol per minute.

Enzymatic hydrolysis of CWM's

An amount of CWM corresponding to 10 mg NSP was weighed into Eppendorf-vials and suspended in sodium acetate buffer (0.05M, pH 5.0, containing 0.1g litre^{-1} NaN$_3$, final volume 0.5ml). Enzyme addition was standardised on amount of protein: 50μg was added per incubation with crude preparations; fractions were added in amounts proportional to their concentration in the parent preparations (e.g. 'D3' comprises 74% of total protein recovered after anion-exchange chromatography of crude preparation 'D'; based on an addition of 50μg protein of the 'crude' preparation, 37μg of fraction 'D3' was added). The vials were incubated for 72h, at 38°C, rotating 'head over tail'. To stop the reaction, they were placed in a boiling water bath (10min). The digests were collected after centrifugation at 18,000 x g (7min). Residues were washed twice with buffer and water and finally freeze dried.

Fractionation of reaction products

Digests from scaled-up incubations of the CWM's with fractions 'E1', 'E2', 'D1', 'D3' and 'G3' were concentrated to about 10mg NSP ml^{-1}, applied to a BioGel P2 column (BioRad, 200-400 mesh, 100cm x 1.6cm) and eluted with water (60°C, containing 0.1g litre^{-1} NaN$_3$) at a flow rate of 14 ml h^{-1}. Calibration was performed using dextran 150, raffinose, maltose and glucose. The eluates were monitored for total sugar (orcinol-sulphuric acid-test (Tollier and Robin 1979) and uronic acids (3-phenylphenol-test (Thibault 1979). The sulfuric acid used in the latter test contained 0.0125M sodium tetraborate. With this concentration, glucuronic acid and galacturonic acid (used for calibration) gave equal responses. Neutral sugar contents were corrected for the interference of uronic acids and vice versa. Major peaks were pooled and concentrated in a stream of air.

HPLC of reaction products

Digests and oligomer-fractions isolated from size-exclusion chromatography were analysed by high-performance anion-exchange chromatography (HPAEC) on a DIONEX (Sunnyvale, CA, USA) system, composed of an Eluant Degas Module, a Gradient Pump Module (GMP2) and an Electrochemical Detector (PED). Separation of either neutral or acidic reaction products was achieved on a PA1 column (250mm x 4mm), equipped with a CarboPac PA guard column (25mm x 3mm), running linear gradients of eluant A (100mM NaOH) and eluant B (1M

75

sodium acetate/100mM NaOH). Elution was monitored by triple-pulsed amperometric detection (PAD) using a gold electrode. Digests of xylan, mannan, arabinan and pectin were used to determine the retention times of monomers and oligomer-series of increasing degree of polymerisation (DP).

General Analytical methods

The amounts of neutral sugars in CWM's, residues, digests and oligomer-pools were measured by GLC as alditol acetates. Insoluble samples were hydrolysed by sulfuric acid (72%, 1h, 30°C), followed by dilution to 1M concentration and further hydrolysis for 3h at 100°C (Saeman 1954). Other samples were hydrolysed by 2M TFA (121°C, 1h). Derivatisation to alditol acetates was performed according to Englyst and Cummings (1984).
The protein content of enzyme preparations and subfractions was determined by a modification of the Lowry-method (Bensadoun and Winterstein, 1976) with bovine serum albumine as standard.

RESULTS

Polysaccharidase activities in crude preparations and fractions

The crude preparations 'E' and 'D' both contained a wide range of polysaccharidase activities. They significantly differed in the level of pectolytic and cellulolytic activities, prevailing in 'E' and 'D', respectively. The effect of these multi-component preparations is likely to be the result of the cooperative action of their various constituent enzymes. In order to distinguish between groups of enzymic activities, like 'pectolytic', 'xylanolytic', 'mannanolytic' etc.. and to assign an observed effect to a particular activity, fractionation was attempted by anion-exchange chromatography. An example of this fractionation (crude preparation 'E') is shown in Fig.1. Of the multiple fractions obtained only those which still achieved considerable solubilisation of the CWM's are reported here: These were ; 'E1' and 'E2', 'D1' and 'D3' for sunflower and 'E2', 'D3' and 'G3' for palm-kernel incubations. Although, with few exceptions, none of the fractions obtained was totally devoid of any of the other activities, an enrichment in certain activities had taken place (Table 1). Thus, 'E1', in comparison to 'E2', contains a surplus of CMCase and xylanase activity, while mannanase and polygalacturonase activities were mainly recovered in 'E2'. The glycosidase activities were similarly distributed between both fractions. For 'D', polygalacturonase activity (higher in
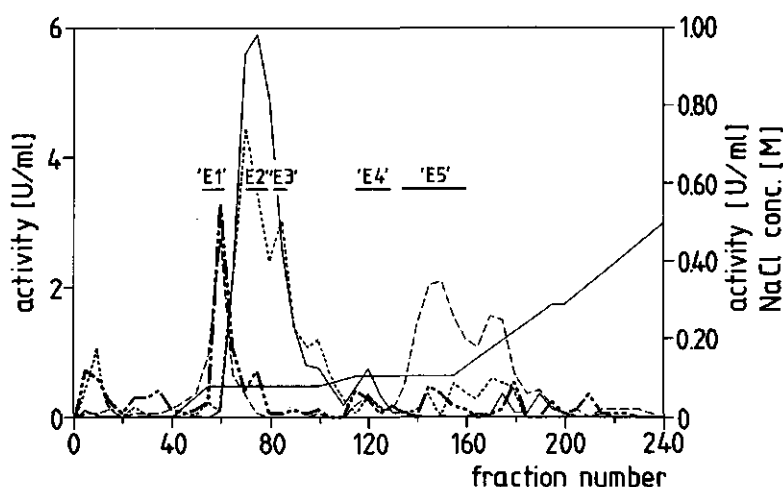
Figure 1: Fractionation of crude preparation 'E' by anion-exchange chromatography. Elution pattern of activities and pooled fractions. Plotted against left y-axis: —— polygalacturonase activity, plotted against right y-axis:---xylanase-,－－CMCase-, ----mannanase-activity [U/ml],—— NaCl gradient [M]

Table 1

Glycanase and glycosidase activities of crude enzyme preparations 'E', 'D', 'G' and their most relevant fractions (expressed as Units/mg protein)

substrate	'E'	'E1'	'E2'	'D'	'D1'	'D3'	'G'	'G3'
CMC	0.29	0.76	0.18	1.37	0.17	0.19	0.05	0.04
Avicel	0.02	0.02	0.01	0.05	0.17	0.04	0.00	n.dt
mannan	1.45	0.09	1.65	0.92	0.00	0.36	25.00	33.33
xylan	1.25	0.59	0.06	2.00	4.92	0.85	0.17	0.04
polygal. acid	21.55	0.22	31.55	0.92	5.16	0.18	0.00	n.dt
arabinan	0.25	0.05	0.10	0.81	3.33	0.12	0.00	0.00
pnp-ß-D-glc$p$	0.10	0.37	0.05	0.07	0.25	0.03	0.02	0.00
pnp-ß-D-gal$p$	0.17	0.31	0.03	0.05	0.00	0.09	0.01	0.00
pnp-α-D-gal$p$	0.27	0.02	0.27	0.05	0.00	0.08	0.02	0.00
pnp-ß-D-xyl$p$	0.02	0.02	0.00	0.02	0.00	0.00	0.00	n.dt
pnp-α-L-fuc$p$	0.00	n.dt	n.dt	0.01	n.dt	n.dt	0.00	n.dt
pnp-ß-D-man$p$	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
pnp-α-L-ara$f$	0.20	0.00	0.17	0.10	0.17	0.10	n.dt.	n.dt
% of protein[a]	100	18	63	100	11	74	100	30

[a] Protein present in enzyme fraction expressed as percentage of total protein recovered in all fractions

'D1'), mannanase activity (exclusively present in 'D3') and arabinanase (prevailing in 'D1') were "separated". By anion-exchange chromatography of the mannanase-preparation 'G', the levels of 'contaminating' side-activities in resulting fraction 'G3' (xylanase, CMCase, ß-mannosidase and α-galactosidase) were reduced.

Solubilisation of non-starch polysaccharides

The solubilisation of NSP achieved by the crude enzyme preparations and their most relevant fractions amounted to 14 -30% for sunflower and 19-44% for palm-kernel CWM (Table 2). In general, the fractions released less NSP than did the corresponding crude preparations. As an exception, 'D3' solubilised even higher amounts from palm-kernel CWM than the crude preparation. Glucose, uronic acids and arabinose were the major polysaccharide constituents solubilised from sunflower CWM by any preparation or enzyme fraction. The proportions released by the various fractions differed markedly, reflecting their prevailing activities. This difference is even more pronounced when regarding the relative solubilisation of each individual sugar (Table 2, data in parentheses). Comparing the effect of crude preparation 'D' with its fractions demonstrates very well the absence of mannanase activity in fraction 'D1'. Similar features can be deduced for the other sugar residues.

A very uniform composition of reaction products was observed in palm-kernel digests, with mannose as major constituent and small but similar proportions of other sugars released by any preparation or fraction. The presence of cellulose-degrading activities in 'E' and 'D' is reflected by the higher amounts of glucose released. Although xylanase activity was higher in these preparations as well, this did not influence the proportion of xylose released.

For both substrates, xylose and glucose were the least solubilised compounds (the data for rhamnose in palm-kernel, being very low, are subject to high standard errors and are therefore not taken into consideration). The resistance of these two polysaccharide constituents to enzymic attack was confirmed by their accumulation in the residues (data not shown). In contrast, the solubilisation of uronic acids, galactose, arabinose and mannose (in sunflower CWM also rhamnose) was better, the highest values being obtained in sunflower CWM.

Partial characterisation of reaction products

The digests obtained from incubation with the enzyme fractions, known to contain mono- to small oligomeric material (Düsterhöft et al 1993a), were fractionated by chromatography on BioGel P2. In Fig.2 typical chromatograms are shown. Further resolution of the various components present in the isolated P2-

Table 2

Amount[a] and composition of NSP solubilised from CWM's by crude enzyme preparations and fractions and relative amounts solubilised per monosaccharide (data in parentheses)[b]

enzyme	solubilisation[a]	rha	ara	xyl	man	gal	glc	aua
					mol per 100 mol			
				sunflower CWM				
'E'	29	4 (100)	23 (58)	8 (9)	7 (71)	5 (56)	26 (15)	26 (51)
'E1'	14	5 (58)	33 (41)	8 (5)	7 (24)	6 (32)	14 (4)	28 (27)
'E2'	18	5 (76)	27 (43)	6 (4)	11 (52)	6 (39)	12 (4)	33 (41)
'D'	30	3 (73)	22 (59)	9 (11)	9 (70)	5 (56)	32 (20)	21 (45)
'D1'	19	5 (75)	28 (47)	10 (8)	2 (11)	6 (45)	20 (8)	30 (40)
'D3'	20	3 (47)	16 (29)	7 (6)	11 (57)	5 (36)	35 (14)	23 (32)
				palm-kernel CWM				
'E'	29	0	2 (23)	1 (4)	83 (30)	3 (25)	9 (21)	2 (31)
'E2'	21	0	2 (18)	0 (0)	85 (22)	3 (16)	7 (11)	3 (37)
'D'	34	0	2 (28)	1 (5)	82 (35)	4 (41)	10 (25)	2 (37)
'D3'	38	0	2 (24)	0 (0)	83 (39)	4 (42)	10 (31)	2 (31)
'G'	44	0	1 (22)	1 (10)	91 (50)	4 (48)	2 (6)	2 (32)
'G3'	19	0	1 (6)	0 (0)	91 (21)	3 (15)	4 (5)	2 (16)

[a] Amount of total NSP in the substrate which is solubilised by enzyme fractions [g per 100g], corrected for blank.
[b] Amount of initial content of a monosaccharide in the substrate which is solubilised by the enzyme fraction [g per 100g], corrected for blank.
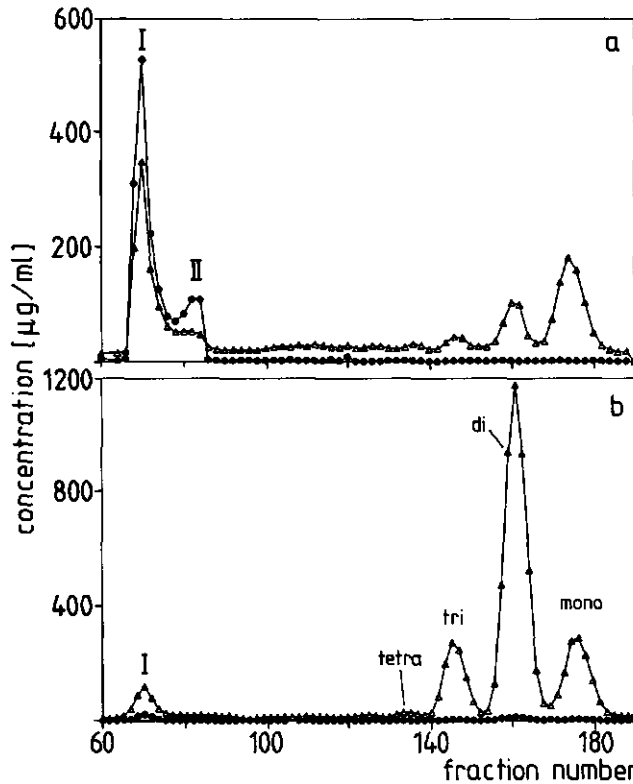
Figure 2: Size-exclusion chromatography (BioGel P2) of reaction products from enzymic hydrolysis of CWM's. (a) digest from sunflower CWM, incubated with 'E2', (b) digest from palm-kernel CWM, incubated with 'D3'. △ neutral sugar, ● uronic acids

pools and in the original digests was achieved by high-performance anion-exchange chromatography (Fig.s 3 and 4). The elution conditions applied were chosen to achieve optimal separation of neutral oligomers, at the cost of resolution of monomers. The elution order of oligosaccharides with equal DP is dependent on their sugar composition and does, in the case of mixed oligomers, not necessarily follow a strict order of increasing molecular weight (Lee 1990).

Oligomer references available from our laboratory eluted in the following order of increasing retention times: mannobiose < mannotriose < xylobiose < cellobiose < xylotriose << arabinobiose < galacturonic acid < arabinotriose < arabinotetraose < di-galacturonic acid. Due to the lack of suitable standards, we were not able to identify any heterogeneous dimers, like xyl-glc or glc-man, ara-xyl for example, which might theoretically be present. Tentative identifications given below for sunflower digests are based on retention times (Fig.3) and sugar composition (Table 3) of the isolated compounds.

Figure 3: HPAEC-elution pattern of reaction products solubilised from sunflower CWM by enzymatic treatment. Comparison of digests obtained with crude preparations or enzyme fractions and of selected oligomer pools. (1): arabinose, (2): glucose, (3): mannobiose, (4): mannotriose (?), (5): xylobiose, (6): cellobiose, (7): xylotriose, (8): arabinobiose, (9): galacturonic acid, (10): arabinotriose, (11): arabinotetraose. The gradient applied for elution is shown by the hatched line.

Different monomers, of which only the major ones could be identified by HPAEC (arabinose (1), glucose (2), galacturonic acid (9), Fig.3), were released from sunflower CWM. Neutral mono- and dimers each represented about 10 - 20% of total solubilised NSP. Dimers of mannose (3), xylose (5), glucose (6) and arabinose (8) may be tentatively assigned. It is unclear whether peak (4) of the dimer fraction and peak (4?) of the trimer fraction are identical compounds as their retention times would propose. Very small amounts of trimers and tetramers were recovered from digests obtained with some enzyme fractions. The major peak of the trimer-pool (4?) corresponds in retention time (HPAEC) to mannotriose.

Table 3

Yields[a] and sugar composition[b] of BioGel P2-pools containing monomers, oligomers and high polymeric/acidic material produced after 72h incubation of sunflower CWM with enzyme fractions 'E1', 'E2', 'D1' and 'D3'

	yield[a] %	rha	fuc	ara	xyl	man	gal	glc	aua
					sunflower CWM				
E1 mono	12	1	0	3	5	1	3	86	
E2 mono	21	2	1	61	1	4	8	24	
D1 mono	17	0	0	32	9	1	4	53	
D3 mono	12	0	1	4	4	3	7	80	
E1 di	5	0	0	12	39	27	0	22	
E2 di	13	0	0	15	11	56	0	19	
D1 di	13	0	0	12	34	5	5	44	
D3 di	21	0	0	5	13	64	3	16	
E2 tri	3	0	0	37	3	48	0	12	
D3 tri	7	0	0	3	22	21	0	54	
E2 tetra	2	0	0	53	8	15	0	23	
D1 tetra	3	0	0	56	7	7	14	15	
E1 -I	83	9	0	41	8	1	8	5	28
E2 -I	53	12	0	13	12	1	12	7	43
E2 -II	8	1	0	19	11	0	1	3	65
D1 -I	65	9	0	29	10	1	7	5	39
D1 -II	3	0	0	24	1	0	1	2	72
D3 -I	60	8	0	31	7	1	7	7	39

[a] g NSP per 100g NSP applied to BioGel P2
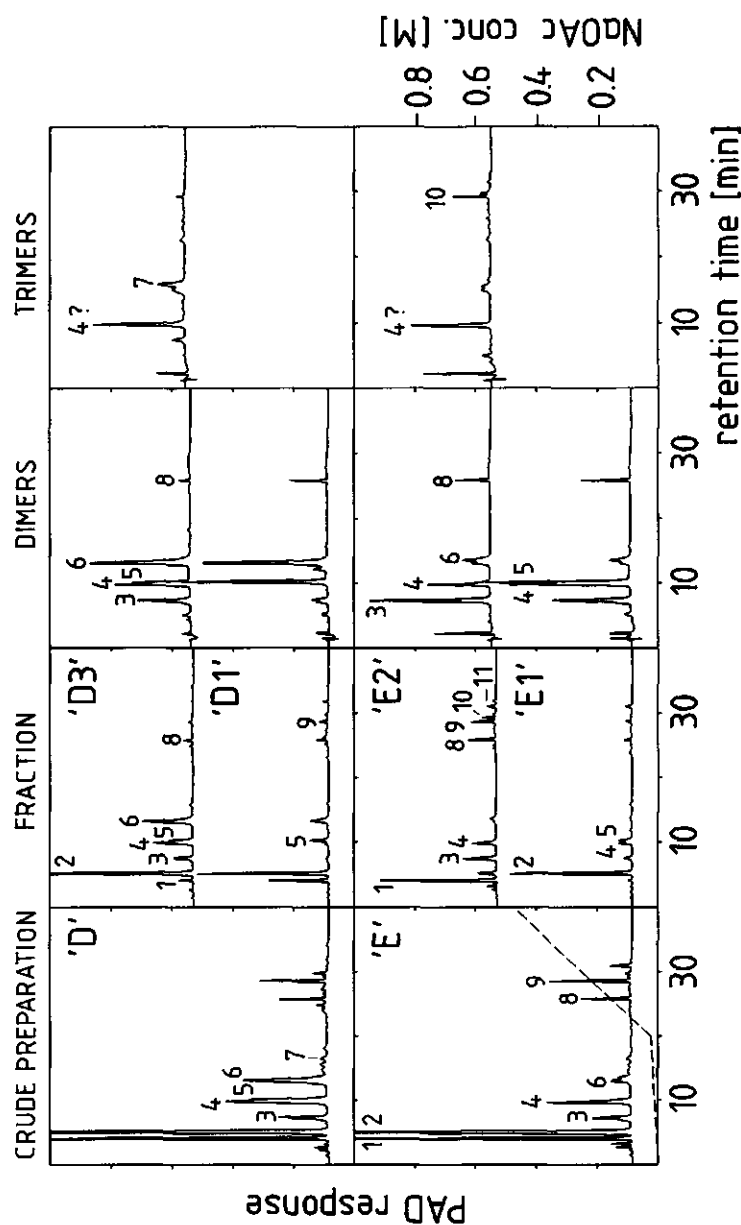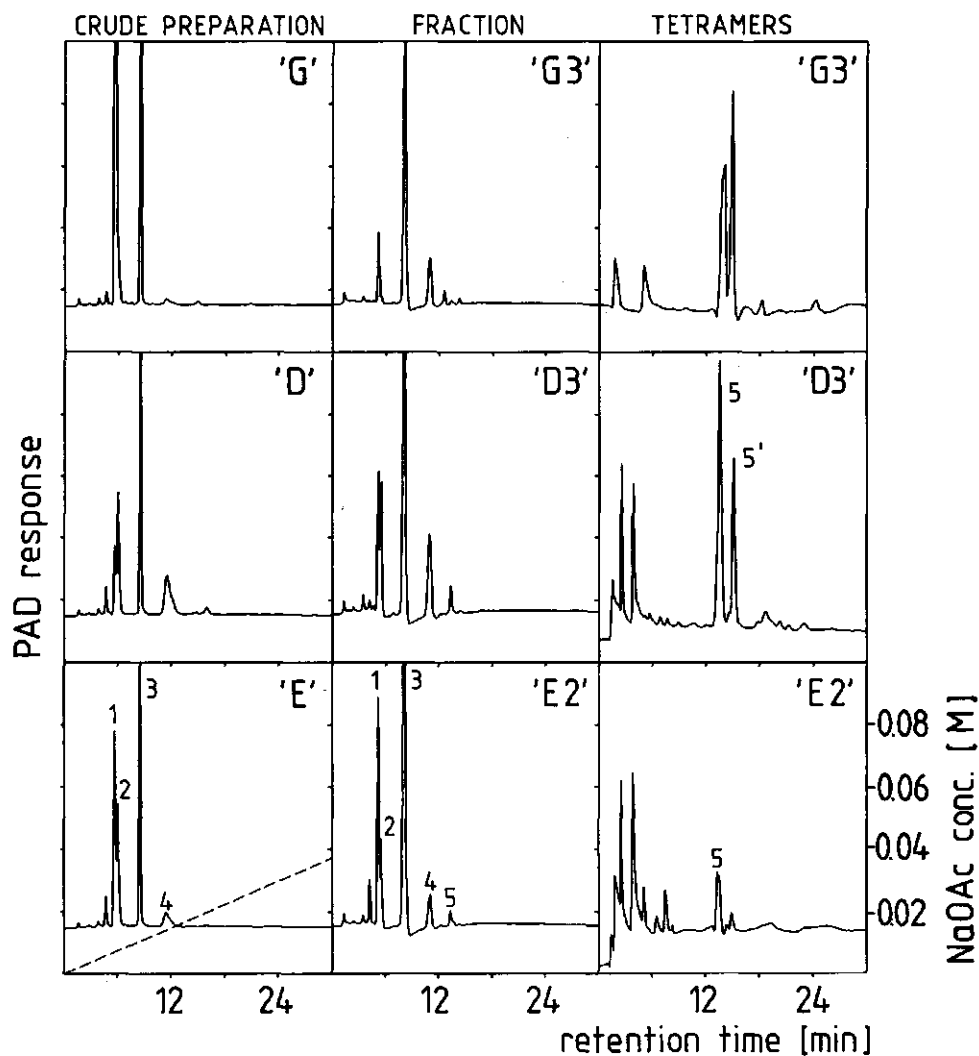[b] expressed as rel. mol-%

Figure 4: HPAEC-elution pattern of reaction products solubilised from palm-kernel CWM by enzymatic treatment. Comparison of digests obtained with crude preparations or enzyme fractions and of selected oligomer pools. (1): mannose, (2): glucose, (3): mannobiose, (4): mannotriose, (5) and (5'): tetramers. The gradient applied for elution is shown by the hatched line.

However, this identification is unlikely regarding the sugar composition of the fractions. In tetramer fractions arabinotetraose and two unidentified peaks were present in about equal amounts (not shown). The major part of the solubilised material (60% - 80%) was eluted in the void volume (Fig.2a, I). Its elution-behaviour is most probably caused by the acidic nature rather than by a high molecular weight. As could be deduced from a control experiment, about half of this material was already soluble without enzyme addition. Those enzyme fractions containing the bulk of pectolytic activity ('E2', 'D1') produced a second peak eluting close to the void volume (II, Fig. 2a). Both peaks were high in uronide content and arabinose, the voids having high proportions of rhamnose and galactose and lower proportions of galacturonic acid than the slightly retarded fractions (II). The molecular size of the acidic material as estimated by HPAEC (chromatograms not shown) indicated that galA-dimer and -trimer were prevailing constituents in voids from fractions of 'D', while fractions of 'E' released mono-galacturonic acid

Table 4

Yields[a] and sugar composition[b] of BioGel P2-pools containing monomers, oligomers and high polymeric/acidic material produced after 72h incubation of palm-kernel CWM with enzyme fractions 'E2', 'D3' and 'G3'.

	yield[a] %	rha	fuc	ara	xyl	man	gal	glc	aua
				palm-kernel CWM					
E2 mono	15	0	0	17	1	45	12	26	
D3 mono	11	0	0	3	6	54	8	29	
G3 mono	7	0	0	0	2	88	4	6	
E2 di	75	0	0	2	2	93	0	3	
D3 di	71	0	0	1	2	91	0	7	
G3 di	73	0	0	0	2	95	0	3	
E2 tri	10	0	0	0	1	96	0	3	
D3 tri	14	0	0	0	1	92	4	4	
G3 tri	15	0	0	1	2	90	6	2	
D3 tetra	1	0	0	5	2	74	13	6	
G3 tetra	1	0	0	0	2	79	12	7	
D3 -I	3	6	0	31	6	11	19	5	23
G3 -I	5	4	0	38	4	8	25	11	11

[a] g NSP per 100g NSP applied to BioGel P2
[b] expressed as rel. mol-%

as major low molecular weight end product. For palm-kernel CWM, the mannose-dimer, peak (3), was major reaction product of the fractions, while by the crude preparations comparatively higher amounts of monomeric mannose (1) and glucose (2) were formed (Fig.4). Dimers and trimers almost exclusively were composed of mannose residues (Table 4). According to the elution pattern, two types of tetramers (5 and 5'), were formed in very low amounts. They contained, next to mannose, significant amounts of galactose. Based on their retention time, the dimers, trimers (chromatograms not shown) and tetramers formed by all preparations and fractions were identical. Low amounts of uronic acid containing material were excluded from the gel-filtration column and eluted in the void volume (Fig.2b, I). The sugar composition of these fractions, next to some glucose and mannose, is characteristic of pectic compounds.

A more precise identification of the degraded compounds might be achieved by glycosyl-linkage analysis of the residues (Gordon *et al* 1983). Attempts to methylate this residual material in the present study, however, were unsuccessful, as significant amounts of xylose and glucose were recovered in unmethylated form (results not shown).
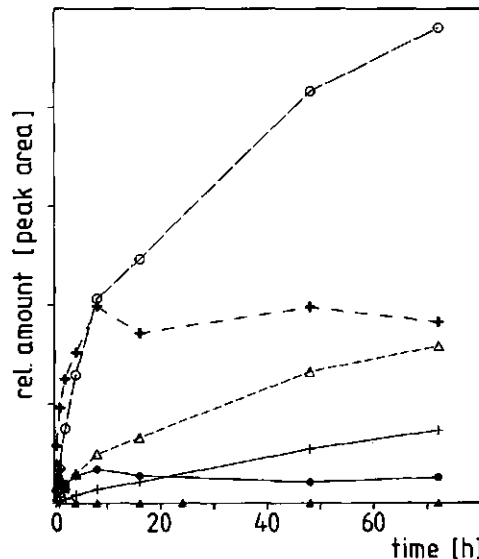

Figure 5: Formation of mannose, glucose and manno-oligomers during prolonged incubation of palm-kernel CWM with preparation 'D'. + mannose, △ glucose, ○ mannobiose, + mannotriose, ● tetramer, ▲ pentamer

Kinetics of mannan degradation

As an exact identification of the oligomers produced from palm-kernel CWM was obtained, the kinetics of their formation could be investigated during 72 hours incubation. A typical time course of the release of monomers and oligomers of DP 2 to 5 is shown in Fig.5 (incubation with 'D3'). The release of the major reaction product, mannobiose, follows a biphasic curve, which was also typical for the release of total NSP (Düsterhöft *et al* 1993a). Low amounts of trimer, tetramer and pentamer were formed in the first phase of incubation; they remained at a constant, low level during prolonged hydrolysis and were not further degraded. Similar patterns were obtained with enzyme preparations 'E' and 'G', the latter preparation releasing monomeric mannose as major end product (not shown).

DISCUSSION

The by-products investigated in the present study both derive from oleogeneous crops. They differ considerably in botanical origin and in morphological and chemical composition. The cell wall material from sunflower meal (dicotyledon) consists mainly of thick secondary walls from the hull and the thin-walled parenchyma tissue from the seed. Cellulose, (4-*O*-methyl)-glucuronoxylans and pectic compounds were found as major constituent polysaccharides. Palm-kernel CWM (monocotyledon) consists mainly of endosperm tissue and minor amounts of highly lignified endocarp (Vaughan 1970, Düsterhöft *et al* 1991). Mannans and cellulose were identified as prevailing polysaccharides (Düsterhöft *et al* 1992). The results obtained from enzymic hydrolysis of these materials need to be discussed in relation to these characteristics.

In general, a low solubilisation of xylose- and, though varying with different enzyme-substrate-combination, glucose-containing polysaccharides was observed in both materials. In contrast, arabinose-, uronic acid-, galactose- and mannose-containing polysaccharides were solubilised to much higher extents. These constituents, except mannose-based polysaccharides, may largely derive from pectic compounds. The different susceptibility of the afore mentioned compounds to enzymic degradation has frequently been observed when different plant materials were digested *in-vivo* or *in-vitro* by rumen microorganisms (Gordon *et al* 1983), Åman and Nordkvist 1983, Nordkvist and Åman 1986) or cell free enzyme preparations (Sinoquet 1989). These apparent differences in the degradability of the

cell wall polymers have been shown to be largely caused by the morphological composition of the (heterogeneous) substrates, while the chemical composition of the polymers seems to be of secondary importance (Hatfield 1989, Chesson 1993). Mesophyll cells and parenchyma tissues containing primary cell walls are more readily degraded by rumen microorganisms than heavily lignified sclerenchyma and xylem (Akin 1989). It is therefore likely to assume that the pectic constituents solubilised in sunflower meal derive from parenchyma tissues of the seed, while the less solubilised constituents (presumably xylans, cellulose) derive from the hull material. The sugar composition of different botanical fractions of sunflower (Sabir *et al* 1975) is in agreement with this assumption. Attempts to study this phenomenon by light- or electron-microscopy, however, were inconclusive, as the integrity of especially the parenchyma tissues in sunflower CWM was destroyed upon industrial processing and did not allow either the identification of particular structures or the observation of their disintegration by enzymic action. In palm-kernel CWM, the morphological characteristics were preserved better and enzymic action was demonstrated in the cell contents of endosperm cells and in different cell wall layers (Düsterhöft *et al* 1993a).

Next to pectic compounds, low amounts of xylose and glucose were released from the CWM's. This may indicate the partial hydrolysis of xyloglucans, arabinoxylans or (gluco)mannans, all of which were encountered in minor amounts in the CWM of either sunflower or palmkernel meals (Düsterhöft *et al* 1992). As constituents of primary cell walls, xyloglucans and arabinoxylans are more readily degradable than cellulose or (glucurono)xylans, the major constituents of secondary thickened walls (Chesson *et al* 1986).

In palm-kernel meal, as much as 25% to 30% of the glucose initially present could be solubilised by preparation 'D' or fraction 'D3'. As cellulose is the only glucose-containing polysaccharide found to be present in this material in considerable amounts, the major part of released glucose must derive from cellulose hydrolysis. Mannan, the prevailing polymer of palm-kernel meal, could maximally be solubilised to about half of its amount, notably by preparation 'G', possessing the least side activities. Comparing the release of glucose (cellulose) and mannose (mannan) from palm-kernel meal, we conclude that neither cellulose hydrolysis enhances mannan-degradation, nor vice versa.

While the experimental design of the present study does not allow the detection of truly synergistic effects occurring between different enzymes, the results confirm, that the presence or absence of certain activities (or the concentration at which they are present) clearly influence the rate and extent of hydrolysis of the target polymers. Thus the low release of monomeric mannose

from palm-kernel CWM by the fractions if compared to the crude preparations reflects the removal of ß-mannosidase or of other activities able to release the monomer. The largest neutral oligomers produced in appropriate amounts were tetramers. Endo-mannanases from *A. niger* and *I. lacteus* have been reported to have 5 significant binding subsites (McCleary and Matheson 1983). This explains their inactivity on mannotriose and low activity towards mannotetraose. The finding in this study, that these oligomers remained essentially undegraded after extended times of hydrolysis, indicates the lack of enzyme activities for complete conversion to monomers. Thus the few galactose-substituted sites in the palm-kernel mannans (Düsterhöft *et al* 1992, Daud and Jarvis 1992) were partly left undegraded, particularly when the level of α-galactosidase was low ('G3'). However, it is unlikely that the overall degradation was limited by these structures, as they were only encountered in small amounts.

Characteristically the average molecular size of the reaction products formed from both CWM's was very small (DP 1 to 2). This was not only due to prolonged times of incubation but was found generally during the entire course of hydrolysis (Düsterhöft *et al* 1993a). It might be speculated here, that any potentially soluble and accessible material (like pectic compounds or highly substituted galactomannans) would be attacked in the initial stages of the reaction and, dependent on the presence of appropriate activities, degraded to monomers. After depletion of this type of easily accessible material, more crystalline or lignified structures and relatively larger particles remain to be degraded. Our findings indicate that after the initial phase of the reaction, enzymic attack on these substrates occurred by the direct release of mono- or dimeric products, without the intermediate formation of soluble polymeric material. Various factors, like those proposed for the enzymic hydrolysis of crystalline cellulose, e.g. adsorption characteristics and synergism of enzyme components, or the capacity of endo-acting enzymes to release monomers (Klyosov 1990) still need to be studied in the degradation of insoluble xylans and linear mannans, in order to evaluate whether the hydrolysis of this type of substrates may further be enhanced.

The saccharification of constituent polysaccharides from the lignocellulosic substrates could be achieved to only limited extents. In contrast, less or non-lignified substrates, like carrot fibres (Massiot *et al* 1989), sugar beet (Beldman 1984, Sinoquet 1989, Thibault and Rouau 1990) or wheat aleurone (Mulder *et al* 1991) could be degraded very effectively by commercial polysaccharidases. As in the present study it was found that especially the major polysaccharides were either the least degraded (glucuronoxylan and cellulose in sunflower) or of low susceptibility to hydrolysis (mannan in palm-kernel), the total efficiency, in terms

of energy gain, will be low. Enhancing cellulose and mannan hydrolysis would therefore be major targets for improvement. Similar findings have recently been reported by Savory (1992), studying the degradation and the metabolism of (enzyme supplemented) cell wall substrates in fowls. Enzyme supplementation of (monogastric) diets, however, may influence the utilisation of nutrients and NSP by other effects than direct energy gain. Thus, pentosans in cereal based diets can be selectively hydrolysed and deleterious effects they exert be removed (Choct and Annison 1992, Groot Wassink *et al* 1989, Petterson and Åman 1991). The production of large amounts of monomeric pentoses or uronic acids might reduce the performance of monogastric animals (Longstaff *et al* 1988, Schutte *et al* 1991). Non-starch polysaccharides influence nutrient absorption, transit time, and bacterial microflora (Graham and Åman 1991) and these parameters may likewise be influenced by degradation products formed upon enzymic treatment. It is obvious that monitoring the formation of reaction products and their characterisation as reported in the present investigation are essential for an understanding of the effects of enzymic treatment observed in *in-vivo* studies. Experiments using further purified enzyme fractions of known specificity will be necessary to identify relevant activities and to evaluate, whether and how the production of desired end products can be guided by variation in enzyme formulation.

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CHAPTER 6

THE ROLE OF FUNGAL POLYSACCHARIDASES IN THE HYDROLYSIS OF CELL WALL MATERIALS FROM SUNFLOWER AND PALM-KERNEL MEALS

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ABSTRACT

Main fractions from multi-component polysaccharidase preparations (Driselase, Gamanase and an experimental preparation of fungal origin), previously sed for the enzymic treatment of cell wall materials from sunflower and palm-kernel meals, were subfractionated by different chromatographic techniques in order to evaluate the contribution of their constituent activities in cell wall degradation. Based on activity measurements, 5- to 10-fold purification was achieved for the major enzymes, but residual side-activities were still detectable in most subfractions. The solubilisation of non-starch polysaccharides from the cell wall materials by the resulting pectolytic, xylanolytic, cellulolytic and mannanolytic subfractions and by highly purified glucanases, arabinanases and xylanases was, when acting individually, very low (1% - 5% of NSP). With few exceptions, the solubilising effect of the main fractions could only slightly be enhanced by supplementation with pectolytic, cellulolytic or mannanolytic subfractions or by highly purified enzymes. It remained mostly lower than the sum of both individually obtained values. In the degradation of palm-kernel cell wall material, however, synergistic action of mannanases and glucanases was observed. The hydrolysis of pectic compounds in sunflower cell wall material was most effective when polygalacturonases, arabinanases and rhamnogalacturonan-degrading activities were applied together. The resistance of 4-O-methyl-glucuronoxylan, major hemicellulosic polymer in sunflower CWM, to enzymic hydrolysis was not only caused by its location in the cell wall or interlinkage to other polymers but also by its primary structure. Neither purified endo-xylanase nor the crude parent preparation were able to achieve complete hydrolysis of the polysaccharide after extraction.

INTRODUCTION

Plant cell walls contain a variety of polysaccharides, the distribution of which varies within primary and secondary cell walls and between mono- and dicotyledenous plants. The polymers are interlinked with each other by covalent or non-covalent linkages or via non-carbohydrate compounds, like phenolic acids, lignin or protein (Fry 1986, Jeffries 1990). Depending on the primary structure of polysaccharides, various enzyme activities are required to hydrolyse the main chain and to remove substituents. The consecutive action of different enzymes may also

improve the accessibility of the substrates if it affects the interlinkages of different polysaccharides within the cell wall matrix. Examples are the enhancement of hemicellulose- and cellulose hydrolysis by pectolytic enzymes (Voragen *et al* 1980, Ben Shalom 1986), or the synergistic action of pectolytic enzymes and glucanases in the hydrolysis of xyloglucan/pectin complexes in the primary cell walls of apples (Renard *et al* 1991). Lignification of cell walls and the crystalline structure of some polysaccharides, like cellulose, impose structural barriers by which plants gain mechanical stability and are protected from pathogens, thereby forming major obstacles to enzymic hydrolysis.

In earlier reports we have investigated the enzymic degradation of sunflower and palm-kernel meals, lignocellulosic by-products from the production of edible oil, with the aim of enhancing the utilisation of their non-starch polysaccharides (NSP). Upon treatment of the finely ground cell wall materials (CWM's) with multi-component enzyme preparations, the major polysaccharides, (4-*O*-methyl)-glucuronoxylans in sunflower-, mannans in palm-kernel and cellulose in both meals (Düsterhöft *et al* 1992) were found to be solubilised to only limited extents while the constituent sugars of pectic compounds and mannan-containing polysaccharides (in sunflower CWM) were well degraded (Düsterhöft *et al* 1993b).

Aim of the present paper was the identification in some technical enzyme preparations of the most relevant enzyme activities for the effective solubilisation of NSP from sunflower and palm-kernel CWM. This information can contribute to an optimisation of enzymic treatment in terms of saccharification yield. It is essential for controlled product formation, which, due to the different physiological fates of mono-, oligo- and polysaccharides (Graham and Åman 1991, Mathers 1991), is of importance in feed applications. Multi-component polysaccharidase preparations were therefore subfractionated with the aim of purifying their major activities. Hydrolysis by the resulting (partially) purified fractions or by pure enzymes from other fungal sources (glucanases, xylanase, arabinanase), separately or in combination, allowed to evaluate qualitatively and quantitatively the contribution of individual enzymes to the solubilisation of the CWM's. In addition, the enzymic hydrolysis of isolated (4-*O*-methyl)-glucuronoxylan from sunflower meal was compared with its *in-situ*-degradation in order to evaluate the influence of its primary structure and of cell wall architecture on enzymic action.

MATERIAL and METHODS

Materials

Cell wall materials (CWM) from sunflower (*Helianthus annuus* L.) and palm-kernel (*Elaeis guineensis* Jacq) meals were prepared as described previously (Düsterhöft *et al* 1991). They were milled to a fine powder, using a freezer mill (Spex Ind., Edison, USA). (4-*O*-methyl)-glucuronoxylan was extracted by 1M KOH from delignified sunflower CWM (Düsterhöft *et al* 1991). The isolated polysaccharide fraction had a xylose/4-*O*-methyl-glucuronic acid-ratio of 9:1 (Düsterhöft *et al* 1992). Rhamnogalacturonan (modified hairy regions, MHR) from apple was prepared in our laboratory according to Schols *et al* (1990).

Enzymes and their fractionation

Multi-component polysaccharidase preparations Driselase ('D', from *Irpex lacteus*, Sigma, St. Louis, MO, USA), an experimental preparation of fungal origin ('E', Novo Nordisk, Bagsvaerd, Denmark) and Gamanase ('G', from *Aspergillus niger*, Novo Nordisk) were fractionated by anion-exchange chromatography to yield 'fractions' enriched in certain activities (Düsterhöft *et al* 1993b). Selected fractions were subjected to further purification on columns of CM BioGel A, BioGel HTP (BioRad, Richmond, LA, USA), MONO Q (Pharmacia LKB, Uppsala, Sweden) or crosslinked alginate (Rombouts *et al* 1979) as specified in Figures 1 to 3. In the following, the resulting fractions will be referred to as 'subfractions'. All buffers contained 0.01% NaN₃ to prevent microbial growth. Purified enzymes from other sources: endo-glucanases I and IV, cellobiohydrolase (CBH) (ex *Trichoderma viride*, Beldman *et al* 1985), endo-xylanase I (ex *Aspergillus awamori*, Kormelink *et al* 1992), endo- and exo-arabinanase (ex *A. niger*, Rombouts *et al* 1988) were obtained from our laboratory collection. α-glucuronidase was partially purified from a cellulase preparation (ex *T. viride*, Gist Brocades, Delft, The Netherlands) by successive gel filtration (BioGel P10), cation-exchange (CM-BioGel A) and gel filtration chromatography (BioGel P-100). It still contained minor amounts of ß-xylosidase activity (Kroef, to be published).

Characterisation of (partially) purified enzymes (subfractions)

a) Determination of glycanase and glycosidase activities. Glycanase activities were measured against CM-cellulose (type AF 0305, Enka B.V., Arnhem, The Netherlands), Avicel (Serva, Heidelberg, FRG), xylan (ex oat spelt) and polygalacturonic acid (both from Sigma, St.Louis, MO, USA), mannan (ex palm-kernel meal (Düsterhöft *et al* 1991) and linear, dyed arabinan (Megazyme, North

Rocks, N.S.W., Australia). For the determination of arabinanase activity, substrate (10g litre^{-1}) was incubated with enzyme in 150mM sodium acetate buffer, pH 5.0 at 30°C (1h). The reaction was stopped by addition of ethanol. From the absorption of the supernatant at 520nm, the activity was calculated by reference to a standard curve obtained with a well-defined pure endo-arabinanase. Other glycanase activities were calculated from the formation of reducing end-groups, released after 1h incubation of substrate (1g litre^{-1}) in 0.05M sodium acetate buffer (pH 5.0) at 30°C (Somogyi 1952). In order to standardise activity measurements on partially insoluble substrates (mannan, xylan), of which it is known that activity is not linearly correlated with enzyme concentration (Khan *et al* 1986), activities were calculated from experiments conducted with enzyme concentrations that achieved approximately 5% conversion of these substrates in the given time. Glucuronidase activity was measured by a modification of the Somogyi-method (Milner and Avigad 1967). The substrate, 4-*O*-methyl-glucuronoxylan from birchwood, pretreated with endo-xylanase I (ex *A. awamori*), was incubated with enzyme for 1h at 40°C in 0.05M sodium acetate buffer (pH 5.0) and the supernatant was assayed for the amount of reducing end-groups deriving specifically from released uronic acids. Other glycosidase activities were measured by the release of *p*-nitrophenol from the corresponding derivatives of ß-D-mannopyranose, α-L-arabinofuranose, ß-D-xylopyranose, ß-D-glucopyranose, α/ß-D-galactopyranose and α-L-fucopyranose. Except for the substrate concentration (0.2g litre^{-1}), incubation conditions were identical with glycanase assays. One Unit [U] of activity is defined as the amount of enzyme that catalyses the release of one μmole of reducing sugar or *p*-nitrophenol per minute.

b) Detection of rhamnogalacturonase-activity. The presence of rhamnogalacturonase activity in subfractions was investigated by high-performance size-exclusion chromatography, monitoring the change in molecular weight distribution of saponified MHR upon incubation with enzyme fractions (2h and 24 h in sodium acetate buffer, pH 5.0, at 30°C) (Schols *et al* 1990). Digests were analysed on BioGel TSK 40, 30, 20XL columns (BioRad), coupled in series, with 0.4M sodium acetate (adjusted to pH 3.0) as eluent, at a flow rate of 0.8ml min^{-1} and a temperature of 30 °C.

c) Homogeneity of enzyme fractions. The (apparent) homogeneity of enzyme fractions was inspected by SDS-gel electrophoresis (10 -15% polyacrylamide gels) on a Phast System (Pharmacia LKB) according to the procedure of Laemmli (1970). Gels were stained with silver according to the procedure of the supplier.

d) Protein content. The protein content of solutions was measured by a modification of the Lowry method (Bensadoun and Winterstein 1976).

Enzymic hydrolysis of CWMs and of isolated polysaccharides
CWM's were incubated with (a) enzyme fractions 'E1', 'E2', 'D1', 'D3', 'G3' alone, (b) fractions in combination with each other (for palm-kernel incubations) or in combination with subfractions or purified enzymes (both CWM's) and (c) subfractions or purified enzymes alone (both CWM's), (see Fig's. 1 - 3 for origin of the enzyme fractions).
Typically, an amount of (cryo-milled) CWM corresponding to 10 mg NSP was incubated in Eppendorf-vials in 0.5ml of 0.05M sodium acetate buffer (pH 5.0, containing 0.01% NaN$_3$) for 72h, at 38°C, rotating 'head over tail'. For sunflower incubations, the amount of an enzyme fraction added was proportional to its concentration in the 'parent' preparation (e.g. 'D3' comprises 74% of total protein recovered after anion-exchange chromatography of crude preparation 'D' (Düsterhöft *et al* 1993b); based on an addition of 50μg protein of the 'crude' preparation, 37μg of fraction 'D3' was added). For palm-kernel incubations, addition of mannanase-containing enzyme fractions was standardised on mannanase activity (50mU); main fraction 'G1' was added in amounts corresponding to 15mU ß-D-mannosidase activity. Addition of other subfractions and of pure enzymes to both substrates was based on their major activities; the amounts added were theoretically sufficient to degrade the target polymer within 12 - 24h. Table 1 gives an overview of the amounts of main (bold printed) and side activities applied in experiments with different enzyme fractions. For combined incubation with different fractions, the individual values have to be added. Endo-glucanase and CBH were added in a ratio, which was reported to give maximal synergistic effect (Beldman *et al* 1985). Reactions were stopped by placing the vials in a boiling water bath (10min), supernatants were removed by centrifugation (18,000 x *g*, 7min) and appropriately diluted for further analysis.
Hydrolysis of isolated (4-*O*-methyl)-glucuronoxylan (in 0.05M sodium acetate buffer, pH 5.0 at 30°C, for 48h) was performed with the crude preparations 'E' and 'D', fractions 'D1', 'D3' and 'E1' and with the xylanase-subfraction 'D1$_1$'. Equal amounts of xylanase activity, theoretically sufficient to hydrolyse the polymer completely within 12h, were added individually or in combination with 64 mU of α-glucuronidase activity to the reaction mixtures. The reaction was stopped as described above.

Fractionation, isolation and characterisation of degradation products from hydrolysis of (4-O-methyl)-glucuronoxylan
The molecular size distribution of digests was inspected by HPSEC (BioGel TSK 40,30,20 XL) under conditions given above (Characterisation of Enzyme

Table 1

Amount of polysaccharidase activity added in incubation of CWM's with different enzyme fractions [mU/incubation]

enzyme fraction	CMCase	Avice-lase	manna-nase	xyla-nase	polyga-lacturon.	arabi-nanase
main fractions						
			palm-kernel CWM			
'E2'	5.4	0.3	**50.0**	1.8	956.0	3.0
'D3'	26.4	5.5	**50.0**	118.0	25.0	16.7
'G3'	0.0	n.dt	**50.0**	0.0	n.dt	0.0
			sunflower CWM			
'E1'	7.5	0.2	0.9	5.8	2.2	0.5
'E2'	3.5	0.2	32.0	1.2	612.2	1.9
'D1'	0.9	0.9	0.0	26.6	27.9	17.8
'D3'	7.0	1.5	13.3	31.4	6.6	4.4
subfractions and pure enzymes						
			'pectolytic'			
'E2-II'	1.2	n.dt.	0.0	0.2	**100.0**	0.0
'D1$_2$'	0.0	n.dt.	0.0	12.8	**100.0**	126
'D1$_3$'	0.0	n.dt.	0.0	1.2	**100.0**	27.6
'E2-I$_3$'	3.0	n.dt.	6.0	3.0	0.0	0.0
endo/exo arab.						**20.0**
			'cellulolytic'			
'E5'	**25.0**	n.dt.	4.6	2.0	3.9	0.0
'D3$_3$'	**25.0**	n.dt.	56.6	50.0	4.3	n.dt.
Endogluc I/IV	**25.0**					
			'xylanolytic'			
'D1$_1$'	0.2	n.dt.	0.2	**50.0**	0.8	1.0
'D3$_2$'	0.0	n.dt.	1.5	**50.0**	1.5	3.2
endo xyl I				**50.0**		
			'mannanolytic'			
'E2-I$_2$' [a]	1.9	n.dt.	**25.0**	1.9	0.0	0.0
'D3$_1$' [a]	0.5	n.dt.	**25.0**	1.0	1.0	0.0

[a] values for incubation of sunflower CWM. The double amount was added for palm-kernel CWM (50mU mannanase activity).

fractions). Oligomeric reaction products in digests were analysed by high-performance anion-exchange chromatography (HPAEC) on a Dionex System as described previously (Düsterhöft *et al* 1993b). Samples were chromatographed on a PA-100 column (4mm x 250mm) running a linear gradient of eluent A (100mM

NaOH) and B (1M NaOAc/100mM NaOH) as specified in Fig. 8. The major acidic end product of prolonged hydrolysis of (4-O-methyl)-glucuronoxylan by subfraction 'D1$_1$' was isolated by HPAEC with a PA1 column (9mm x 250mm). The effluent was post-column neutralised with 1M acetic acid. The pooled fraction was desalted on Dowex 50W X8 (BioRad) and acetic acid was removed by repeated co-evaporation with methanol. The final product was freeze dried and its structure determined by NMR. Spectra were recorded with a Bruker WM 600 and a Varian Unity 400 spectrometer at 30°C for a solution of 2mg in 0.6ml D$_2$0. Trimethylsilylpropanoate-d4 was used as reference. 2D-NMR spectroscopy was performed with standard COSY, TOCSY, and heteronuclear correlated C/H pulse sequences using indirect detection (HMQC and HMBC) (Venekamp, to be published).
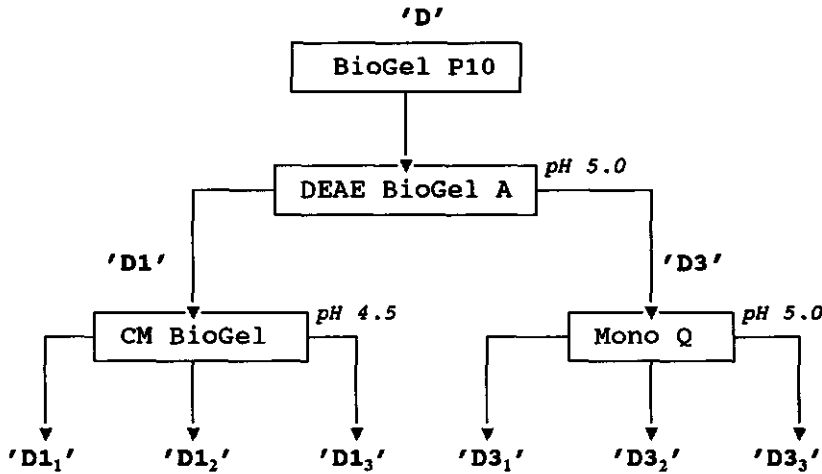
General analytical methods

The amounts of neutral sugars and uronic acids released upon enzymic hydrolysis of CWM's (experiment (a) and (b)) were measured by automated methods of the orcinol-sulfuric acid-test (Tollier and Robin 1979) and the 3-phenyl-phenol-test (Thibault 1979). Mannose was used as standard in palm-kernel incubations, equal amounts of arabinose and glucose in sunflower incubations. Corrections were made for the mutual interferences of both methods. The 'solubilisation' of NSP is defined as the sum of released neutral sugars and uronic acids, after correction for the control.

Sugar composition and -contents in digests, obtained with purified enzymes or subfractions, (experiment (c)) were determined by GLC. For the conversion to alditol acetates, digests were hydrolysed by 2M TFA (1h, 121°C) and the released monosaccharides were derivatised according to Englyst and Cummings (1984).

RESULTS

Fractionation and partial purification of crude enzyme preparations

To investigate the contribution of their constituent enzymes to the hydrolysis of the CWM's, the crude preparations were fractionated by anion-exchange chromatography into fractions enriched in, -or devoid of-, certain groups of activities, like cellulolytic, mannanolytic or pectolytic activities (Düsterhöft *et al* 1993b). Those fractions exhibiting the relatively largest solubilising effect on the cell wall materials were further fractionated with the aim of purifying their major
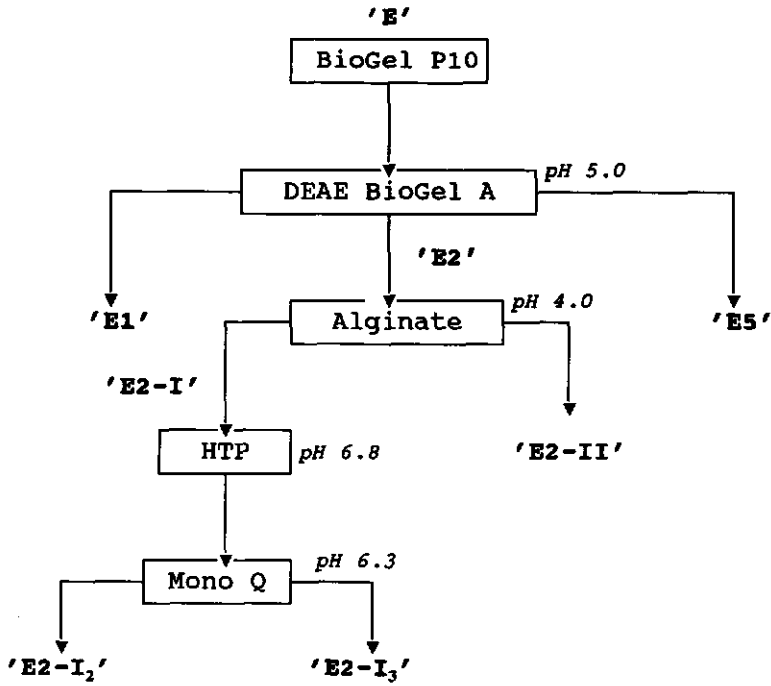
'D'

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┌──────────────┐
│  BioGel P10  │
└──────────────┘
```



```
        pH 5.0
┌──────────────────┐
│  DEAE BioGel A   │
└──────────────────┘
```

'D1'   **'D3'**

```
        pH 4.5                          pH 5.0
┌──────────────┐                 ┌──────────────┐
│  CM BioGel   │                 │   Mono Q     │
└──────────────┘                 └──────────────┘
```

'D1₁'   **'D1₂'**   **'D1₃'**   **'D3₁'**   **'D3₂'**   **'D3₃'**

Spec. activities (U/mg)

substrate	crude prep. 'D'[a]	fractions 'D1'[a]	'D3'[a]	subfractions 'D1₁'	'D1₂'	'D1₃'	'D3₁'	'D3₂'	'D3₃'
CMC	1.37	0.17	0.19	0.04	0.00	0.00	0.04	0.00	**0.40**
Avicel	0.05	0.17	0.04	-	-	-	0.04		
mannan	0.92	0.00	0.36	0.04	0.00	0.00	**2.07**	0.09	**0.92**
xylan	2.00	4.92	0.85	**11.80**	1.04	0.66	0.08	**2.94**	**0.81**
polygal.	0.92	5.17	0.18	0.02	**8.12**	**55.30**	0.08	0.09	0.07
arabinan	0.81	3.30	0.12	0.24	**10.17**	**15.30**	0.00	0.19	-
MHR	-	-	-	-	++	++	-	-	-
pnp-ß-D-glc$p$	0.07	0.25	0.03	-	-	-	-	-	-
pnp-ß-D-gal$p$	0.05	0.00	0.09	-	-	-	-	-	-
pnp-α-D-gal$p$	0.05	0.00	0.08	-	-	-	-	-	-
pnp-ß-D-xyl$p$	0.02	0.00	0.00	0.00	-	-	-	0.03	-
pnp-α-L-fuc$p$	0.01	-	-	-	-	-	-	-	-
pnp-ß-D-man$p$	0.00	0.00	0.00	-	-	-	0.00	-	-
pnp-α-L-ara$f$	0.10	0.17	0.10	0.07	0.55	0.02	-	-	-

[a] from: Düsterhöft *et al* 1993b

-: not determined

Figure 1: Fractionation scheme of enzyme preparation 'D' and specific activities of resulting fractions ('D1', 'D3') and subfractions ('D1₂,...etc.). ++ denotes: present as major activity.

'E'

| BioGel P10 |

| DEAE BioGel A | pH 5.0

'E2'

'E1' | Alginate | pH 4.0 | 'E5'

'E2-I'

| HTP | pH 6.8 | 'E2-II'

| Mono Q | pH 6.3

'E2-I₂' | 'E2-I₃'

Spec. activities (U/mg)

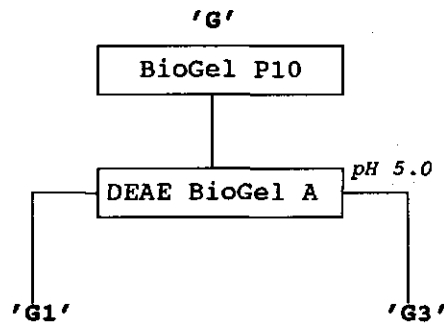| substrate	crude prep. 'E'[a]	fractions 'E1'[a]	'E2'[a]	'E5'	subfractions 'E2-II'	'E2-I$_2$'	'E2-I$_3$'
CMC	0.29	0.76	0.18	3.80	0.83	0.50	0.20
Avicel	0.02	0.02	0.01	-	-	-	-
mannan	1.45	0.09	1.65	0.70	0.17	7.00	0.40
xylan	1.25	0.59	0.06	0.30	0.17	0.50	0.20
polygal.	21.55	0.22	31.55	0.60	71.70	0.00	0.00
arabinan	0.25	0.05	0.10	-	0.00	0.00	0.00
MHR	-	-	-	-	-	-	+ +
pnp-ß-D-glc$p$	0.10	0.37	0.05	-	-	-	-
pnp-ß-D-gal$p$	0.17	0.31	0.03	-	-	-	-
pnp-α-D-gal$p$	0.27	0.02	0.27	-	-	-	-
pnp-ß-D-xyl$p$	0.02	0.02	0.00	-	-	-	-
pnp-α-L-fuc$p$	0.00	-	-	-	-	-	-
pnp-ß-D-man$p$	0.00	0.00	0.00	-	-	0.00	-
pnp-α-L-ara$f$	0.20	0.00	0.17	-	-	-	-

[a] from: Düsterhöft *et al* 1993b
- : not determined

Figure 2: Fractionation scheme of enzyme preparation 'E' and specific activities of resulting fractions ('E1',...etc,) and subfractions ('E2-II',...etc.). + + denotes: present as major activity

activities as far as possible. Fig.s 1 - 3 show the different fractionation schemes for preparations 'D', 'E' and 'G', and the specific activities measured in 'fractions' and 'subfractions'.

Of the most relevant enzyme fractions obtained from Driselase (see Fig.1), 'D1', which did not bind to the anion-exchanger, was further fractionated by cation-exchange chromatography, separating xylanolytic ('D1$_1$') and pectolytic ('D1$_2$', 'D1$_3$') subfractions. As estimated by SDS-PAGE, all these subfractions contained 4 - 5 protein bands (not shown).

'G'

```
            ┌──────────────┐
            │  BioGel P10  │
            └──────┬───────┘
                   │
                   │              pH 5.0
       ┌───────────┴───────┐
   ┌───┤ DEAE BioGel A      ├───┐
   │   └───────────────────┘    │
   │                            │
 'G1'                         'G3'
```

Spec. activities (U/mg protein)

	crude prep.	fractions	
substrate	'G'[a]	'G1'	'G3'[a]
CMC	0.05	0.17	0.04
Avicel	0.00	-	-
mannan	25.00	0.38	33.3
xylan	0.17	0.00	0.04
polygal.	0.00	0.42	-
arabinan	0.00	-	0.00
ß-D-glc$p$	0.02	-	0.00
ß-D-gal$p$	0.01	-	0.00
α-D-gal$p$	0.02	0.21	0.00
ß-D-xyl$p$	0.00	-	-
α-L-fuc$p$	0.00	-	-
ß-D-man$p$	0.01	0.63	0.00
α-L-ara$f$	-	-	-

[a] from: Düsterhöft et al 1993b
-: not determined

Figure 3: Fractionation scheme of enzyme preparation 'G' and specific activities of resulting fractions ('G1', 'G3')

From fraction 'D3', (released from DEAE at about 0.15M NaCl concentration), subfractions containing mannanase ('D3$_1$', 1 protein band on SDS-PAGE, Fig.4), xylanase ('D3$_2$') and a subfraction containing xylanase, cellulase and mannanase activities ('D3$_3$'), were obtained.

From enzyme preparation 'E' (Fig.2), the two most relevant fractions did bind to the anion-exchanger and were eluted close to each other at a NaCl-concentration of about 0.1M. From fraction 'E2', a polygalacturonase was purified by chromatography on crosslinked alginate (E2-II, Fig.4). The fraction which did not bind to crosslinked alginate could be fractionated by two further steps into a mannanase-containing subfraction ('E2-I$_2$', Fig.4) and into a subfraction with major activity on rhamnogalacturonan ('E2-I$_3$'). Fraction 'E5' was used as a pool enriched in cellulase activity.

The fractions from 'G' (Fig.3), 'G3' (mannanase, see also Fig.4) and 'G1' (ß-D-mannosidase and α-D-galactosidase), were used directly in experiments because sufficient separation of their activities was already achieved after the first chromatography step.
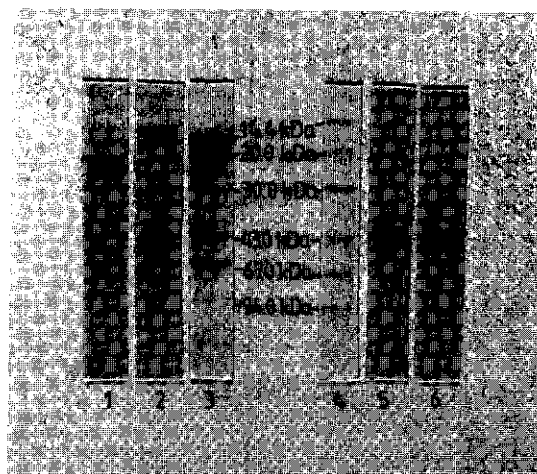

Figure 4: SDS-PAGE (silver staining) of enzyme fractions 'D3$_1$' (lane 1), 'G3' (lane 2), standards (lane 3 and lane 4), 'E2-II' (lane 5), 'E2-I$_2$' (lane 6).

Table 2

Solubilisation[a] of NSP from sunflower CWM by subfractions or pure enzymes. Sugar composition of supernatant (mol-%) and relative solubilisation[b] per monosaccharide (data in parentheses).

| | cellulolytic | | xylanolytic | | mannanolytic | | |
	glucanase (endo I)	glucanase (endo I/CBH)	$'DI_1'$	$'D3_2'$	$'D3_3'$	$'E2\text{-}I_2'$	$'DI_3'$
rha	0.7 (1.8)	1.2 (3.8)	4.7 (24.8)	7.3 (38.8)	2.8 (20.6)	5.7 (14.0)	5.5 (49.2)
ara	5.9 (1.6)	8.0 (2.6)	29.7 (16.7)	34.4 (19.6)	15.4 (12.2)	20.9 (5.5)	45.7 (44.1)
xyl	6.8 (0.8)	7.0 (1.0)	27.0 (7.0)	24.3 (6.4)	11.9 (4.3)	0.0 (0.0)	5.4 (2.4)
man	15.0 (11.7)	13.6 (13.0)	2.0 (3.3)	2.8 (4.7)	17.0 (38.5)	52.0 (40.3)	0.0 (0.1)
gal	3.7 (4.3)	4.3 (6.1)	5.8 (14.4)	5.6 (13.9)	4.2 (14.5)	6.1 (7.1)	4.1 (17.5)
glc	53.9 (3.3)	54.0 (4.1)	11.4 (1.5)	0.0 (0.0)	38.4 (7.0)	9.2 (0.6)	0.0 (0.0)
aua	13.9 (2.9)	11.8 (3.1)	19.4 (8.7)	25.6 (11.6)	10.2 (6.4)	6.0 (1.3)	39.1 (29.9)
total NSP[a]	2.9	3.6	5.7	5.7	8.4	2.8	10.0

| | pectolytic | | | | | | |
	arabinanase (endo)	arabinanase (exo)	arabinanase (endo-exo)	$'E2\text{-}II'$	$'E2\text{-}I_3'$	$'DI_2'$	$'DI_3'$
rha	1.0 (1.7)	0.0 (0.0)	1.4 (3.5)	7.5 (20.3)	8.8 (49.8)	6.3 (76.7)	5.5 (49.2)
ara	41.9 (7.0)	69.4 (3.5)	56.3 (15.4)	22.5 (6.5)	40.8 (24.8)	35.8 (47.1)	45.7 (44.1)
xyl	4.3 (0.3)	1.0 (0.0)	3.2 (0.4)	0.0 (0.0)	7.0 (2.0)	11.3 (6.8)	5.4 (2.4)
man	13.7 (6.7)	1.7 (0.2)	8.3 (6.5)	0.9 (0.8)	4.4 (7.8)	0.3 (1.2)	0.0 (0.1)
gal	4.6 (3.4)	2.6 (0.6)	3.6 (4.3)	3.3 (4.2)	5.2 (14.0)	4.5 (25.7)	4.1 (17.5)
glc	26.1 (1.0)	12.4 (0.1)	21.8 (1.3)	0.0 (0.0)	4.0 (0.6)	5.4 (1.6)	0.0 (0.0)
aua	8.2 (1.1)	13.0 (0.5)	5.3 (1.1)	65.8 (15.2)	29.6 (14.3)	36.4 (38.1)	39.1 (29.9)
total NSP[a]	1.7	0.5	2.7	3.3	6.3	13.7	10.0

[a] Percent of initial amount of NSP (w/w), which is solubilised
[b] Percent of initial amount of monosaccharide (w/w), which is solubilised

Effect of (partially) purified enzyme preparations on enzymic hydrolysis of CWM's.

a) sunflower meal

In general the solubilisation achieved by either (partially) purified subfractions or single enzymes was very low. The less pure subfractions (e.g. 'D3$_1$', 'D1$_2$' and 'D1$_3$', see Table 2 and Fig.5) did achieve comparatively higher values, however not more than 50% of the amount which was solubilised by the parent fraction. The molar sugar composition of the digests generally reflected the major enzyme activities by which they were obtained. Less pure enzyme fractions, in addition, released significant proportions of sugars other than those originating from their target substrates. However, even the electrophoretically pure endo-arabinanase and endo-glucanase showed this effect (Table 2).

Supplementation of main enzyme fractions with (partially) purified subfractions which were arbitrarily classified into cellulolytic, mannanolytic or pectolytic groups, or with pure enzymes, had different effects ranging from a decrease of maximally 17% (combination 'E1'/endo-xyl) to more than additional increase (70% by combination 'E1'/'D3$_3$')(Fig.5). In most cases, however, improvements of solubilisation remained less than additional. The effect of fractions 'D1' and 'D3' could slightly be improved by supplementation with pectolytic or mannanolytic subfractions; it was unaffected by the cellulolytic subfractions tested. Addition of endo-xylanase from *A. awamori* typically resulted in slightly decreased solubilisation values for all preparations, other xylanolytic subfractions ('D1$_1$', D3$_1$') had almost no effect on 'D1' and 'D3', but did improve solubilisation by fractions from 'E' by approximately 20%.

b) palm-kernel meal

Upon incubation with the partially purified mannanolytic subfractions 'E2-I$_2$' and 'D3$_1$', 5% and 15% less solubilisation was achieved, as compared to incubation with equal amounts of mannanase activity in their parent fractions 'E2' and 'D3' (Table 3 and Fig.6). The sugar composition of digests revealed that this decrease was largely accounted for by mannose and that the major difference in composition of reaction products was the proportion of glucose, being lower for the mannanase-subfractions (not shown). 'G3', achieving 20% solubilisation of NSP, was the least effective of all enzyme fractions, when tested at equal mannanase concentration.

Pure endo-glucanases were inactive towards isolated palm-kernel mannan. When acting alone on the CWM, they released low amounts of material consisting
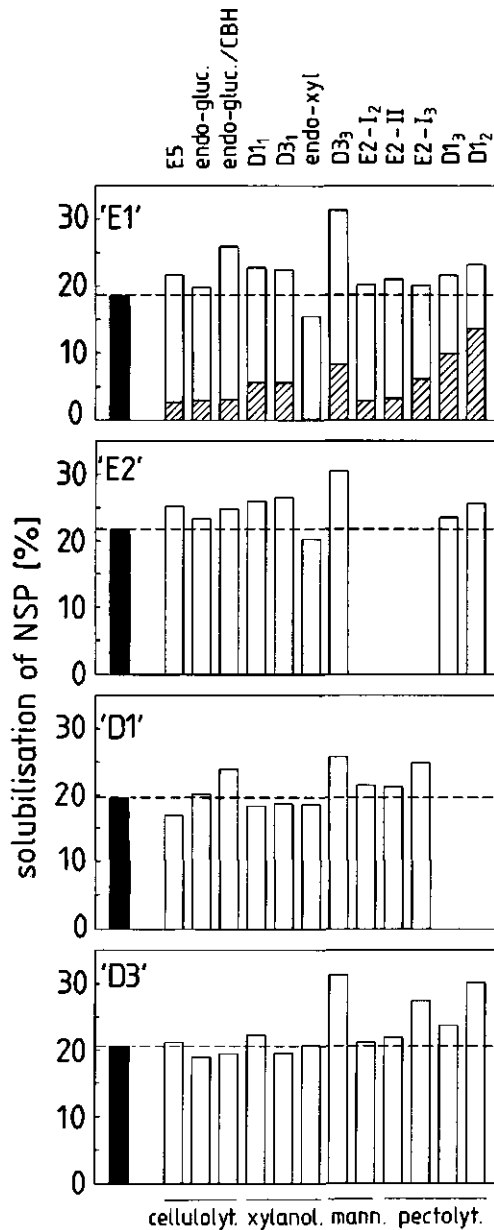
Figure 5: Solubilisation of NSP from sunflower CWM by major enzyme fractions alone and after supplementation with cellulolytic, xylanolytic, mannanolytic and pectolytic subfractions.
■ solubilisation by fraction, ▨ solubilisation by subfraction alone, □ solubilisation by combined incubation.

primarily of mannose (Table 3). Combined action of endoglucanase and CBH relatively enhanced the release of glucose as compared to their individual effects. Combinations of any of the main fractions with each other or with cellulolytic subfractions were relatively most effective for 'G3' (Fig.6). Thus, supplementation of 'G3' with glucanases resulted in more than additional improvements. In contrast, for any combination of fractions 'E2' and 'D3', the solubilisation was always less than the sum of the amounts released by the individual fractions and never exceeded an upper level of 40% to 45%. Here, a synergistic effect by supplementation with glucanases was not observed. Similarly, the presence of ß-mannosidase or α-galactosidase (combinations with 'G1') did not significantly influence the effect of any fraction.
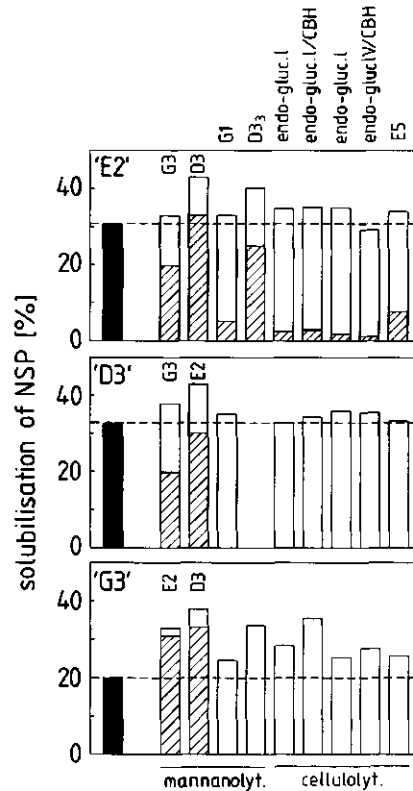

Figure 6: Solubilisation of NSP from palm-kernel CWM by major enzyme fractions alone and after supplementation with cellulolytic and mannanolytic subfractions.
◼ solubilisation by fraction, ▨ solubilisation by subfraction alone,
▭ solubilisation by combined incubation.

Table 3

Solubilisation of NSP[a] from palm-kernel CWM by subfractions or pure enzymes. Sugar composition of supernatant (mol-%) and relative solubilisation[b] per monosaccharide (data in parentheses).

| | mannanolytic | | cellulolytic | | | |
	'D3$_1$'	'E2-I$_2$'	glucanase (endo I)	glucanase (endo I/CBH)	glucanase (endo IV)	glucanase (endo IV/CBH)
rha	0.0 (0.0)	0.0 (0.0)	0.5 (6.9)	0.5 (10.4)	0.5 (5.5)	1.6 (12.1)
ara	1.4 (15.2)	1.6 (18.5)	0.0 (0.0)	0.6 (0.7)	7.2 (5.2)	7.1 (3.3)
xyl	2.1 (11.9)	0.9 (18.5)	0.0 (0.0)	0.0 (0.0)	3.0 (1.1)	4.0 (0.9)
man	85.5 (30.6)	91.9 (35.0)	94.3 (2.8)	86.0 (3.1)	73.0 (1.7)	58.1 (0.9)
gal	6.0 (51.5)	3.6 (32.8)	2.7 (1.9)	2.8 (2.4)	3.6 (2.0)	2.3 (0.8)
glc	4.9 (11.2)	2.0 (4.8)	2.5 (0.5)	10.0 (2.3)	12.7 (1.9)	26.9 (2.5)
total NSP[a]	28.4	30.3	2.4	2.9	1.8	1.2

[a] Percent of initial amount of NSP (w/w) which is solubilised.
[b] Percent of initial amount of monosaccharide (w/w) which is solubilised.

Degradation of isolated (4-*O*-methyl)-glucuronoxylan

The degradation patterns of the isolated polysaccharide by the crude preparation 'D', fractions 'D1', 'D3' and xylanolytic subfraction 'D1$_1$' on the isolated polysaccharide were found to be very similar (Fig.7). An identical pattern was obtained by incubation with endo xylanase I (*A. awamori*) or fractions 'E1' and 'E2' (not shown). The polymer was partially degraded to low molecular weight fractions (retention time > 30 min). Only 20% to 35% of the theoretically present glycosidic linkages were split and a fraction of polymeric material (ret. time 27 - 28 min) remained undegraded after extensive incubation.
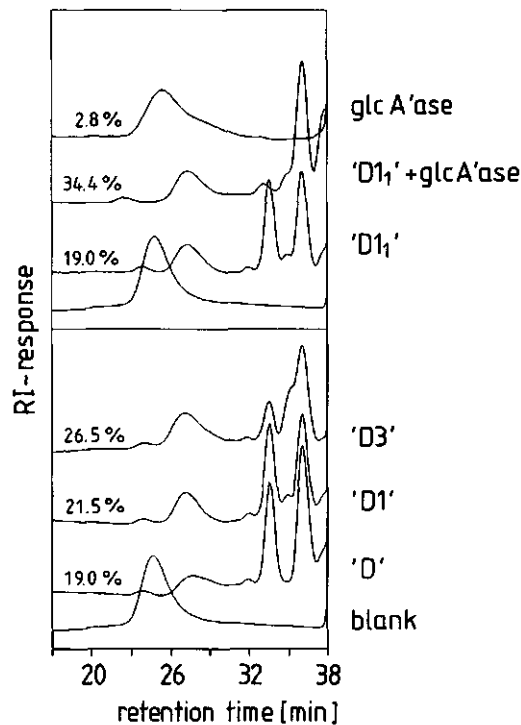
Figure 7: Molecular size distribution of (4-*O*-methyl)-glucuronoxylan from sunflower CWM and of its digests obtained from incubation with xylanolytic enzyme fractions of differing purity (HPSEC). Percentages (left) indicate the theoretical extent of hydrolysis (calculated as reducing end-groups).

The major acidic end-product formed by Driselase or its subfractions (Fig.8, retention time 34min) was identified by NMR as a tetramer (4-*O*-methyl-α-D-glucopyranosyl-(1→2)-*O*-ß-D-xylopyranosyl-(1→4)-*O*-ß-D-xylopyranosyl-(1→4)-*O*
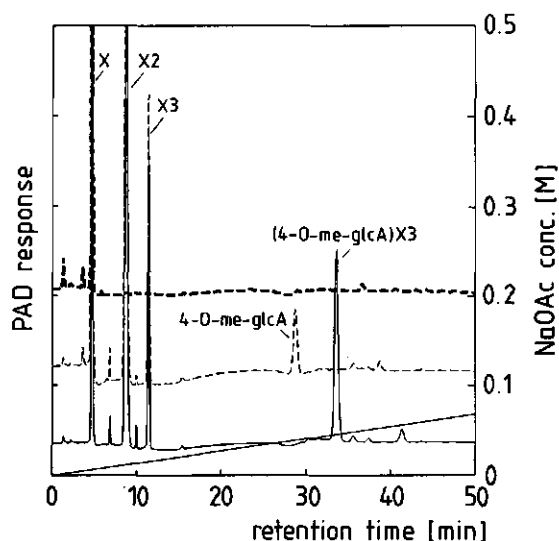
Figure 8:Reaction end-products obtained from incubation of (4-O-methyl)-glucuronoxylan with subfraction 'D1$_1$' (——), with 'D1$_1$' + α-glucuronidase (----), and with α-glucuronidase alone (– – –); NaCl gradient (——)
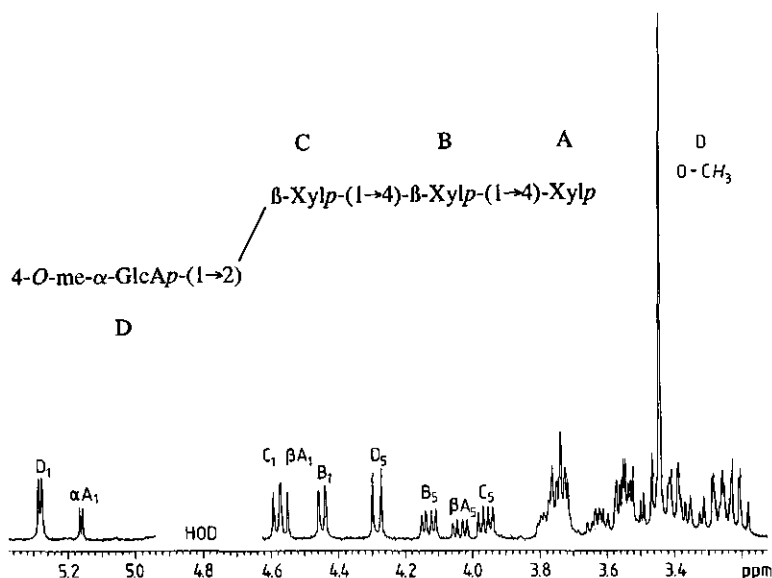

Figure 9:400-MHz ^{1}H NMR spectrum of acidic reaction product from enzymic degradation of (4-O-methyl)-glucuronoxylan. The chemical shifts (in ppm relative to the signal of TSP d_4 set at 0.00 ppm) were assigned as follows: αA$_1$: 5.17, αA$_2$: 3.54, αA$_3$: 3.74, αA$_4$: 3.74, αA$_5$: 3.80, αA$_5$,: 3.74, ßA$_1$: 4.57, ßA$_2$: 3.24, ßA$_3$: 3.53, ßA$_4$: 3.77, ßA$_5$: 4.05, ßA$_5$,: 3.37, B$_1$: 4.45, B$_2$: 3.26, B$_3$: 3.57, B$_4$: 3.77, B$_5$: 4.14, B$_5$,: 3.43, C$_1$:4.58, C$_2$: 3.39, C$_3$: 3.48, C$_4$: 3.63, C$_5$: 3.97, C$_5$,: 3.30, D$_1$: 5.29, D$_2$: 3.57, D$_3$: 3.75, D$_4$: 3.21, D$_5$: 4.28, OMe: 3.44.

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α,β-D-xylopyranoside). A detailed analysis of the ^{1}H- and ^{13}C-NMR spectra of the isolated compound in D_2O solution was achieved by the use of 2-dimensional techniques. The ^{1}H NMR data are shown in Fig.9. Xylobiose, xylose and xylotriose were encountered in decreasing amounts as neutral end products. The acidic tetramer could effectively be hydrolysed by an α-glucuronidase, as indicated by the disappearance of the tetramer-peak and the occurrence of 4-O-methyl-glucuronic acid (Fig.8).

DISCUSSION

Cellulose, (4-O-methyl)-glucuronoxylans and pectic compounds in sunflower CWM, and cellulose and mannans in palm-kernel CWM are the most relevant substrates for polysaccharidase action (Düsterhöft et al 1991, 1993b). In our studies on the effect of various enzymes in commercial broad-range preparations in cell wall degradation, we therefore focussed on activities known to attack these types of polymers. The most straightforward way to study the mode of action, product formation and synergistic effects is the use of highly purified enzymes. As shown in this study, however, the effect of single activities on insoluble, lignocellulosic substrates is very limited and can only be satisfactorily investigated by the (combined) use of relatively large amounts of pure enzymes. These, however, are not readily available. Rather than aiming at the complete purification of a whole range of cell wall degrading enzymes, our approach was to fractionate some effective crude preparations into several subfractions, enriched in 'main activities'. Thus, only few of the subfractions described here are electrophoretically pure ('E2-II', 'E2-I$_2$', 'D3$_1$'); most still contain multiple protein bands. However, as judged by activity measurements, the 'main activities' present in the subfractions have generally been purified 5- to 10-fold.

Several reports in literature describe the purification and characterisation of enzymes from $Aspergillus$ $niger$ and $Irpex$ $lacteus$, that are also used in this study. ß-D-mannanases have been purified from both microorganisms (McCleary 1979, McCleary and Matheson 1983). These enzymes have molecular masses of 53 kDa ($I.$ $lacteus)$ and 45 kDa ($A.$ $niger)$ and isolelectric points of 5.0/5.5 and 4.0, respectively. The estimated molecular masses of the (partially) purified mannanases in this report were 23.0 kDa ($I.$ $lacteus$, 'D3$_1$') and 48.0 kDa ($A.$ $niger$, 'G3') and indicate that the ß-mannanase from $I.$ $lacteus$ present in fraction 'D3$_1$' is different from the earlier described enzyme, while the ß-mannanase present in fraction 'G3'

resembles the one isolated by McCleary and Matheson (1983) from the same organism. An α-galactosidase, purified from *A. niger* (Adya and Elbein 1977), was found to be most active towards oligosaccharides but also hydrolysed polymeric substrates like guar flour and locust bean gum.

Several endo-xylanases have been purified from *I. lacteus* (Hoebler and Brillouet 1984, Brillouet 1985, Kanda *et al* 1985). According to its electrophoretic behaviour and reaction end products, the xylanase present in subfraction 'D1$_1$' has great similarities with xylanase I, described by Kanda *et al* (1985) and Hoebler and Brillouet (1984). This endo-xylanase was reported to hydrolyse larchwood glucuronoxylan to about 30%, yielding xylobiose, xylotriose and small amounts of xylose. Another xylanase (III), purified from Driselase, exhibited cellulolytic activity (Kanda *et al* 1985). This aspecific xylanase might be present in subfraction 'D3$_3$', a subfraction with xylanolytic, cellulolytic and mannanolytic activities which we did not succeed to fractionate further on the basis of charge characteristics.

I. lacteus has also been reported to produce several cellulase components, including endo-cellulases (one of which is also active on xylan, Kanda *et al* 1980, Kubo and Nisizawa 1983) and at least one exo-cellulase with high activity towards Avicel (Kanda *et al* 1978). The latter protein might be present in fraction 'D3$_3$'.
The specificities and characteristics of the above cited enzymes can be used to interpret the effects of the respective partially purified subfractions used in the present study on the cell wall materials.

We have taken into account that the use of only partially purified enzyme fractions limits the unambiguous interpretation of results. Therefore, relevant enzymes, which were available in highly purified form, though derived from different microorganisms, were included in this study.

Cellulolytic enzymes, when working individually, had little solubilising effect (Fig.s 5, 6; hatched area) and released, in addition to glucose also mannose from the CWM's (Table 2, 3). Neither endo-glucanase IV (a non-specific glucanase with activity towards xylans, Beldman *et al* 1985) nor endo-glucanase I showed activity towards the extracted palm-kernel mannan. These results suggest that the hydrolytic action of the tested glucanases on the CWM preferably takes place in non-cellulosic hetero-glucans, like glucomannans and only secondly in cellulose. Synergistic action with other polysaccharidases was only observed, when glucanases were used in combination with the mannanase-fraction 'G3' (palm-kernel CWM), achieving higher solubilisation than the sum of the individual values (Fig.6). Here, the hydrolysis of cellulose (or other glucose-containing polysaccharides) obviously enhanced the solubilisation of total NSP.

Subfraction 'D3$_3$', representing cellulolytic, mannanolytic and xylanolytic

activities of Driselase and being essentially devoid of pectolytic activity, was relatively effective on both CWM's; alone, as well as in combination with other fractions. Its superior activity might be inherent to the xylanolytic/cellulolytic enzyme system produced by this fungus (Kanda *et al* 1985), which, as outlined above, is expected to be present in this subfraction. A cellulose-degrading enzyme system with strong Avicelase-activity is likely to be superior in attacking crystalline polysaccharide structures as those present in the CWM's.

Xylanolytic activities are needed for the hydrolysis of the (4-*O*-methyl)-glucuronoxylans, which constitute a large proportion of NSP in sunflower CWM. A pure endo-xylanase I from *A. awamori* was inactive towards the CWM but able to partially hydrolyse isolated xylans from this substrate. Less purified xylanolytic subfractions solubilised about 7% of total xylose from the CWM, as compared to 11% by the crude preparations (Düsterhöft *et al* 1993b). In order to evaluate the reasons for this resistance to enzymic degradation, we also studied the hydrolysis of the isolated polysaccharide. Earlier investigations (Düsterhöft *et al* 1992) indicated that this alkali-extracted polysaccharide (with an average glucuronosyl-substitution of 10%) was composed of several populations differing in uronic acid content. Neither the xylanolytic subfraction nor the complete xylanolytic system of Driselase were able to completely hydrolyse this substrate. The accumulation of an acidic tetramer as end product indicates the lack of α-glucuronidase activity in the crude preparation. Like other α-glucuronidases described in literature (Korte 1990, Smith and Forsberg 1991), the enzyme from *T. viride* used in this study was obviously only active on oligomeric substrates. It effectively hydrolysed the tetramer, but left unattacked a residual fraction of the xylan of apparent higher molecular size. It thus did not enhance markedly the total hydrolysis. Bazus *et al* (1992) reported that the glucuronic acid substitution in xylans from sunflower hulls occurred in two distribution modes. Those fractions with highest degree of substitution (23%) were left unattacked by an endo-xylanase from *Clostridium thermolacticum*. In line with these findings, a very high degree of glucuronic acid substitution in the unattacked xylan fraction in the present study could explain its resistance to enzymic attack, as it would hinder the action of endo-xylanase and consequently that of α-glucuronidase. In the cell wall, xylans may be bound by hydrogen-bonds to cellulose, be covalently linked to lignin and carry acetyl-substituents, rendering them even more resistant to enzymic hydrolysis. The findings of Tenkanen *et al* (1992), that endo-xylanases from *T. reesei* had only little activity towards unsubstituted and consequently poorly soluble xylans, imply that insolubility represents another important limitation to xylan hydrolysis which might play a role in the CWM's.

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Mannose-based polysaccharides in sunflower CWM, where they represent a minor constituent of NSP, were quite well degraded by the mannanolytic subfractions. In palm-kernel meal, mannans of different physical structure, characterised by a high crystallinity (Daud and Jarvis 1992) occur. Regarding the reaction products formed from the latter substrate, the mannanases present in 'E2', 'D3' or 'G3' and recovered in the subfractions are very similar (Düsterhöft *et al* 1993b), all belonging to the class of endo-acting enzymes. However, when the CWM's were incubated with equal amounts of mannanase activity from different fractions, mannose-solubilisation (and consequently total NSP-solubilisation) from palm-kernel CWM decreased with increasing purity of the mannanase fraction: 'E2', 'D3' > 'E2-I_2', 'D3$_1$' > 'G3'. Two reasons might explain this finding: (i) the removal/lack of unidentified activities essential for the degradation of native mannans or (ii) synergistic effects of cellulose- and mannan-hydrolysis, like observed in combinations of 'G3' with glucanases, which are minimized or eliminated in highly purified mannanase-preparations. As supplementation of the mannanase fractions with ß-mannosidase and α-galactosidase ('G1') did not enhance the solubilisation, we conclude that neither accumulation of mannobiose nor galactose substitution hindered the overall hydrolysis of the substrate.

Pectolytic enzymes are mainly responsible for NSP solubilisation in sunflower CWM. Like other pure enzymes, the polygalacturonase 'E2-II' and arabinanases released only minor amounts of NSP. When however, polygalacturonase, arabinanases and rhamnogalacturonan-degrading fractions were added simultaneously (e.g. 'D1$_3$', 'D1$_1$'), high extents of solubilisation were obtained. Thus fraction 'D1$_3$', though devoid of cellulase activity, released as much as 13% of NSP. Obviously, the cooperative action of the different pectolytic enzymes is needed to effectively degrade the pectic compounds present in sunflower CWM.

The results obtained from supplementation experiments depend on the initial level at which enzyme activities with the same or similar specificity as the supplementing fraction are already present. Thus, combination of two pure enzymes (with very low individual effectivity) generally resulted in additional amounts of solubilisation (not shown). If, in contrast, the initial solubilisation by one enzyme fraction was moderate (though not maximal, e.g. in 'E2', 'D3'), effects may be less expressed or masked. Still, the present set of data allows to conclude that cooperative (synergistic) action of different enzymes in the hydrolysis of pectic compounds from sunflower meal and in the solubilisation of palm-kernel NSP occurs. On the other hand, by in- or exclusion of certain (groups of) enzyme activities, the hydrolysis of their target substrates can markedly be enhanced or suppressed, often without influencing the solubilisation of others. Key-enzymes

with major implications for the effectivity of the hydrolysis of the two CWM's were the pectolytic activities (polygalacturonase, rhamnogalacturonase), arabinanase, cellulases and probably mannanases with activities towards crystalline, insoluble substrates. Xylanolytic activities able to attack highly substituted or insoluble xylans and α-glucuronidases active towards polymeric substrates are believed to be able to improve the hydrolysis of the xylans.

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EVALUATION OF EXPERIMENTAL APPROACH AND IMPLICATIONS FOR DIFFERENT FIELDS OF APPLICATION

The central question which this thesis tries to answer is whether and how the enzymic solubilisation of structural polysaccharides from lignocellulosic plant residues can be enhanced and controlled. With sunflower meal and palm-kernel meal as representatives of this kind of by-products, the topic was approached along three lines:
1) detailed characterisation of the substrate
2) screening and fractionation of enzymes
3) study of enzyme-substrate interaction
In the following, important aspects concerning these three points and the implications of the results for different applications and raw materials will be discussed.

Substrate characterisation. Information in literature about the composition of various by-products is mainly restricted to the gross chemical composition (crude protein, crude fat, crude fibre, ash) or to a classification of the fibre moiety (hemicellulose, cellulose, lignin (Wolters *et al* 1992). For a number of products, additionally the non-starch polysaccharide composition of the insoluble cell wall residue has been determined (Carré and Brillouet 1986, Brillouet *et al* 1988). It is, however, impossible from this information to unambigously identify the types of polysaccharides present in a sample. Arabinose, xylose and uronic acid for example, can originate from pectic compounds, arabinoxylans or glucuronoxylans or from a mixture of them. The actual structure has important consequences concerning the choice of suitable enzyme activities for its degradation. Determination of the glycosidic linkage composition allows more precise conclusions to be drawn. The technique used, methylation analysis, was adapted by Lomax *et al* (1983) to analyse complete cell walls, rather than isolated polysaccharides. It thus provided a time-saving method, avoiding any chemical pretreatment (extraction) of the cell wall, which bears the risk of artefact formation. While Lomax *et al* (1983) and his colleagues (Gordon *et al* 1983) reported complete methylation and recovery (which they determined by the use of an internal standard) of samples from rye grass, straw and beechwood, our results obtained with different substrates, in contrast, revealed important limitations (Chapter 3). Complete methylation of the essentially insoluble samples was difficult to achieve and modifications were necessary. Moreover, if methylated samples are worked up by extraction with an organic solvent (the preferred method used by the majority of scientists), unmethylated compounds are excluded from analysis because they preferentially solubilise in the water phase. Consequently erroneous results will be obtained. Although we cannot explain the divergence of our results

with those from Lomax and co-workers other than by being strongly substrate-dependent (mannans in palm-kernel meal for example, unlike cellulose, were extremely resistant to methylation), we recommend the use of dialysis instead of phase-partitioning to ensure exact presentation of results.

In this thesis, results were further confirmed by parallel analysis of extracted polysaccharides. Comparing these results enabled not only the identification of all major polysaccharides present in the meals but also a realistic estimation of their quantities.

Enzyme characterisation. To identify major enzyme activities relevant for the degradation of the meals, several commercial enzyme preparations were selected after a preliminary screening. They were fractionated by ion-exchange chromatography and the major activities of the most relevant fractions were further purified (Chapter 6). Rather than aiming at the purification to homogeneity of a whole range of activities, our approach was to fractionate into 'groups' of NSP-degrading enzymes, like cellulolytic, mannanolytic, pectolytic and xylanolytic. Comparing the solubilising effect of crude preparations, fractions and (partially) purified subfractions enabled to evaluate the contribution of each of these 'groups' to the overall solubilisation. Only few of the subfractions, however, were electrophoretically pure and consequently the unambiguous interpretation of results was limited by the presence of side-activities. The alternative, the use of highly purified enzymes, was unfeasible for two reasons: Amounts and different types of highly purified enzymes needed for the degradation of the complex insoluble substrates are not normally available and, as became clear from the results obtained with a few highly purified enzymes from other sources, these were very ineffective, even in combination. Moreover, new enzymes with as yet unidentified specificities can only be detected by the former approach.

Enzyme-substrate interaction. The study of major reaction parameters, like the kinetics of degradation, influence of enzyme concentration and particle size of the substrates, gave results which are characteristic for the enzymic degradation of lignocellulosic materials: The accessibility of the substrate was a major factor limiting enzymic degradation (Chapter 4). This implies that physical or chemical pretreatments are necessary to achieve significant improvements in total solubilisation. The maximum degree of solubilisation determined in this study is an overestimation of what realistically may be expected under industrial conditions. Milling to a particle size as fine as in the present study is unfeasible on a technical scale and can only serve for analytical purposes. Surprisingly, in contrast to many

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reports in literature (Ford 1978, Morrison 1988 and 1991), the high lignification of the substrates did not directly influence enzymic degradation. Poor correlation between degree of lignification and degradability was also observed by Al Katrib *et al* (1988) in a study on wheat straw. This leads us to conclude that there is a high variability in the susceptibility of different raw materials to pretreatment methods.

Besides the above mentioned pretreatments, we think that an optimisation of the enzyme composition might enhance the effectivity of NSP hydrolysis. The investigation of reaction products revealed that especially the major constituents (cellulose, mannans and (4-*O*-methyl)-glucuronoxylans) were least degradable (Chapter 4 and 5). These polysaccharides may be classified as linear, insoluble polymers with little substitution and with a high ability to associate or, as in the case of cellulose and mannans, to adopt crystalline conformations. To attack these structures, enzyme systems with high specificity for the degradation of such resistant substrates are required. The degradation of cellulose has been extensively studied and enzyme components with high specificities for the crystalline regions ('Avicelase') have been identified (Coughlan 1990 and references therein). To date, this information is lacking for xylan and mannan-degrading enzymes. Screening methods mostly rely on (partially) soluble substrates (e.g. galactomannans, xylan from oat spelt). While it is realised that the complexity of xylan structure due to various side-chains inhibits the action of endo-xylanases and requires the presence of several side-chain degrading activities (Biely 1985), only recently the resistance of linear, unsubstituted (and presumably insoluble) xylans has been addressed (Tenkanen *et al* 1992). However, this might be one of the most important features for the enzymic saccharification of (lignocellulosic) biomass. In this study, enzymic treatment was performed at slightly acidic conditions (pH 5), as preliminary studies had shown a broad pH-optimum of the multi-component enzyme preparations. Regarding our results, however, a process under slightly alkaline conditions, combining a mild pretreatment effect with enzymic degradation, might enhance the efficacy of the treatment. For such processes, enzymes with alkaline pH-optima, to date mainly used in the detergent and pulp and paper industry, need to be selected and produced. Also, screening for enzymes with very low molecular masses seems promising, as these would perhaps be superiour in attacking and penetrating the firm cell wall structures investigated here. However, in only one instance so far a xylanase of very low molecular mass (5kDa to 9kDa) has been found (Iowa State University Research Foundation Inc. 1988).

Implications for different fields of application

The benefit obtained from enzymic treatment of the meals in terms of

saccharification yield, which is the most important parameter for a use as chemical feedstock or as direct energy source, is marginal, especially at larger particle-size. Important implications may, however, be expected in the field of animal nutrition. Although we have not conducted animal trials, some aspects will be discussed on the basis of recent literature. As outlined above, the direct benefit of enzyme supplementation of the meals in terms of metabolisable energy will be small and depend on the way of application: as a pretreatment or as a feed additive with action in the alimentary tract of animals. Xylose, arabinose and uronic acids cannot be utilised effectively by monogastric animals and may even exert detrimental effects in poultry and pig diets (Longstaff *et al* 1988, Schutte *et al* 1991 and 1992). Enzyme supplementation of diets with a high percentage of sunflower meal might even enhance these negative effects. The results presented in Chapter 6 indicate that, in sunflower meal, the degradation of different types of polysaccharides occurred almost independently from each other. It thus should be possible to circumvent the production of monosaccharides deriving from pectic compounds by exclusion of pectolytic enzymes without severely inhibiting the degradation of other polysaccharides.

Only recently, increasing evidence has been presented for working mechanisms of exogeneous enzymes other than the direct release of metabolisable sugars. Best described is the antinutritional effect of arabinoxylans and ß-glucans in cereal-based diets. This can effectively be overcome by supplementation with 'pentosanases' and ß-glucanases (Annison and Choct 1991, Petterson and Åman 1988). Morgan *et al* (1992) have emphasised the importance of oligosaccharides and their physiological effects for human and animal nutrition. Dietary oligosaccharides have been shown to selectively stimulate the growth of beneficial bacterial species in the gastrointestinal tract and to act as receptor analogues to which pathogens or enterotoxins might bind competitively. In the context of this thesis especially the effect of mannose and manno-oligomers on the intestinal microflora is interesting (Oyofo *et al* 1989, Izat *et al* 1990).

The examples discussed here show that the formulation of enzyme preparations is very important if type and extent of NSP hydrolysis are to be controlled, and that the development of tailored products merits attention. Monitoring the product formation upon enzymic treatment of feed compounds and characterisation of reaction products are thus essential. For this, high-performance anion-exchange chromatography, as used in this study, is a very versatile tool enabling analytical and preparative work with an outstanding resolution.

Finally, the question arises, whether and how the present study can account for other raw materials than the ones described here. Undoubtedly, the studies

presented in this thesis are labourious and not readily repeated with a large number of by-products. Although we tried to gain representative information by choosing raw materials from different botanical classes, the diversity encountered in the NSP composition of by-products limits the generalisation of our results. However, by gathering data from literature, much information about major classes of polysaccharides, their structure and enzymic degradation can be obtained. For those raw materials where information is lacking, sugar composition and linkage analysis are minimum information needed. Despite the diversity, however, some general principles have emerged from the present study, which will facilitate the evaluation of enzymic treatment for any raw material. The amounts and types of soluble and insoluble NSP and the type of botanical tissues present are the major parameters to be measured. They will essentially govern the enzymic hydrolysis. The determination of these parameters can be achieved in reasonable time-scales though it requires the use of sophisticated and expensive instrumentation.

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SUMMARY/ SAMENVATTING

Non-starch polysaccharides (NSP) constitute a potentially valuable part of plant by-products deriving from the food and agricultural industries. Their use for various applications (fuel, feed, food) requires the degradation and modification of the complex plant materials. This can be achieved by enzymatic processes which, in comparison with chemical or physical methods, are regarded as energy-saving and non-polluting. However, a major disadvantage of enzymic processes often is their low effectivity and consequently high costs.

The investigations described in this thesis were conducted to understand the reasons for the low susceptibility to enzymic hydrolysis of such by-products and, in particular, of their non-starch polysaccharides, and to find out whether and how the efficacy of enzymic treatment could be enhanced. The studies should provide information necessary for the development of polysaccharidase-preparations, tailored for the use in different applications.

Sunflower (*Helianthus annuus* L.) meal and palm-kernel (*Elaeis guineensis* Jacq) meal, by-products from the production of edible oil and used as animal feed compounds, were chosen for our studies.

In **chapter 1** an introduction is given to the macroscopic and microscopic structure of the raw materials, to plant cell walls and their constituent polymers. The biodegradation of cell walls and its limitations are briefly reviewed and major non-starch polysaccharide degrading enzymes are summarised. Chapter 1 closes with an outline of the thesis.

For a detailed study of type and structure of the non-starch polysaccharides, cell wall materials (CWM) were prepared from the meals by enzymatic digestion of protein and removal of small amounts of buffer-extractable material (**chapter 2**). The resulting CWM's were enriched in NSP (55% of sunflower CWM and 75% of palm-kernel CWM) and had a high lignin content. Two different chemical methods, sequential extraction with alkali and sodium chlorite and treatment with 4-methylmorpholine-N-oxide (MMNO) were tested to extract all constituent polysaccharides. Almost complete dissolution could be achieved by a combination of both methods, but the recovery of sugars, especially during MMNO treatment, was low. From the sugar composition of polysaccharide fractions, obtained by sequential chemical extraction, a tentative identification of major polysaccharides was achieved. Their distribution in different botanical fractions of the meals could be deduced by comparison with data from literature (sunflower) or by own experiments (palm-kernel).

The polysaccharide extracts of different purity were further fractionated by graded precipitation with ethanol, size-exclusion or adsorption-chromatography. By determination of the sugar- and glycosidic linkage composition of extracts,

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(partially) purified subfractions and intact cell wall materials, the identification, partial characterisation and quantification of major non-starch polysaccharides were achieved (**chapter 3**). In sunflower meal, cellulose (42% of NSP), pectic polysaccharides (24%) and (4-*O*-methyl)-glucuronoxylans (24%) with about 10% glucuronosyl-substitution were major constituents. Minor amounts of (gluco)mannans (5%) and fucoxyloglucans (4.5%) were also identified. Major polysaccharides in palm-kernel meal were mannans (78% of NSP) with very low degrees of galactose-substitution and of apparently small molecular size (DP 12 to 14), and cellulose (12%). Arabinoxylans (3%) and (4-*O*-methyl)-glucuronoxylans (3%), deriving from the endocarp fraction of the meal, were present in low amounts in this monocotyledenous material.

For a study of the enzymic hydrolysis of the cell wall materials (**chapter 4**), three multi-component enzyme preparations were chosen. Solubilisation occurred as a bi-phasic process with high reaction velocities in the first stage of the incubation and only slow progress during extended incubation up to 72h. The solubilisation could markedly be improved by reduction in particle size; partial delignification or increasing enzyme concentration, however, had almost no effect. Maximally 30% of NSP in sunflower meal and 50% in palm-kernel meal could be solubilised from the finely milled CWM's. Although the composition of the enzyme preparations was found to influence the type of reaction products, the extent of their release and, as observed by transmission electron microscopy, the site of enzymic attack in different cell wall layers, our results suggested that substrate accessibility was the major factor limiting enzymic hydrolysis.

A detailed study of the reaction products obtained by incubation with the crude enzyme preparations or fractions thereof (prepared by anion-exchange chromatography) revealed, that pectic compounds and mannose-containing polysaccharides in sunflower CWM were readily degradable (**chapter 5**). The hydrolysis of mannans in palm-kernel CWM varied from 20% to 50%. In both CWM's, xylans and cellulose were most resistant to hydrolysis. The results indicate the preferential degradation of parenchyma and endosperm tissues and the resistance of hull and endocarp fractions to enzymic hydrolysis. The reaction products formed during all stages of the treatment were of small oligomeric and monomeric size.

The contribution of different enzyme activities to the total solubilisation achieved by the heterogeneous enzyme preparations was studied with (partially) purified subfractions which were prepared by various chromatographic techniques from the crude preparations, and with highly purified enzymes from other microbial sources (**chapter 6**). In general, the effect of these purified enzyme

fractions was low (solubilisation of NSP: 1% to 5%). Supplementation of main enzyme fractions with pectolytic, cellulolytic or mannanolytic subfractions did only slightly enhance the total solubilisation. Synergistic action was observed between glucanases and mannanases in palm-kernel incubations and between arabinanases, polygalacturonases and rhamnogalacturonan-degrading enzyme fractions in the hydrolysis of pectic polysaccharides in sunflower CWM. The enzymic hydrolysis of (4-O-methyl)-glucuronoxylans was studied *in-situ* and with the isolated polysaccharide. The results indicated that the resistance of the xylans to enzymic degradation is not only due to their interlinkage with other polymers and location in the cell wall but also to their primary structure.

In **chapter 7**, important aspects concerning the approach and the methodology used are discussed. Implications arising for different fields of application are shown and suggestions for the formulation of enzyme preparations, which merit further research, are made.

SAMENVATTING

Niet-zetmeel polysacchariden (NSP) zijn een potentieel waardevol bestanddeel van bijprodukten uit de levensmiddelenindustrie en de agrarische sektor. Om de NSP te kunnen benutten voor diverse doeleinden (zoals brandstof, veevoer, ingrediënt voor levensmiddelen) is het noodzakelijk, om de komplexe planteweefsels af te breken of te modificeren. Dit kan bereikt worden door enzymatische processen, die, in vergelijking met chemische of fysische methodes, het voordeel hebben dat zij weinig energie vergen en niet milieu-belastend zijn. Een belangrijk nadeel van enzymatische omzettingen is dat zij vaak weinig effektief zijn, veel tijd vereisen en daarom met hoge kosten gepaard gaan.

Het in dit proefschrift beschreven onderzoek werd uitgevoerd om 1) inzicht te verkrijgen in de oorzaken voor de geringe enzymatische afbreekbaarheid van zulke bijprodukten, en met name van de niet-zetmeel polysacchariden hierin en 2) te bestuderen of en hoe de effektiviteit van de enzymatische hydrolyse verhoogd kan worden. Zodoende trachtten wij informatie te verkrijgen, die het mogelijk zou maken om voor verschillende doeleinden 'op maat' samengestelde enzympreparaten te ontwikkelen.

Zonnepitschroot (*Helianthus annuus* L) en palmpitschroot (*Elaeis guineensis* Jacq), bijprodukten die ontstaan bij de produktie van plantaardige spijsoliën en die onder andere als veevoederbestanddeel gebruikt worden, werden als grondstoffen voor dit onderzoek gekozen.

In **hoofdstuk 1** wordt een introduktie gegeven over de makroskopische en mikroskopische struktuur van de grondstoffen, over plantecelwanden en de polymeren waaruit zij opgebouwd zijn. Zowel de biodegradatie van celwanden en belemmerende faktoren hierbij, als ook de benodigde niet-zetmeel polysaccharide splitsende enzymen worden kort beschreven. Het hoofdstuk sluit met een overzicht van de hoofdlijnen van het hierna beschreven onderzoek.

Om de verschillende soorten NSP en hun struktuur goed te kunnen bestuderen werd door middel van enzymatische eiwithydrolyse en het uitwassen van kleine hoeveelheden buffer-extraheerbare komponenten een zogenoemd "celwandmateriaal" (CWM) bereid (**hoofdstuk 2**). Deze CWM's werden gekenmerkt door een verhoogd gehalte aan NSP (55% in zonnepitschroot en 75% in palmpitschroot) en lignine. Om alle in het CWM aanwezige polysacchariden te extraheren en in oplossing te brengen, zijn twee methodes getest: sequentiële extraktie met alkali en natriumchloriet, en een behandeling met 4-methylmorpholine N-oxide (MMNO). Bijna volledige oplossing van de CWM's kon door een kombinatie van beide methodes bereikt worden, maar de recovery van de suikers,

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met name bij de MMNO behandeling, was laag. Door bestuderen van de suikersamenstelling van de verschillende, door sequentiele extraktie verkregen polysaccharide frakties werd een tentatieve identificatie van de meest belangrijke polysacchariden in de grondstoffen mogelijk. De verdeling van deze polysacchariden in verschillende botanische frakties van de grondstoffen kon afgeleid worden uit literatuurgegevens (voor zonnepitschroot) en eigen aanvullende experimenten (voor palmpitschroot).

Polysaccharide-frakties van uiteenlopende zuiverheid werden verder gefraktioneerd m.b.v. ethanol precipitatie, gelfiltratie- of adsorptie-chromatografie. Door bepaling van de suikersamenstelling en van de types en hoeveelheden glycosidische bindingen in extrakten, partieel gezuiverde subfrakties of in het intakte celwandmateriaal werd het mogelijk, de belangrijkste polysacchariden in de grondstoffen te identificeren, gedeeltelijk te karakteriseren en te kwantificeren (**hoofdstuk 3**). Hoofdbestanddelen van NSP in zonnepitschroot waren cellulose (42% van de totale hoeveelheid NSP), pektine bevattende polysacchariden (24%) en (4-O-methyl)-glucuronoxylanen (24%), die voor ongeveer 10% gesubstitueerd waren. (Gluco)mannanen en fucoxyloglucanen werden eveneens, maar in lage concentratie, aangetoond (5% en 4.5%). De meest belangrijke polysacchariden in palmpitschroot waren mannanen (78% van NSP) met zeer geringe galactose-substitutie en bijzonder laag molekuulgewicht (polymerisatiegraad 10-14), en cellulose (12%). Arabinoxylanen (3%) en (4-O-methyl)-glucuronoxylanen (3%), beiden afkomstig uit de endocarp fraktie van het schroot, konden eveneens aangetoond worden.

Om de enzymatische afbraak van de celwandmaterialen te bestuderen (**hoofdstuk 4**), werden drie commerciële polysaccharidase-preparaten met breed werkingsspectrum gekozen. Het in oplossing brengen van de suikers verliep in twee fases, gekenmerkt door een hoge initiële reaktiesnelheid en slechts geringe toename van reaktieprodukten in latere stadia van de incubatie (tot 72 uur). Het oplossend vermogen kon duidelijk verbeterd worden door verkleining van de deeltjesgrootte van de substraten. Gedeeltelijke verwijdering van lignine of verhoging van de enzymkoncentratie daarentegen hadden nauwelijks effekt. Maximaal 30% van de NSP van zonnepit-CWM en 50% van palmpit-CWM konden uit zeer fijngemalen substraten in oplossing gebracht worden. Alhoewel de samenstelling van de enzympreparaten duidelijk het soort en de hoeveelheid reaktieprodukten en, zoals bleek uit elektronen-mikroskopische opnames, de plaats van de enzymwerking in verschillende celwandlagen beïnvloedde, konkluderen we uit de resultaten dat de beperkte toegankelijkheid van de substraten voor de enzymen de meest limiterende faktor was.

Een gedetailleerd onderzoek naar de reaktieprodukten die ontstaan na incubatie met de technische enzympreparaten of met frakties ervan (door ionenwisselingschromatografie verkregen) liet zien dat pektine- en mannose-bevattende polysacchariden (in zonnepit-CWM) het best afbreekbaar waren (**hoofdstuk 5**). De hydrolyse van mannanen uit palmpit-CWM varieerde van 20% tot maximaal 50%. In beide CWM's behoren xylanen en cellulose tot de moeilijkst afbreekbare polysacchariden. De verkregen resultaten wijzen er op, dat enzymatische werking met voorkeur in parenchym- en endosperm-celwanden plaatsvindt en dat de dop-fraktie in zonnepit-CWM en de endocarp-fraktie van het palmpit-CWM slechts matig afbreekbaar waren. De reaktieprodukten die gevormd werden hadden gedurende de gehele incubatie-periode een gemiddelde molecuulgrootte van 1 tot 3 suikereenheden.

Om de bijdrage van de onderlinge enzymaktiviteiten in een commerciëel enzympreparaat aan de enzymatische hydrolyse te evalueren, werden de meest effektieve enzym-frakties verder gezuiverd m.b.v. verschillende chromatografische technieken, en de CWM's opnieuw met deze subfrakties en ook met reeds gezuiverde cellulases, xylanases en arabanases van andere mikrobiële afkomst geïncubeerd (**hoofdstuk 6**). In het algemeen was de werking van deze frakties zeer gering: slechts 1% tot 5% van het NSP kon in oplossing gebracht worden. Ook supplementering van enzymfrakties met pektolytische, cellulolytische en mannanolytische subfrakties kon de werking nauwelijks verbeteren. Bij de enzymatische hydrolyse van palmpit-CWM werd synergistische werking tussen glucanases en mannanases geconstateerd. Ook arabinanases, polygalacturonases en rhamnogalacturonan-afbrekende enzymaktiviteiten bleken in zonnepit-CWM synergistisch te werken bij de hydrolyse van de pektine bevattende polysacchariden. De enzymatische hydrolyse van (4-*O*-methyl)-glucuronoxylanen werd *in-situ* én met het geëxtraheerde polysaccharide bestudeerd. De verkregen resultaten duiden erop dat de geringe enzymatische afbreekbaarheid van deze polysacchariden niet alleen veroorzaakt wordt door hun lokatie in de celwand en de interakties met andere celwandpolymeren, maar ook door hun primaire struktuur.

In **hoofdstuk 7** tenslotte, worden belangrijke aspekten betreffende de onderzoeksaanpak en de gebruikte analysemethodes aan de orde gesteld. De betekenis van de in dit proefschrift beschreven resultaten voor verschillende toepassingsgebieden (o.a. de veevoeding) wordt bediscussieerd en suggesties voor de samenstelling van enzympreparaten, die verdere aandacht en onderzoek verdienen, worden gegeven.

Curriculum Vitae

The author of this dissertation was born 7 may 1958 in Berlin-Dahlem, Germany. In 1978 she finished school at Altkönig-Gymnasium (Kronberg/Ts., Germany) with the final exam. The same year the author started to study Food Technology at University Hohenheim, Germany. In 1985 this study was completed with receiving the degree "Diplom-Lebensmittel-Ingenieur". From 1985 to 1987 she joined the pet-food producing company EFFEM GmbH (Minden, Germany) as R & D employee, working in the fields of product development and quality assurance. From 1987 to 1991 the author was affiliated with HENDRIX Nutrition Nederland B.V., (Boxmeer, The Netherlands). In the same period the research described in this thesis was carried out at the Department of Food Chemistry and -Microbiology, (Agricultural University, Wageningen) where the author was stationed. Since July 1991, she is employee of the Agricultural University Wageningen, working on a short term project (post-doc) in the field of enzymology at the Department of Food Chemistry- and Microbiology.