Isolation and properties of a feruloyl esterase from *Aureobasidium pullulans* and its mechanism in lignocellulose degradation

by

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Promoter: Professor Bernard Prior

Co-promoter: Professor Karl-Heinz Robra

Declaration

I, the undersigned hereby declare that the work contained in this dissertation is my original work and I have not previously in its entirety or in part been submitted it to any university for a degree.

Abstract

The production, purification and functional characterisation of feruloyl esterase from *Aureobasidium pullulans* were set as the primary objectives of this study. A further objective was to investigate a possible co-operative effect with other selected lignocellulolytic enzymes on substrates relevant to industry.

In a comprehensive review, feruloyl esterases from various micro-organisms were compared both functionally and with regard to their primary structure, where applicable. Feruloyl esterases show intriguing differences in substrate specificity and sequence structure. Enzymes that are closely related regarding their amino acid sequence exhibit different substrate specificities. Sequence similarities can be found with a range of other enzyme families, including serine esterases, acetyl xylan esterases, lipases, tannases, glycosyl hydrolases and xylanases. More data on the three dimensional structure of feruloyl esterases as well as an examination of all available feruloyl esterases with the same substrates is necessary before structure-function relationships can be established and before the feruloyl esterases can be organized into discrete families based on ancestral origins.

The highest production levels of feruloyl esterase by *A. pullulans* are achieved when grown on birchwood xylan. Expression was not repressed when glucose or xylose was present in the medium. However, free ferulic acid supplemented to the medium affected fungal growth and therefore did not increase feruloyl esterase activity. It is also suggested that the synthesis of feruloyl esterase is independently regulated from xylanase synthesis. Feruloyl

esterase from *A. pullulans* acts on α- and β-naphthyl acetate, as well as naphthol AS-D chloroacetate as substrates.

Feruloyl esterase from *A. pullulans* was purified to homogeneity using ultrafiltration with high molecular weight cut-off, anion exchange, hydrophobic interaction and ultimately gel filtration chromatography. With a molecular weight of 210 kDa, the enzyme is the largest of the feruloyl esterases reported to date. Kinetic data was produced using both synthetic and natural substrates. *A. pullulans* feruloyl esterase shows properties similar to other fungal feruloyl esterases, especially from *Aspergillus niger* cinnamic acid esterase and *Penicillium funiculosum* feruloyl esterase B. The N-terminal sequence of *A. pullulans* feruloyl esterase was identified, but no similarities to known enzyme families were found. Peptide mass mapping did not reveal structural information.

In an effort to evaluate the significance of feruloyl esterase from *A. pullulans* in the degradation of lignocellulose, dissolving pulp and sugar cane bagasse were selectively treated using feruloyl esterase and hemicellulolytic enzymes. The enzymatic degradation reaction was monitored using microdialysis sampling, anion exchange chromatography, online desalting and mass spectrometry. It has been shown, that feruloyl esterase activity together with xylanase activity releases monosaccharides from both substrates. Sugars of higher degree of polymerisation were not released, giving evidence for the recalcitrance of the material. The fibre architecture of the substrates was apparently not accessible to the enzymes and therefore complete hydrolysis was hindered.

Opsomming

Die produksie, suiwering en funksionele karakterisering van feruloïel esterase afkomstig van *Aureobasidium pullulans* was die primêre doelwitte van hierdie studie. `n Verdere doelwit was om vas te stel of daar `n koöperatiewe effek met ander geselekteerde lignosellulitiese ensieme op substrate wat industrie-relevant is, bestaan.

Die feruloïel esterase van verskillende mikro-organismes is vanuit die oogpunt van funksie en primêre struktuur omvattend met mekaar vergelyk, waar toepaslik. Interessante verskille tussen die substraat spesifisiteit en volgorde-struktuur van feruloïel esterase kan waargeneem word. Ensieme wat nou aanmekaar verwant is wat hul aminosuurvolgorde betref, het duidelik verskillende substraatspesifiteite. Volgordeverwantskap kan in `n reeks van ander ensiemfamilies, insluitende serienesterase, asetielxilaanesterase, lipases, tannases, glikosielhidrolases en xilanases vasgestel word. Meer inligting oor die driedimensionele struktuur van feruloïel esterase asook `n analise van al die beskikbare feruloïel esterase met dieselfde substrate is nodig voordat struktuur-funksie verwantskappe vasgestel kan word en voordat die feruloïel esterases in eie families op die grond van hul oorsprong georganiseer kan word.

Die hoogste produksie vlakke deur feruloïel esterase van *A. pullulans* word bekom deur dit op berkhoutxilaan te groei. Ekspressie was nie onderdruk wanneer glukose of xilose in die medium aanwesig was nie. Wanneer vrye feruliensuur by die medium bygevoeg is, is die fungale groei beïnloed en het die feruloïel esterase aktiwiteit nie vermeerder nie. Dit word ook voorgestel

dat die sintese van feruloïel esterase onafhanklik deur xilanase sintese gereguleer word. Feruloïel esterase van *A. pullulans* reageer op α- en β-naftolasetaat, asook naftol AS-D chloroasetaat as substrate. Feruloïel esterase van *A. pullulans* is tot homogeniteit deur ultrafiltrering met `n hoë molekulêre gewiggrens, anioonuitruiling, hidrofobiese interaksie en eindelik gelfiltrasie-chromatografie gesuiwer. Met 'n molekulêre gewig van 210 kDa, is die ensiem die grootste van die feruloïel esterases tot dusver beskryf. Kinetiese data is met behulp van sintetiese en natuurlike substrate geproduseer. *A. pullulans* feruloïel esterase het eienskappe wat vergelykbaar is aan die van ander fungal feruloïel esterases, veral die wat afkomstig is van *Aspergillus niger* sinnamiensuur esterase en *Penicillium funiculosum* feruloïel esterase B. Die N-terminale volgorde van *A. pullulans* feruloïel esterase is identifiseer maar geen ooreenkoms aan bekende ensiemfamilies kon vasgestel word nie. Peptiedmassakaartering kon ook geen strukturele inligting gee nie.

Oplosbare pulp en suikerrietbagasse is geselekteerd met behulp van feruloïel esterase en lignosellulitiese ensieme behandel om die belang van feruloïel esterase van *A. pullulans* in die afbraak van lignosellulose vas te stel. Die hidroliese-reaksie is deur mikrodialise monsterneming, anioonuitruilings-chromatografie, oplyn ontsouting en massaspektrometrie gemonitor. Wanneer die aktiwiteit van feruloïel esterase met die van xilanase gekombineer is, is monosakkariede deur albei substrate afgeskei. Suikers met 'n hoër graad van polimerisering is nie afgeskei nie, wat 'n bewys van die materiaal se weerstandbiedendheid is. Dit het geblyk asof die vesel-argitektuur van die

verbruikte substraat nie toeganklik was vir ensieme nie en dus is algehele hidroliese verhinder.

Biographical sketch

Karl Rumbold was born in Klagenfurt, Austria, on March the 6th, 1973, where he completed primary and secondary education in 1991. He studied Biology at the Universität Graz, Austria, and received his MSc in 1999.

List of manuscripts

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CHAPTER 1

Introduction

Scope

Plant biomass is the only foreseeable sustainable source of organic fuels, chemicals and materials (Lynd et al., 1999). As the primary component of the biosphere, biomass is also an industrial raw material compatible with human and other life forms. Because of the CO_2 consuming character of plant growth, biomass-based processes and products can be incorporated into nature's photosynthesis driven carbon cycle with lifecycle greenhouse gas emissions approaching zero. The products of biotechnological biomass processing are typically biodegradable and non-toxic.

Cellulosic biomass (e.g. grass or woody materials) is particularly well suited for production of commodity products because of its low price and large potential supply as compared to grains or cane sugar. However, the recalcitrance of cellulosic biomass makes it more challenging to process in a cost-effective manner (Perez et al., 2002). Possible sources of cellulosic biomass include residues arising from the agricultural or forest products industries, and "energy crops" grown primarily for use as industrial raw materials.

South Africa has a long tradition in the biotechnological research of biomass conversion, which can be traced back to the early 1970s. During that time, the motivation for research was a response to targeted sanctions against the Apartheid regime and the oil crisis, with the objective of finding ways of making the country independent of oil imports. Since the beginning of the 1990s, with the changing political climate and the lifting of sanctions, a redirection of the research effort took place mainly to introduce

biotechnological applications to benefit the country's large pulp and paper industry as well as the animal feed industry (Christov and Prior, 1998).

Initially, South African research efforts in the field of lignocellulose biotechnology could be described as isolated. However, there has been a marked increase in international co-operation and networking with worldwide bio-energy research groups, which has led to another turning point in South African biotechnology since the late 1990s. The main topic of these various forms of co-operation has been the attempt to identify biotechnological bottlenecks in lignocellulose degradation and optimising the technology for various industries (Lynd et al., 2002).

Aim and outline

The outline for this thesis was conceived during a period of increased co-operation (IEA Bioenergy Conference, South Africa, 1999) and represents an effort to contribute to the issues of characterising enzymes produced by plant-invading fungi, enzymatic degradation of recalcitrant lignocellulose and analysis of carbohydrates. The aim of the study is to investigate the effectiveness of the black-yeast *Aureobasidium pullulans* in the degradation of lignocellulose. *A. pullulans* belongs to the phylum Ascomycota and is an ubiquitous saprophyte found in many different habitats. Under the microscope, *A. pullulans* shows chains of 1- to 2-celled, darkly pigmented arthroconidia and numerous hyaline, single-celled, ovoid-shaped conidia (ameroconidia), which are produced on short denticles. Colonies are fast growing, smooth, covered with slimy masses of conidia, cream or pink to brown or black, depending on the variety: *A. pullulans* var. *pullulans,* with a colony which remains pink, light brown, or yellow for at least three weeks, and

A. pullulans var. *melanogenum* which soon becomes black or greenish-black due to dark hyphae which often fall apart into separate cells. The growth temperature ranges from 2-35°C, the optimal growth temperature is between 25°C and 29°C (de Hoog and Hermanides-Nijhof, 1977). The fungus is well established in applied biotechnology (Deshpande et al, 1992), mainly in the industrial production of pullulan. *A. pullulans* produces a whole range of hemicellulolytic enzymes, most of which are induced when grown on xylan with xylanase being produced at high level with high specific activity (Leathers, 1986). Previously, this fungus was identified to be valuable for application in biodegradation processes, particularly in the bleaching of sulphite pulp (Christov and Prior, 1996). From all the enzymes produced by *A. pullulans*, i.e. the lignocellulolytic cluster, the main focus of this thesis rests on the feruloyl esterase activity. This enzyme was discovered in this fungus during the course of this study. The purpose of the study is to produce, purify and functionally characterise the enzyme. A further objective is to study a possible co-operative effect with other selected lignocellulolytic enzymes.

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Chapter 2

Feruloyl esterases and their role in lignocellulose breakdown

Introduction

Lignocellulose, an organic substance isolated from plants, mainly consisting of cellulose hemicellulose and lignin, is considered to be the most important energy and commodity source for the future (Berndes et al., 2001). At present, lignocellulose represents a raw material for the paper, pulp and fibre industry, the food industry, and the animal feed industry. The relative abundance of lignocellulose has resulted in extensive biological and chemical investigations on the material. Lignocellulose from wood and from grasses and cereals is in the centre of interest for biotechnological applications. With the advent of enzyme technology, new methods for the extraction of cellulose, the digestion of forages and the production of dietary fibre were created (Breen and Singleton, 1999; Martin et al., 2002; Tengerdy and Szakacs, 2003). One of the key issues in today's investigations is to convert plant biomass to bioenergy and biocommodities (Himmel et al., 1999; Lynd et al., 1999). Thus, lignocellulose degrading microorganisms, their enzyme systems, and the plant cell wall are examined extensively, always with the ultimate motive to apply the knowledge to the numerous existing and prospective industries. A major challenge is always the recalcitrance and poor solubility of plant cell wall material when it comes to the enzymatic degradation of lignocellulose. The extent of wall digestibility by microorganisms may well be dependent upon the cross-linked nature of wall polysaccharides (Hartley and Ford, 1989). Alkaline treatment of the wall dramatically increases the microbial digestibility of graminaceous forage, enhancing the nutritional value of the forage fibre. Cleavage of alkaline-labile linkages has a major role in the

increased digestibility and solubility of lignocellulosic material, suggesting the involvement of ester bonds (Oosterveld et al., 2000; Sun et al., 2002). Hydroxycinnamic acid ester cross-linkages among wall components are present in both the primary and the secondary walls. Therefore, hydroxycinnamic acid-polysaccharide complexes are important in the structure and function of walls during plant development as well as the degradation of the walls for extraction of fibres and for bio-energy. Those complexes restrict complete degradation and represent obstacles in the lignocellulose pulping industry (Christov and Prior, 1996; Gübitz et al., 1998). The formation and nature of covalent cross-links between plant cell wall polymers and their enzymatic degradation will be discussed in the following sections. Feruloyl esterases have been identified to play a major role in the enzymatic decomposition of cell wall polymers. Both microorganisms and the plant itself produce these enzymes for degradation and cell wall extension purposes respectively. Therefore, feruloyl esterases are diverse in their catalytic and structural properties.

The plant cell wall

Morphologically, the plant cell wall consists of three major regions (Figure 2.1; Bacic et al., 1988):

1. Middle lamella - outermost layer, the glue that binds adjacent cells, composed primarily of pectic polysaccharides.

2. Primary wall - wall deposited by cells before and during active growth. The primary wall is comprised of pectic polysaccharides (ca. 30%), cross-linking glycans (hemicellulose; ca 25%), cellulose (15-30%) and protein (ca. 20%). The actual content of the wall components varies with species and age. All plant cells have a middle lamella and primary wall.

3. Secondary Wall - some cells deposit additional layers inside the primary wall. This occurs after growth stops or when the cells begin to differentiate. The secondary wall is mainly for support and is comprised primarily of cellulose and lignin. Three distinct layers can de distinguished, S1, S2 and S3 - which differ in the orientation, or direction, of the cellulose microfibrils.

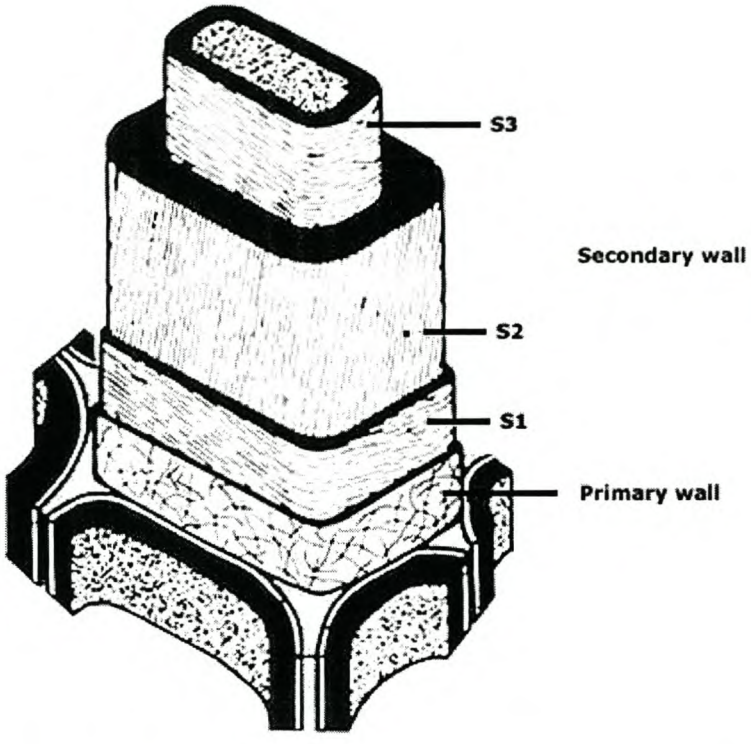


Figure 2.1. Structure of the plant cell wall. Adapted from Bacic et al. (1988). Three distinct layers can de distinguished in the secondary wall - S1, S2 and S3 - which differ in the orientation, or direction, of the cellulose microfibrils.

The main components of cell walls are polysaccharides (or complex carbohydrates or complex sugars), which are built from monosaccharides (or simple sugars). Eleven simple sugars are common in these polysaccharides including glucose and galactose. The interconnections between those polysaccharides are the structural basis for plant cell wall morphology (Figure 2.2). There are a variety of other components in the wall including protein and lignin.

Cellulose or β-1,4-glucan is made of as many as 25,000 individual glucose molecules (Bacic et al., 1988). Every other residue is "upside down".

Cellobiose (glucose-glucose disaccharide) is the basic building block. Cellulose readily forms hydrogen bonds with itself (intra-molecular H-bonds) and with other cellulose chains (inter-molecular H-bonds). A cellulose chain will form hydrogen bonds with about 36 other chains to yield a microfibril. This is somewhat analogous to the formation of a thick rope from thin fibres. Microfibrils are 5-12 nm wide and give the wall strength - they have a tensile strength equivalent to steel. Some regions of the microfibrils are highly crystalline due to the close interactions of individual polymers while others are amorphous, because the intermolecular hydrogen bonds are sub optimal and the individual polymers are accessible to water (Lenting and Warmoeskerken, 2001).

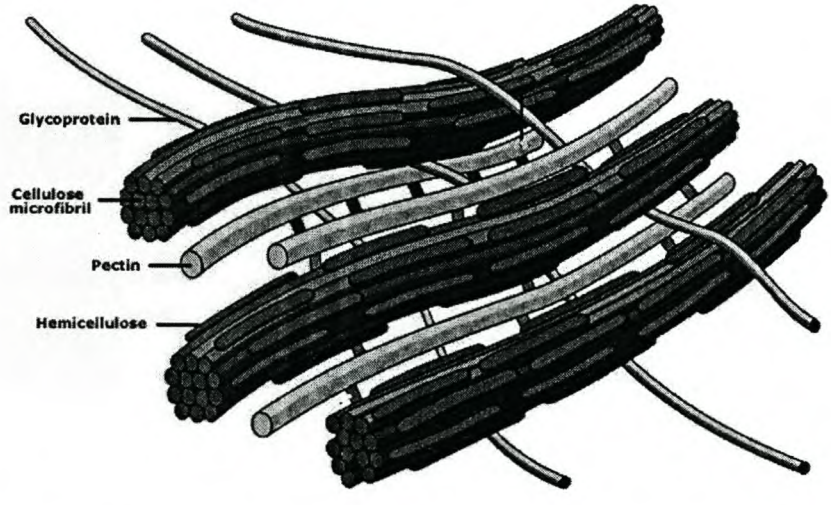


Figure 2.2. Interconnections among plant polysaccharides in plant cell walls. Model adapted from Moore et al. (1998).

The diverse group of carbohydrates forming polymers other than cellulose is called hemicellulose, characterised by being soluble in strong alkali. Hemicellulose forms a supportive matrix for cellulose. Two common types

include xyloglucans and glucuronoarabinoxylans (Figure 2.3). Xyloglucans have a basic structure of linear chains of β-(1,4)-linked D-glucosyl residues with xylosyl residues added at the O6 position of the glucosyl chain. They can bind tightly to the glucan chains of cellulose microfibrils. The majority of dicots and monocots contain xyloglucan in equal proportion to cellulose. In walls of Poaceae, however, glucuronoarabinoxylans are the principal polysaccharides responsible for cross-linking the wall. They are linear polymers of β-(1,4)-linked D-glucosyl residues with single glucosyluronic acid residues at the O2 position and arabinosyl residues at the O3 position of the backbone xylosyl residues (Bacic et al., 1988). Less common forms of hemicellulose include glucomannans, galactoglucomannans, and galactomannans (Lundqvist et al., 2002; Willför et al., 2003). Arabinoxylans are synthesized from UDP-D-xylose and UDP-L-arabinose by the action of glucosyl transferases in the Golgi vesicles. The sugar residues are added onto existing acceptor molecules, which may be oligo-, or polysaccharides. Cereal arabinoxylans also contain ester-linked ferulic and *p*-coumaric acid the biosynthesis of which is not entirely understood. Almost all work on cell wall polysaccharide synthesis has been carried out with crude membrane preparations where many polymers are formed at the same time and endogenous molecules function as acceptors (Ray, 1980; Yalpani, 1987).

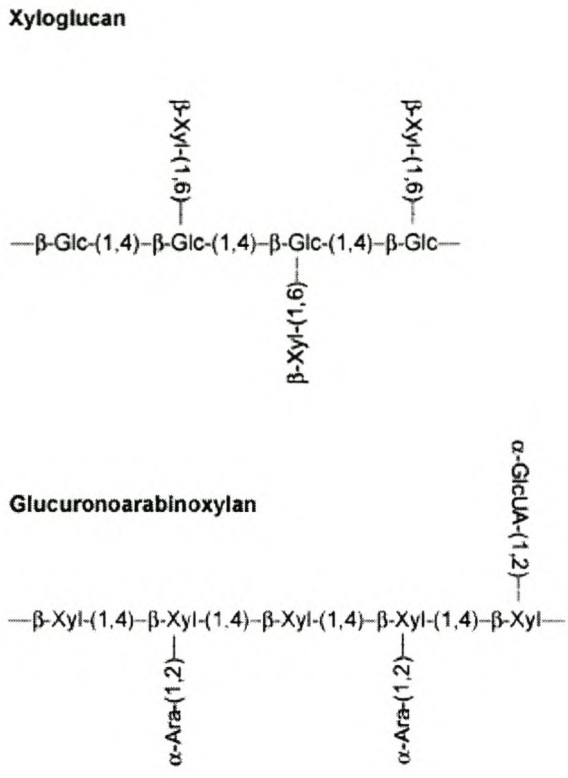


Figure 2.3. Structure of xyloglucan and glucoarabinoxylan (Bacic et al., 1988).

Pectins are common polymers consisting mainly of α-1,4 linked galacturonans called polygalacturosyls. They are the easiest constituents to remove from the plant wall by extraction with hot water or dilute acid or calcium chelators (like EDTA). Divalent cations, like calcium, also form cross-linkages to join adjacent polymers thereby creating a gel. Pectic polysaccharides can also be cross-linked by dihydrocinnamic or diferulic acids. The polygalacturosyls are initially secreted from the Golgi as methylated polymers; the methyl groups are removed by pectin methyl esterase to initiate calcium binding (Mukhiddinov et al., 2000; Ridley et al., 2001). The neutral sugar components of pectins are most likely covalently linked to the main backbone at rhamnose

units. Other pectic acids include rhamnogalacturonan II that contains rhamnose and galacturonic acid in combination with a large diversity of other sugars in varying linkages. Dimers of rhamnogalacturonan II can be cross-linked by boron atoms linked to apiose sugars in a side chain (Ishii and Matsunaga, 1996; Kaneko et al., 1997). Although most pectic polysaccharides are acidic, others are composed of neutral sugars including arabinans and galactans. The pectic polysaccharides serve a variety of functions including the determination of wall porosity; they provide a charged wall surface for cell-cell adhesion (middle lamella), cell-cell recognition and pathogen recognition (Ridley et al., 2001).

Lignin is a highly variable polymer of phenolic monomers, especially phenylpropanoids (*p*-coumaryl, coniferyl and sinapyl alcohol; Figure 2.4), linked by ether and carbon-carbon bonds. Lignin is primarily a strengthening agent in the wall and occurs in supporting and conducting tissues (e.g. xylem). Lignification of the cell wall occurs after the laying down of the polysaccharide components of the wall and after the cessation of cell wall expansion, enabling the plant to grow into large and upright structures and protecting the microfibrils of the wall from chemical, physical and biological attack (Leonowicz et al., 1999).

The wall is largely hydrated and comprised of between 75-80% water. This is responsible for some of the wall properties. For example, hydrated walls have greater flexibility and extensibility than non-hydrated walls (Irwin et al., 1985; Reis et al., 1991).

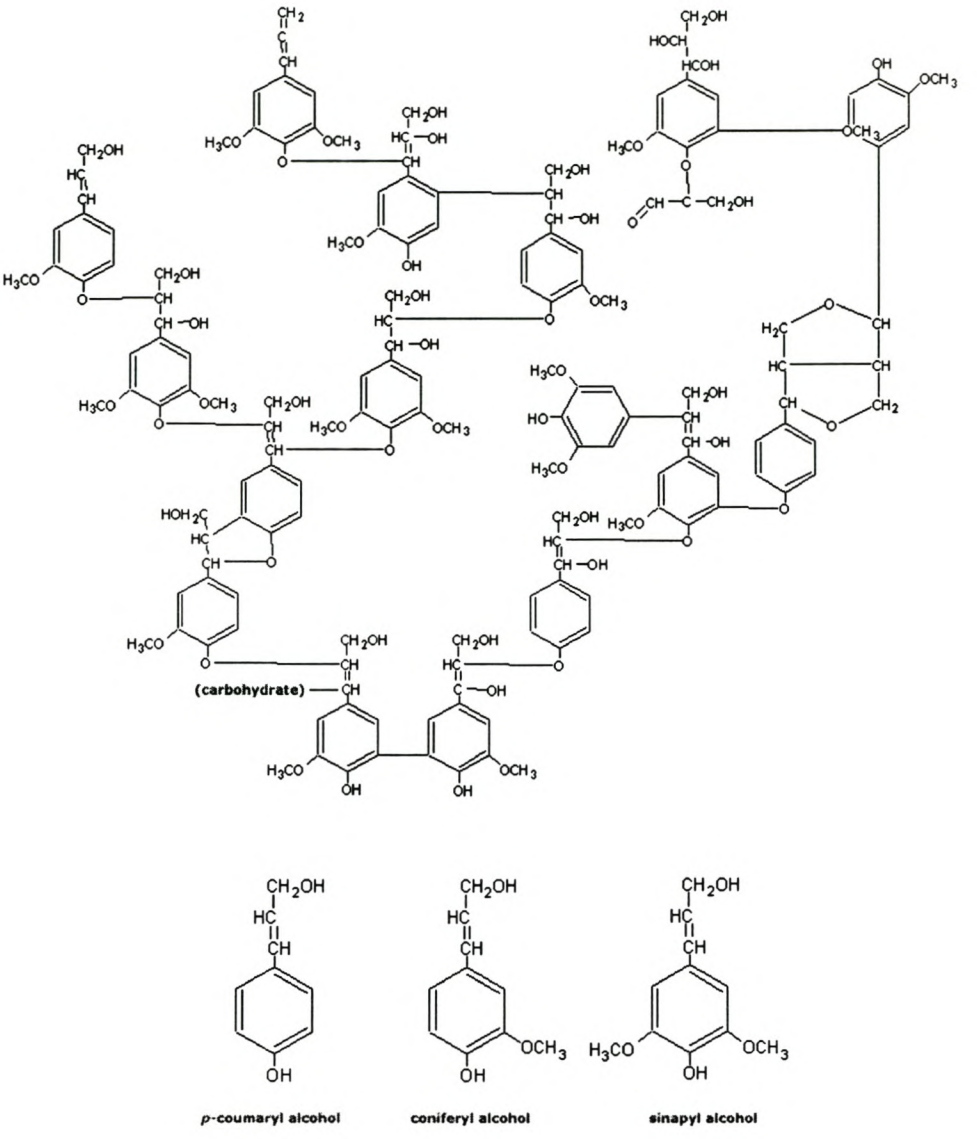


Figure 2.4. Lignin components and structure. *p*-coumaryl, coniferyl and sinapyl alcohol are the building blocks of polymeric lignin. Laccases and peroxidases participate in the construction of the variety of covalent bonds found in lignin (Iiyama et al., 1990).

Cross-links in the cell wall

Plant cell walls are constructed to withstand high turgor pressure. In meristematic and differentiating cells, this pressure can reach values around 3 - 10 bar (Carpita and Gibeaut, 1993; Cosgrove, 1993). The aggregate strength of noncovalent forces between wall polymers appears to make this possible as there is little evidence that covalent cross-linking between polymers is necessary to achieve this stability (Talbott and Ray, 1992). A widespread model of the organisation of polymers in primary cell walls of plants is that cellulosic microfibrils are embedded in a matrix of interwoven noncellulosic polysaccharides and proteins (Talbott and Ray, 1992; Carpita and Gibeaut, 1993). There is good evidence that the microfibril surfaces are coated with noncellulosic polysaccharides such as xyloglucans and arabinoxylans and glucomannans. Further, it is envisaged that a proportion of these polysaccharides are hydrogen bonded to the surfaces of cellulose microfibrils and, by virtue of their length, are able to interact with surfaces of more than one microfibril and so act as an adhesive between them. Other less well-defined component polysaccharides that are hydrogen-bonded or bound through ionic and salt interactions to other polysaccharides or to proteins may be found in the matrix (Reiter, 2002).

Walls must not only resist turgor pressure, they must also allow wall expansion during cell growth. Cosgrove (1993) has discussed a mechanism by which stress relaxation in walls can lead to water uptake by the cell and allow wall expansion that accompanies cell growth. Stress relaxation in a wall composed of noncovalently associated polymers has been postulated to

result from controlled relocation of adhesive associations between microfibrils, for example, by action of the wall-located xyloglucan transferase (Talbott and Ray, 1992).

At the completion of the expansion phase of cell growth, the mechanical properties of walls change and they are no longer capable of stress relaxation. These changes, like those during expansion, are under metabolic control. The end point of the expansion phase may involve creation of permanent (irreversible) associations between wall polymers by formation of covalent cross-links (Reiter, 2002). Many, but not all secondary walls are lignified, reaching levels of 20 – 30 and 7 – 15 % lignin in matured walls of dicotyledons and grasses, respectively. In the case of barley, a graminaceous monocotyledon, cross-linked glucuronoarabinoxylan and esterified phenolic acids contribute to cell wall strength (Shedlitzky et al., 1992) and extension (Sancho et al., 1999).

The following section of the thesis examines reports of covalent linkages between plant cell wall constituents. It consists of three parts: the first part reports on the isolation and characterisation of hydroxycinnamic acids linked to cell wall polysaccharides. The second and third parts summarize reports on how hydroxycinnamic acids are involved in the interconnection of polysaccharides and hydroxycinnamic cross-linkages between polysaccharides and lignin, respectively.

Linkage of hydroxycinnamic acids to cell wall polysaccharides

Hydroxycinnamic acids are readily released by mild alkaline treatment of wall materials, showing that these acids are ester-linked to wall polymers. These

esterified compounds have been detected by spectrophotometry, gas-liquid chromatography (GLC), and high-performance liquid chromatography (HPLC) (Carpita and Gibeaut, 1993). *p*-Coumaric, ferulic and sinapic acids (Figure 2.5) and dehydrodimers, such as diferulate (Hartley and Ford, 1989), disinapate and sinapate-ferulate heterodimers (Bunzel et al., 2003), are identified in the extracts of saponified cell walls.

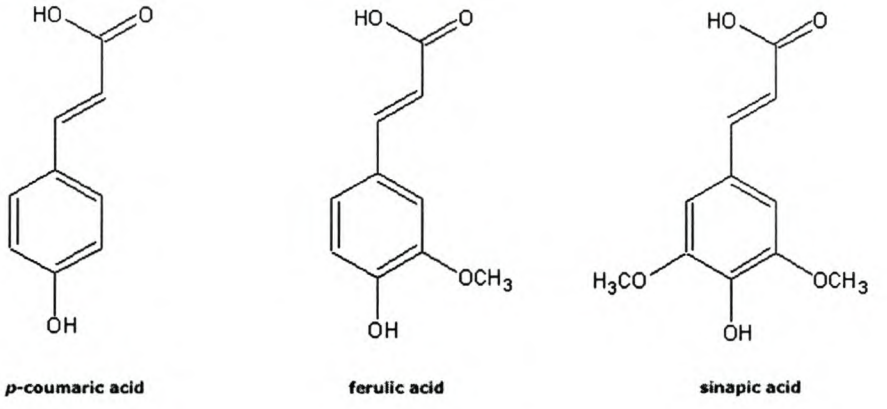


Figure 2.5. Cinnamic acid derivatives released from alkali-treated cell walls (Carpita and Gibeaut, 1993).

The location of hydroxycinnamic acids has been visualized by auto fluorescence of plant tissues excited with ultraviolet light (Hartley, 1973). Fluorescence microscopy of Italian ryegrass (*Lolium multiflorum*) sections showed that ferulic acid is distributed in cell wall preparations from various tissues (Harris and Hartley, 1976; Hartley and Jones, 1976).

The linkage of ferulic acid to wall polysaccharides has been studied by determining the structure of water-soluble fractions released from walls by mild acid hydrolysis (Ralet et al., 1994) or by treatment with a mixture of polysaccharide hydrolysing enzymes without any esterase activities, such as

driselase or celluclast (Fry, 1982; Ishii, 1994). A feruloyl arabinoside, (F-Ara)-(1,5)-(Ara), and a feruloyl galactobiose, (F-Gal)-(1,4)-Gal, were for the first time isolated from the Driselase digest of suspension cultured spinach (*Spinacia oleracea*) cell walls by Fry (1984) and later from the Driselase digest of spinach-leaf walls and sugar beet (*Beta vulgaris*) pulp (Ishii and Tobita, 1993; Colquhoun et al., 1994).

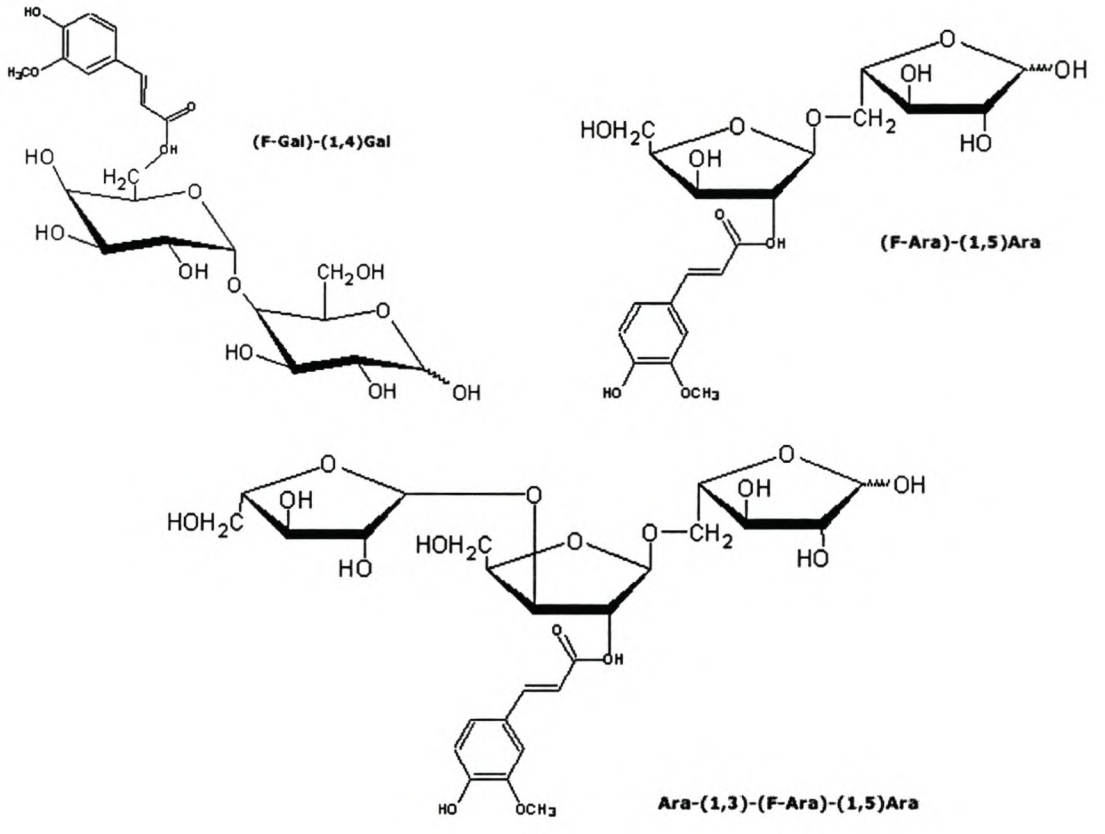


Figure 2.6. Structures of feruloylated oligosaccharides isolated from plant cell walls. Top left: *O*-(6-*O*-*trans*-feruloyl-*β*-D-galactopyranosyl)-(1,4)-D-galactose; top right: *O*-(2-*O*-*trans*-feruloyl-*α*-L-arabinofuranosyl)-(1,5)-L-arabinofuranose; bottom: *O*-*α*-L-arabinofuranosyl-(1,3)-*O*-(2-*O*-*trans*-feruloyl-*α*-L-arabinofuranosyl)-L-arabinose (Fry, 1984).

These were characterised to be *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1,5)-L-arabinofuranose, *O*-α-L-arabinofuranosyl-(1,3)-*O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)- L-arabinose and *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1,4)-D-galactose (Figure 2.6).

A series of feruloyl arabino-oligosaccharides were isolated from sugar beet by mild acid hydrolysis (Ralet et al., 1994). These feruloyl oligosaccharides probably would be derived from feruloylated arabinan and (1,4) linked D-galactosyl oligosaccharide side chains of pectic polysaccharides (Fry, 1986; Ridley et al., 2001). A feruloyl arabinoxylan trisaccharide, (F-Ara)-(1,3)-Xyl-(1,4)-Xyl was isolated from the enzymatic digest of sugar beet pulp (Ishii, 1994) and a feruloyl arabinoxylan disaccharide, (F-Ara)-(1,3) Xyl, was isolated from the enzymatic digest of wheat (*Triticum aestivum*) bran (Figure 2.7; Smith and Hardley, 1983). A feruloyl arabinoxylan trisaccharide (F-Ara)-(1,3)-Xyl-(1,4)-Xyl (Figure 2.7) has been isolated from sugar cane (*Saccharum officinarum*) bagasse (Kato et al., 1983), maize (*Zea mays*) shoot (Kato and Nevins, 1986), barley (*Hordeum vulgare*) straw (Mueller-Harvey et al., 1986), barley aleurone (Gubler et al., 1985) and bamboo (*Phyllostachys edulis*) (Ishii and Hiroi, 1990a). A feruloyl arabinoxylan tetrasaccharide, Xyl-(1,4)-(F-Ara)-(1,3)-Xyl-(1,4)-Xyl, (Figure 2.7) was also isolated from bamboo shoot (Ishii and Hiroi, 1990b) and sugar cane bagasse (Kato et al., 1987). A small amount of *p*-coumaryl arabinoxylan trisaccharide, {(*p*-CA-Ara)-(1,3)-Xyl-(1,4)}-Xyl and tetrasaccharide, Xyl-(1,4)-{(*p*-CA-Ara)-(1,3)-Xyl-(1,4)}-Xyl (Figure 2.7) were isolated from cell walls of barley straw (Mueller-Harvey et al., 1986) and bamboo shoot (Ishii et al., 1990).

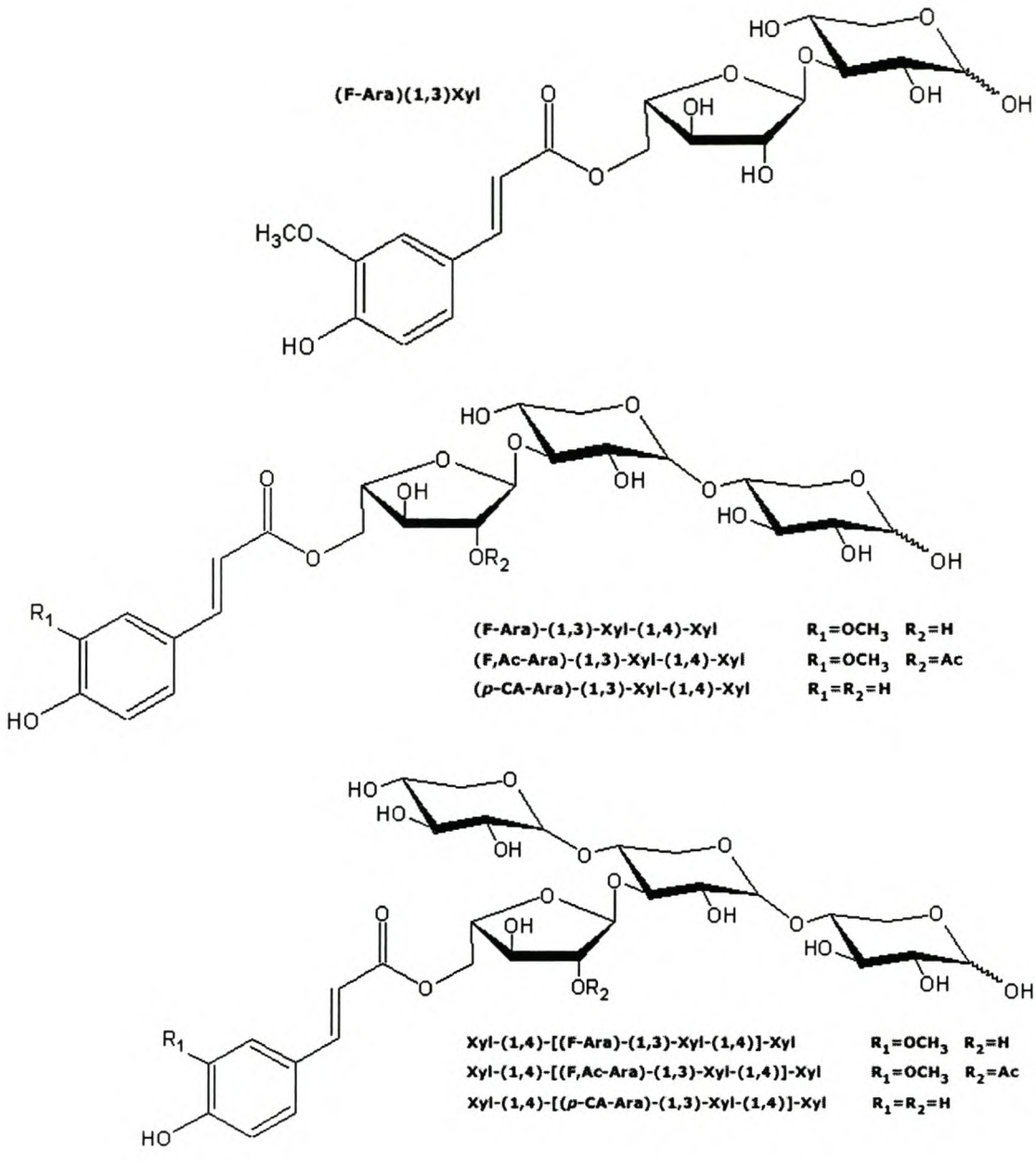


Figure 2.7. Structures of feruloylated, *p*-coumarylated and acetylated oligosaccharides isolated from plant cell walls. Top: feruloyl arabinoxylan disaccharide (Smith and Hartley, 1983); middle: feruloyl/acetyl/*p*-coumaryl arabinoxylan trisaccharide (Kato et al, 1983); bottom: feruloyl/acetyl/*p*-coumaryl arabinoxylan tetrasaccharide (Ishii and Hiroi, 1990b).

An 5-*O*-(*trans*-feruloyl)-L-arabinose, F-Ara, (Saulnier et al., 1995), an *O*-*β*-D-xylopyranosyl-(1,2)-5-*O*-(*trans*-feruloyl)-L-arabinose, Xyl-(1,2)-(F-Ara), (Saulnier et al., 1995; Wende and Fry, 1997), an *O*-*β*-D-xylopyranosyl-(1,3)-*β*-

D-xylopyranosyl-(1,2)-5-*O*-(*trans*-feruloyl)-L-arabinose, Xyl-(1,3)-Xyl-(1,2)-(F-Ara), (Ishii and Hiroi, 1990b), an *O*-L-galactopyranosyl-(1,4)-*O*-D-xylopyranosyl-(1,2)-5-*O*-(*trans*-feruloyl)-L-arabinose, Gal-(1,4)-Xyl-(1,2)-(F-Ara), (Saulnier et al., 1995), and an {[5-*O*-(*trans*-feruloyl)]-[*O*-β-D-xylopyranosyl-(1,2)]-*O*-L-arabinofuranosyl-(1,3)}-β-D-xylopyranosyl-(1,4)-D-xylose, {[F(1,5)] [Xyl-(1,2)]-Ara-(1,3)}-Xyl-(1,4)-Xyl, (Himmelsbach et al., 1994), were isolated from graminaceous monocots by mild acid hydrolysis (Figure 2.8).

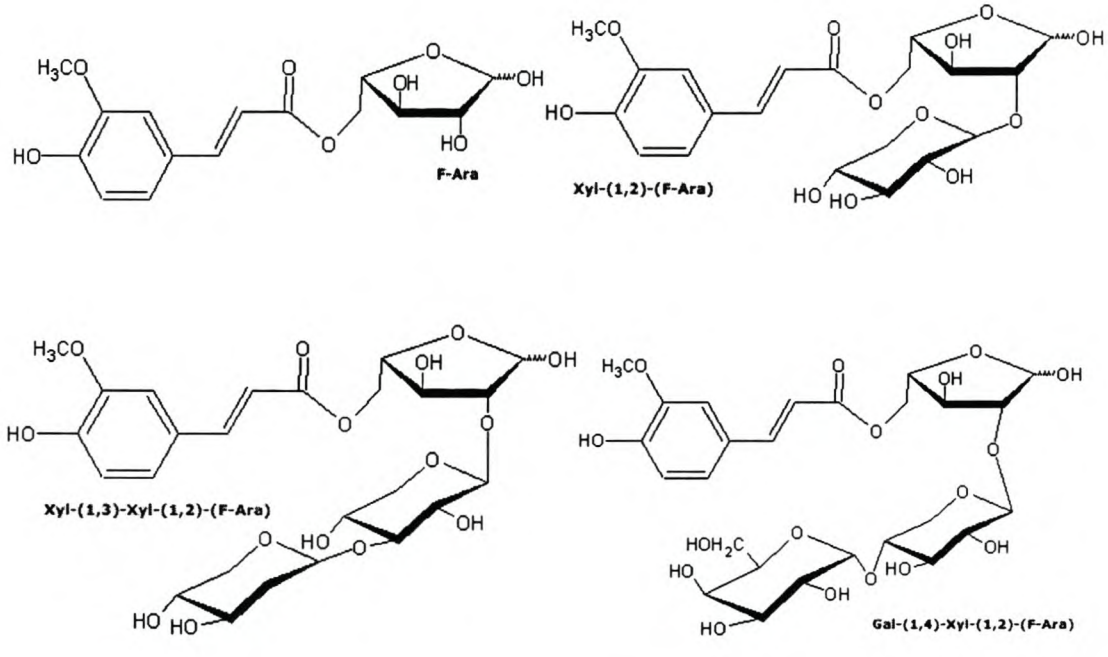
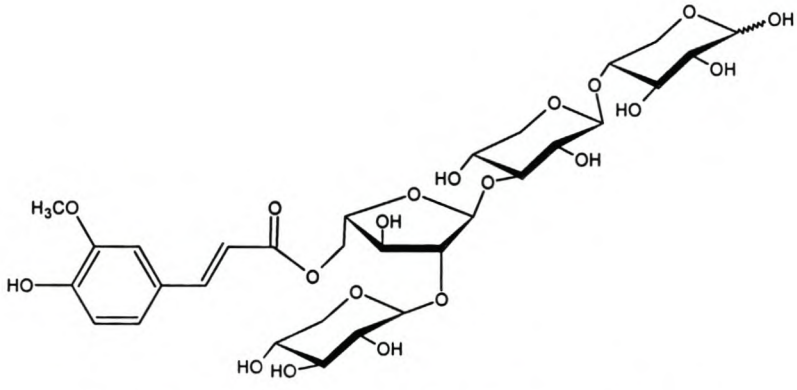


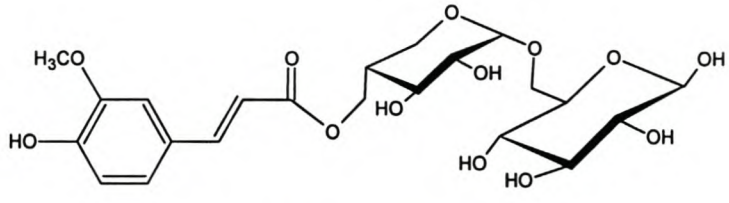
Figure 2.8. Structures of feruloylated oligosaccharides isolated from plant cell walls. Top left: 5-*O*-(*trans*-feruloyl)-L-arabinose (Saulnier et al., 1995); top right: *O*-β-D-xylopyranosyl-(1,2)-5-*O*-(*trans*-feruloyl)-L-arabinose (Saulnier et al., 1995; Wende and Fry, 1997); bottom left: *O*-β-D-xylopyranosyl-(1,3)-β-D-xylopyranosyl-(1,2)-5-*O*-(*trans*-feruloyl)-L-arabinose (Ishii and Hiroi, 1990b); bottom right: *O*-L-galactopyranosyl-(1,4)-*O*-D-xylopyranosyl-(1,2)-5-*O*-(*trans*-feruloyl)-L-arabinose (Saulnier et al., 1995).

An *O*-L-galactopyranosyl-(1,4)-O-D-xylopyranosyl-(1,2)-L-arabinose was previously isolated from side chains of highly branched maize xylan (Whistler and Corbett, 1955). These feruloylated and *p*-coumarylated arabinoxylan oligosaccharides are believed to be derived from arabinoxylan.

The xylosyl residue of monocot xyloglucan was also feruloylated. An *O*-(4-*O*-*trans*-feruloyl-α-D-xylopyranosyl)-(1,6)-D-glucopyranose, (F-Xyl)-(1,6)-Glc, (Figure 2.9) was isolated from the Driselase digest of bamboo shoot walls (Ishii and Hiroi, 1990b). *O*-(α-D-xylopyranosyl)-(1,6)-D-glucose (isoprimeverose) is known to be a component of the xyloglucan molecule (Hayashi, 1989).



{[F(1,5)] [Xyl-(1,2)]-Ara-(1,3)}-Xyl-(1,4)-Xyl



(F-Xyl)-(1,6)-Glc

Figure 2.9. Structures of feruloylated oligosaccharides isolated from plant cell walls. Top: {[5-*O*-(*trans*-feruloyl)]-[*O*-β-D-xylopyranosyl-(1,2)]-*O*-L-arabinofuranosyl-(1,3)}-β-D-xylopyranosyl-

(1,4)-D-xylose (Himmelsbach et al., 1994); *O*-(4-*O*-*trans*-feruloyl-α-D-xylopyranosyl)-(1,6)-D-glucopyranose (Ishii and Hiroi, 1990b).

In summary, the walls of graminaceous monocots typically contain larger amounts of hydroxycinnamic acids than dicotyledons. Feruloylation occurs in monocots on xyloglucan and arabinoxylans. Arabinoxylans are also acylated by less amounts of *p*-coumarate. In dicots, a feruloyl group is attached to the arabinan and (1,4)-linked galactosyl oligosaccharides side chains of pectic polysaccharides and also to the arabinofuranosyl residues of arabinoxylan. There is no report that feruloylation occurs on arabinan and (1,4)-linked galactosyl oligosaccharides of pectic polysaccharides in monocots.

Hydroxycinnamic acid cross-linkages between polysaccharides

The formation of covalent cross-links between plant cell wall polysaccharides involving hydroxycinnamic acid derivatives was first demonstrated by Geissmann and Neukom (1971). It was shown that a water-soluble arabinoxylan from wheat flour could form a gel on addition of peroxidase and H_2O_2. They also demonstrated that the feruloyl residues on arabinoxylan oxidatively coupled to form dehydrodiferulates, and it was subsequently suggested that this reaction is a possible polysaccharide cross-linking mechanism in growing cells (Fry, 1986). The dehydrodimer coupled at 5-5 of the aromatic ring, commonly being referred to as diferulic acid (Figure 2.10) was identified in Italian ryegrass (Hartley and Jones, 1976) and rice (*Oryza sativa*) endosperm (Shibuya, 1984). The existence of the diferuloyl diester cross-link was proven by Ishii (1991), who isolated and characterised a

diferuloyl arabinoxylan hexasaccharide from an enzymatic hydrolysate of bamboo shoot arabinoxylan. The results suggest that dehydrodiferuloyl cross-linking of arabinoxylan occurs naturally in cell walls (Figure 2.11). Besides in arabinoxylan, feruloyl residues are also bound to xyloglucan. It was therefore suggested that feruloylated arabinoxylan and xyloglucan form cross-links with other feruloylated polysaccharides through dehydrodiferuloyl bridges. Furthermore it is possible that a cellulose-xyloglucan or cellulose-arabinoxylan network cross-links other wall polysaccharides such as pectins by formation of dehydrodiferuloyl cross-links. Sugar beet and spinach have feruloylated arabinan and (1,4)-linked galactosyl oligosaccharides. Guillon and Thibault (1990) reported that pectic polysaccharides of sugar beet having ester-linked feruloyl residues on arabinan and (1,4)-linked galactosyl oligosaccharides also formed gels by oxidative cross-linking with peroxidase or ammonium peroxysulphate treatment.

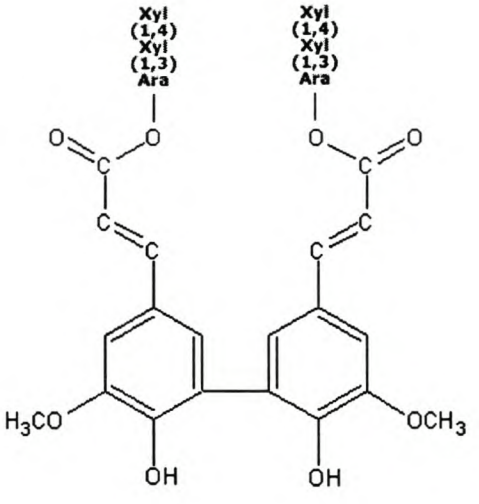


Figure 2.10. Structure of diferuloyl arabinoxylan hexasaccharide isolated from ryegrass. Either feruloyl group is linked to an arabinosyl

on the C-5 residue, which is subsequently (1,3)-linked to a (1,4)-xylobiose. (Hartley and Jones, 1976).

The formation of covalent linkages like dehydrodiferuloyl bridges is believed to terminate the expansion of the cell in grasses. Kamisaka and co-workers (1990) showed that the increase in content of ferulic and dehydrodiferulic acids in oat (*Avena sativa*) coleoptiles after sowing, is closely related to the decrease in wall extensibility and to the increase in minimum stress relaxation time and relaxation rate of walls. Irradiation with white light decreased wall extensibility of rice coleoptiles and that this was correlated with an increase in the contents of ferulic and dehydrodiferulic acids ester-linked to arabinoxylan (Tan et al., 1992).

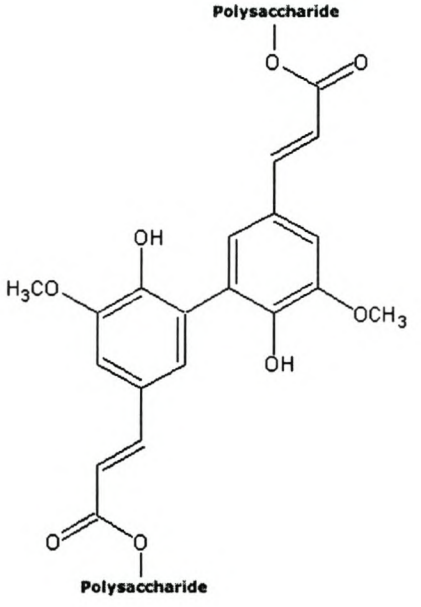


Figure 2.11. Structure of proposed covalent diferulate cross-link between polysaccharides (Ishii, 1991).

Feruloyl and diferuloyl esters between cell wall polysaccharides have been implicated in the aggregation of cultured rice cells. Kato et al. (1994) showed that the content of ferulic and dehydrodiferulic acid in walls of suspension cultured rice depended on the composition of culture medium. When the cells were grown in an amino acid medium, the content of ester-linked hydroxycinnamic acids in the walls decreased in comparison to cells grown in a medium containing NO_3^- and NH_4^+ as nitrogen source. When the cells were grown in the amino acid medium with carboxyesterase, the size of cell aggregates decreased, indicating the involvement of hydroxycinnamic acid esters in the cell aggregation.

Fry (1980) reported that cell expansion of suspension-cultured spinach was strongly promoted by gibberellic acids. He proposed that the promotion of growth by gibberellic acids was inhibited by phenolic substrates and peroxidase, which catalysed oxidative coupling of feruloyl residues of pectins in spinach cell cultures. Fry (1984) also reported that cultured spinach cells very rapidly took up ^{14}C-labelled cinnamate and that the incorporated ^{14}C-labelled products had properties of oxidatively coupled phenolics with highly Driselase-resistant wall components.

Photodimerisation of esterified hydroxycinnamic acids (Figure 2.12) was reported to occur in walls of many grasses (Morrison et al., 1992). Formation of a series of head-to-tail and head-to-head homo- and hetero-cyclobutan type dimers of ester-linked *p*-coumaric and ferulic acids has been demonstrated. Photodimerised hydroxycinnamic acid esters are reported in both unlignified and lignified walls, but not in primary cell walls (Morrison et al., 1992).

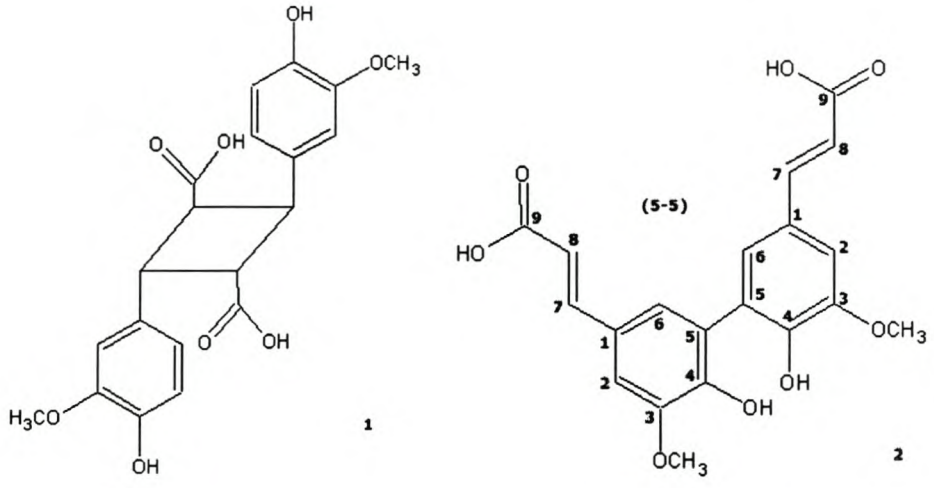


Figure 2.12. Structure of photo-dimer (1) and dehydrodimer (2) of ferulic acid released by alkaline treatment of grass cell walls (Morrison et al., 1992).

Carpita (1986) showed that a significant amount of aromatic acids were incorporated into the cell walls of maize coleoptiles after the elongation stopped. Considerable amounts of ferulic acid and small amounts of diferulic acid were released by saponification, but there was still a significant amount of compounds in the unsaponifiable residues. These could be involved in the cross-linking of the walls.

Hydroxycinnamic acid cross-linkages between polysaccharides and lignin

Several plant tissues, including sclerenchyma, fibre cells, and xylem tracheary elements, contain cells whose walls are functionally involved in mechanical support or in water conduction. The mature cells usually lack protoplasts and the wall is the only significant cellular structure present. Typically, the walls of

these cells consist of a thin primary layer, a thicker multilamellate secondary layer, and sometimes a tertiary layer. The secondary layer is rich in cellulose and the noncellulosic polysaccharides are qualitatively different from those of the primary wall. Many but not all secondary thickened walls are also lignified. The lignin is formed by polymerisation of monolignol precursors after polysaccharide deposition to form the secondary wall layers has begun. Lignin deposition is initiated first at cell corners, then in the middle lamella, and proceeds through the primary wall into the secondary wall. The three monolignol precursors in angiosperms and the two of gymnosperms arise from the phenylpropanoid amino acids phenylalanine and tyrosine and may be delivered to the wall as their soluble β-glycosides (Delmer and Stone, 1988; Iiyama et al., 1993).

In the lignification process the hydrophobic lignin replaces the water in the wall and encrusts the cellulosic and noncellulosic polysaccharides and protein components. As a result, the already thickened walls of high tensile strength - composed largely of highly organized cellulosic microfibrils - are infiltrated with a hydrophobic, phenolic copolymer that effectively makes the wall impermeable as well as imparting extra strength to the mechanical charged cells. The hydrophobic surfaces of the lignin deposits are intimately associated with and overly the surfaces of wall polysaccharides and proteins. At this interface there is an opportunity for covalent cross-linking (Iiyama et al., 1993).

Hydroxycinnamoyl esters of polysaccharides are also encountered in lignified walls from internode and leaf cells of grasses. In addition, hydroxycinnamic acids are also known to be directly esterified or etherified (Figure 2.13). On

proposed that the bifunctional ferulic acid could form covalent ester-ether bridges between polysaccharides and lignins (Figure 2.13).

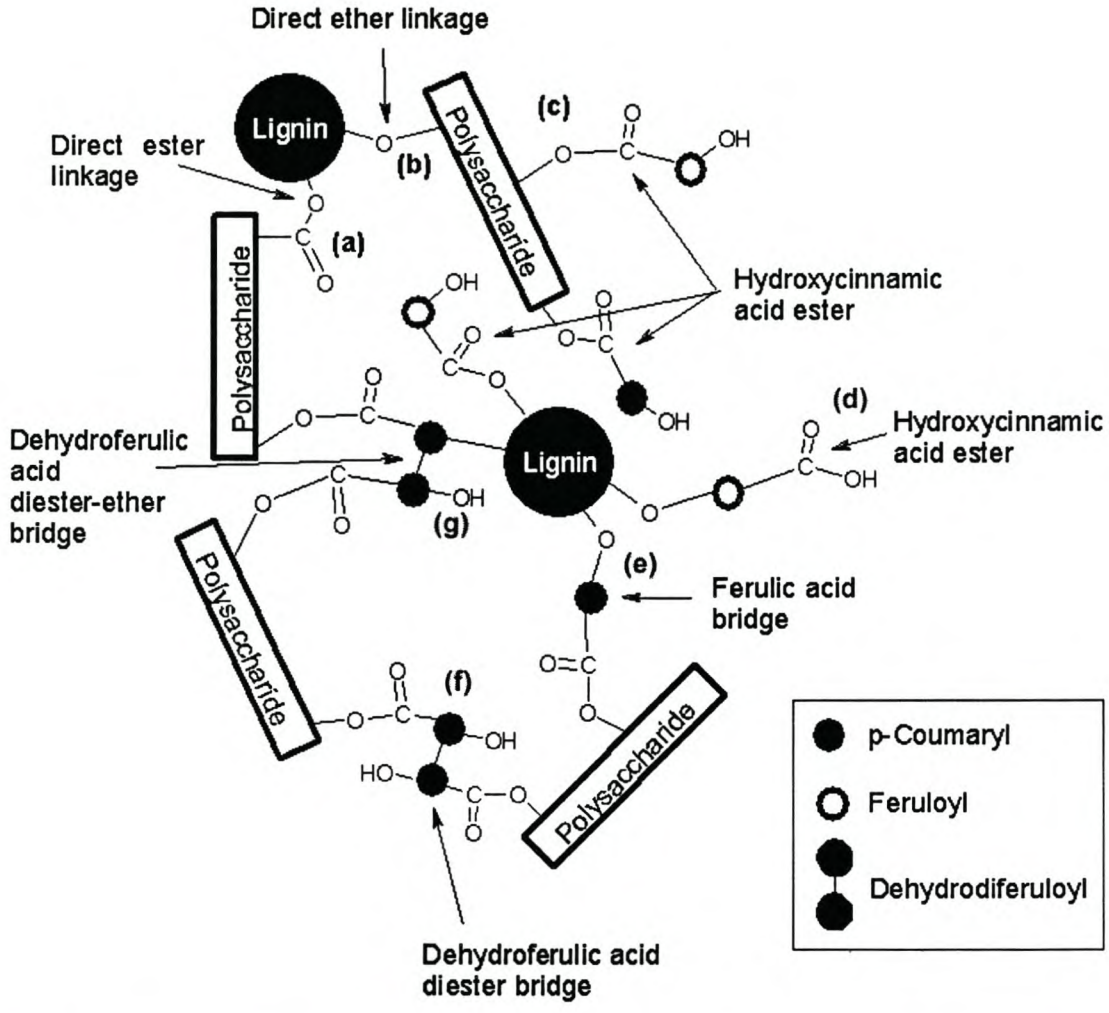


Figure 2.13. Schematic diagram showing possible covalent cross-links between polysaccharides and lignin in walls. (a) direct ester-linkage; (b) direct ether-linkage; (c) hydroxycinnamic acid esterified to polysaccharides; (d) hydroxycinnamic acid esterified to lignin; (e), ferulic acid ester-ether bridge; (f) dehydroferulic acid diester bridge; (g) dehydroferulic acid diester-ether bridge. Adapted from Iiyama et al. (1994).

Circumstantial evidence that such linkages are present in wheat straw has been provided by Scalbert et al. (1985) and Iiyama et al. (1990). It has been

Circumstantial evidence that such linkages are present in wheat straw has been provided by Scalbert et al. (1985) and Iiyama et al. (1990). It has been demonstrated by direct chemical analysis of dioxane-water soluble lignin-polysaccharide complexes from extract free wheat and phalaris internodes that all etherified ferulic acid present is also esterified, presumably to polysaccharide, although lignin-lignin ferulic acid bridges are not excluded (Lam et al., 1992a). There have been no reports that *p*-coumaric acid is involved in similar bridges. Dehydrodiferulic in diester linkage between polysaccharides may also be etherified to lignin (Figure 2.13; Lam et al, 1992b). The involvement of ferulate and 5-5-coupled diferulate in the formation of cross-links between xylan and lignin has been studies by Grabber and co-workers (2002). In model studies of cross-product formation in primary maize cell walls they showed, that mostly 4-O- β' and 8- β' cross-coupled structures are formed. These cross-products formed at the onset of lignification acted as nucleation sites for lignin polymerisation.

Hydroxycinnamoyl residues on polysaccharides can add to quinone methides to give benzaryl ethers (α-ethers), and this was proposed as a mechanism of formation of the ester-ether bridges to lignin (Scalbert et al., 1986). Another route has been demonstrated by Ralph et al. (1992), who showed the incorporation of a feruloyl-arabinose ester into synthetic lignin dehydrogenation polymer (DHP) through a peroxidase-catalysed co-polymerisation. *In vivo*, a composition is envisaged between α-etherification through quinone methide intermediates and incorporation into β-ethers and other products through radical co-polymerisation with lignin monomers. Ralph et al. (1992) point out that the β-etherified ferulic acid may not be easily

identified following its incorporation into the lignin structure. However, Lam et al. (2001), suggest that most of the hydroxycinnamic acids, ferulic and *p*-coumaric acids, are ether-linked to the benzylic and not the *β*-position, challenging the results of Ralph et al. (1992), Ralph and Helm (1992) and Terashima et al. (1993).

Mechanism of enzymatic plant cell wall degradation

The heterogeneous composition of the plant cell wall requires complementary action of several enzymes for its complete degradation. Polysaccharide hydrolases cleave the bonds between the carbohydrate constituents of the polysaccharide chains, whereas esterases remove the organic acids linked to polysaccharides and peroxidases and laccases decompose lignin (Dijkerman et al., 1997; Hrmova et al., 1997; Subramaniyan and Prema, 2002). The degradation of xylan (Figure 2.14) is achieved by the action of endo-*β*-1,4-xylanase (E.C. 3.2.1.8), which cleaves the 1,4 bonds between the constituent xylose residues of the polymeric chain. Substituted and unsubstituted oligosaccharides of variable length are released, serving as substrate for *β*-xylosidase (E.C.3.2.1.37), which removes single xylose moieties from the non-reducing end. The substituents *α*-L-arabinofuranose and 4-O-methylglucuronic acid are removed by the action of *α*-arabinofuranosidase (E.C. 3.2.1.55) and *α*-glucuronidase (E.C. 3.2.1.139), respectively. Acetyl xylan esterases (E.C. 3.1.1.6) hydrolyse the cleavage of ester-bound acetyl groups, substituents on the xylan-backbone, whereas feruloyl esterases (E.C. 3.1.1.73) remove *α*-L-arabinofuranose bound ferulic and *p*-coumaric acids.

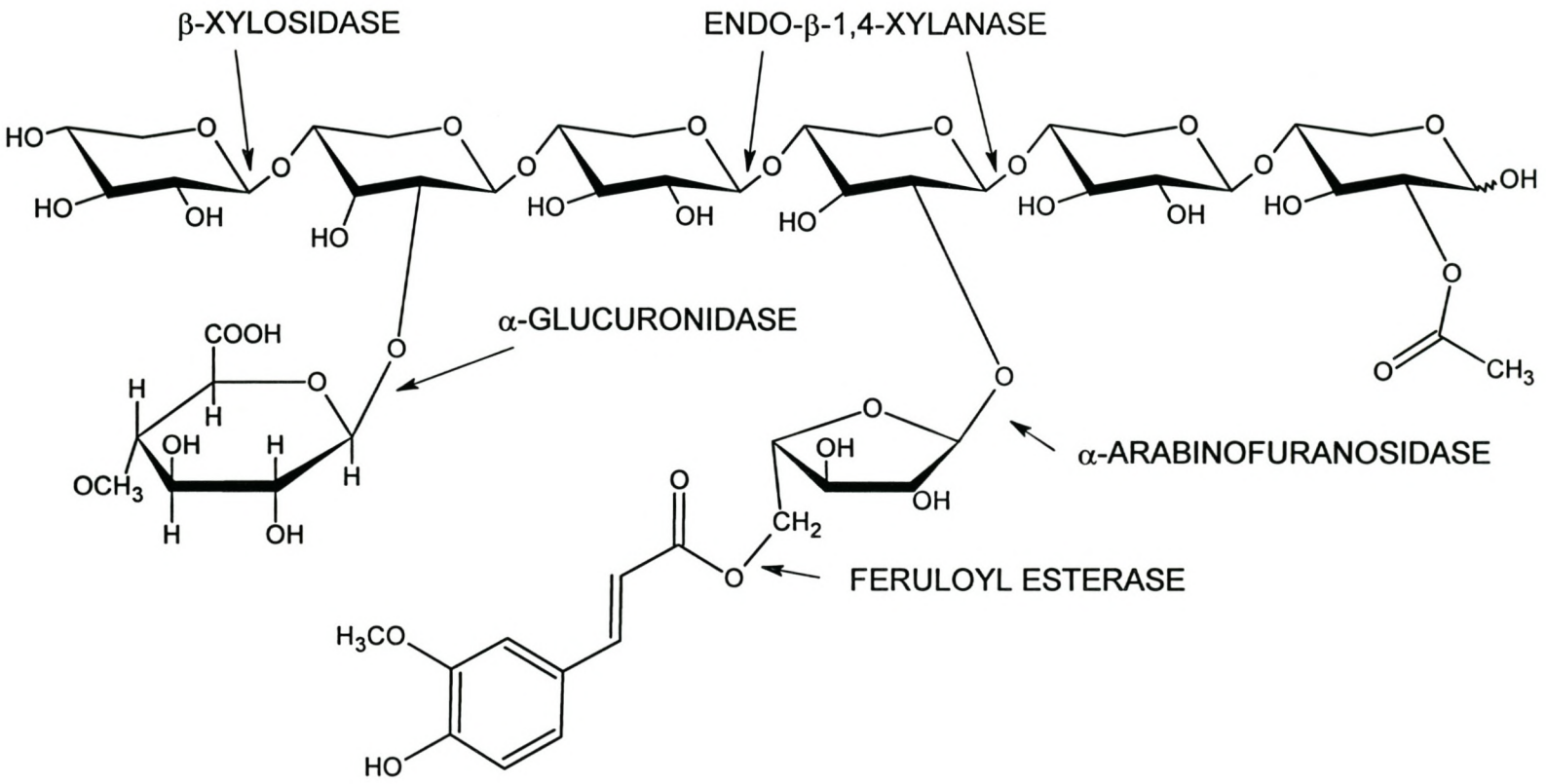


Figure 2.14. Hypothetical xylan structure and mechanism of degradation (Biely et al., 1997).

Carbohydrate esterases, α-arabinofuranosidase and α-glucuronidase are also collectively referred to as xylan-debranching or hemicellulose accessory enzymes (Jeffries, 1990; Eriksson, 1990; Thomson, 1993; Viikari et al., 1994; Williamson et al., 1998b).

Synergy of two or more hemicellulolytic enzymes acting together resulting in a greater rate of xylan degradation than the sum of the rates of degradation when the enzymes act individually, has been extensively reported between most of the accessory enzymes and xylanase. The action of xylanase releases substituted oligosaccharides that are more readily available and have more favourable interactions with the accessory enzymes, while the removal of side-chain branches by the accessory enzymes open up previously unavailable sites for cleavage by xylanases. The release of substituted oligosaccharides by xylanase appears to be of great importance as many of the xylanolytic accessory enzymes have a preference for oligomeric substrates and show very little activity against polymeric xylan. Simultaneously, the enzymic liberation of acetyl and feruloyl groups from xylan through the action of carbohydrate esterases is essential for the efficient action of xylan degrading enzymes (Biely et al., 1993; Viikari et al., 1994; Bartolome et al., 1995; Tenkanen, 1998; de Vries et al., 2000). Biochemical properties and the mode of action in the degradation of plant polysaccharides are described below for xylanases, xylosidases, arabinofuranosidases, glucuronidases and acetyl xylan esterases (Table 2.1).

Regulation of xylanolytic enzyme production

Different opinions exist regarding the regulation of the expression of the xylanolytic enzymes. The xylanolytic systems of most microorganisms appear

to be induced by soluble xylan fragments and xylobiose, but there is evidence that expression of xylanolytic enzymes in certain organisms is controlled by the same inducers as for the cellulolytic enzymes. Repression of xylanolytic genes in the presence of glucose has been assigned to the carbon catabolite repressor protein CreA in *A. niger* (van Peij et al., 1998; de Vries et al., 1999) and its homologue in *A. pullulans* (van den Wymelenberg et al., 1999). This protein is the major factor for carbon catabolite repression. Another mechanism of induction, temporal regulation, was described for *Pseudomonas cellulosa* xylanases (Emami et al., 2002). Microorganisms constitutively produce low levels of xylanolytic enzymes that are able to depolymerise any xylan present in their immediate environment, releasing various oligosaccharide fragments. These fragments are then able to act as inducers for the production of xylanolytic enzymes allowing the organism to utilize the available xylan as a carbon source (Biely et al., 1993; Thomson, 1993; Beg et al., 2001). However, all the different regulation patterns seem to orchestrate all lignocellulolytic enzymes in the synergistic degradation of complex plant cell wall materials.

Endo-β-(1,4)-xylanase

Xylanases are the most intensely studied of the xylanolytic enzymes and have been purified and characterised in several yeasts, foremost *Trichoderma, Penicillium* and *Aspergillus* species, and bacteria, most notably *Bacillus, Clostridium* and *Streptomyces* species. Filamentous fungi are particularly interesting producers of this enzyme from an industrial point of view, due to the fact that they excrete xylanases into the medium. Furthermore, xylanase levels from fungal cultures are generally much higher than those from yeasts

or bacteria. Fungal xylanases are typically small monomeric proteins with molecular weights 20 – 40 kDa. At assay conditions, they exhibit maximal activity at slightly acidic pH, usually around 5, and are stable between pH 3 and 9. Fungal xylanases are usually not very temperature tolerant and are inactivated rapidly above 55°C. (Haltrich et al., 1996; Sunna and Antranikian, 1997). Bacterial xylanases have been divided into two groups based on molecular weight and isoelectric points. Low molecular weight (<30 kDa) enzymes with alkaline pI values and higher molecular weight (40 kDa – 70 kDa) enzymes with neutral or acidic pI have been described. Bacterial xylanases show maximal activity at pH values around 6 (Wong et al., 1988; Eriksson, 1990; Viikari et al., 1994; Sunna and Antranikian, 1997). The low molecular weight xylanases with alkaline isoelectric points have been assigned to the family 11 or G glycosyl hydrolases while the higher molecular weight xylanases with acidic isoelectric points have been assigned to the family 10 or F glycosyl hydrolases based on protein sequence identity (Biely et al., 1997; Coutinho and Henrissat, 1999).

Most xylanases have a preference for polymeric xylan yielding xylobiose, xylotriose and higher xylo-oligosaccharides as the main products and generally show very little activity against xylobiose and xylotriose. Certain purified xylanases additionally show transglycosylation activity, being capable of synthesizing xylo-oligosaccharides from xylose, xylobiose and xylotriose. There is also evidence that some xylanases additionally possess debranching activity, enabling them to remove the arabinose substituents from the xylan polymer (Dekker, 1989; Thomson, 1993).

Table 2.1. Recently isolated xylanolytic enzymes

Organism	M_r (kDa)	pH optimum	Temperature optimum, °C	pI	References
Endo-β-(1,4)-xylanase					
Streptomyces sp. AMT-3	170-700	6	55	ND	Nascimento et al., 2002
Bacillus firmus	45	6 - 8	ND	ND	Tseng et al., 2002
Pycnoporus cinnabarinus	50	5	60	ND	Sigoillot et al., 2002
Rhizopus oryzae	22	4.5	55	ND	Bakir et al., 2001
Aureobasidium pullulans var. *melanigenum*	24	2	50	6.7	Ohta et al., 2001
Staphylococcus sp.	60	7.5	50	ND	Gupta et al., 2000
Geotrichum candidum	60	4	50	3.4	Rodionova et al., 2000
Acrophialophora nainiana	17	6	50	ND	Ximenes et al., 1999
Bacillus sp. BP-7	22	6	55	7	Lopez et al., 1998
Acidobacterium capsulatum	41	5	65	7.3	Inagaki et al., 1998
Streptomyces chattanoogensis	48	6	50	9	Lopez-Fernandez et al., 1998
β-(1,4)-D-xylosidase					
Streptomyces thermoviolaceus	ND	5	60	ND	Tsujibo et al., 2002
Pichia capsulata X91	72	6	50	5.1	Yanai et al., 2000
Fusarium oxysporum	66	6	60	ND	Christakopoulos et al., 2000
Thermobacillus xylanilyticus	56	6	75	ND	Debeche et al., 2000
Streptomyces chartreusis GS901	80	5.5	55	6.6	Matsuo et al., 2000
Aureobasidium pullulans NRRL Y-12974	210	4 - 4.5	75	ND	Saha and Bothast, 1998
α-L-arabinofuranosidase					
Aeromonas caviae ME-1	75	6	50	ND	Suzuki et al., 2001
Candida utilis IFO 0639	92	6	40	5.6	Yanai and Sato, 2001
Phlebia radiata	27	ND	ND	5.9	Rogalski et al., 2001
Hordeum vulgare	66	4.2	60	4.5	Ferré et al., 2000
Streptomyces olivaceoviridis E-86	23	6	60	ND	Kaneko et al., 2000
α-D-glucuronidase					
Aureobasidium pullulans	200	3	40	ND	de Wet, unpublished
Bacillus stearothermophilus T-6	150	5.5 - 6	65	ND	Zaide et al., 2001
Schizophyllum commune	125	4.5 - 5.5	40	3.6	Tenkanen and Siika-aho, 2000
Aspergillus tubingensis	107	4.5 - 6	70	5.2	de Vries et al., 1998

ND=not determined

The production of a multienzyme system of xylanases, in which each enzyme has a special function, is one strategy for a microorganism to achieve effective hydrolysis of xylan. During xylan hydrolysis, synergism has been observed between enzymes acting on the 1,4-β-D-xylan backbone (β-1,4-endoxylanase) and side chain-cleaving enzymes (α-L-arabinofuranosidase, acetyl xylan esterase, and α-glucuronidase) (Beg et al., 2001).

β-(1,4)-D-xylosidase

β-Xylosidases from several fungal and bacterial sources have been purified and characterized, although not as extensively as the xylanases. β-

Xylosidases remove single xylose residues from the non-reducing ends of xylo-oligosaccharides. They show highest activity against xylobiose, and a decrease of activity is observed with increasing degree of polymerisation. The presence of substituents poses a significant obstacle for the degradation of xylo-oligosaccharides by β-xylosidases as most xylanases produce xylo-oligosaccharides with substituents remaining on the non-reducing ends. As for the xylanases, fungal and bacterial xylosidases have pH optima around 5 and 6 respectively. The isoelectric points of the xylosidases isolated so far generally range between 3 and 5 and the molecular weights between 100 kDa and 200 kDa. They can be mono- di- and trimeric. Some xylosidases contain transferase activity, allowing for the synthesis of higher xylo-oligosaccharides from xylose. General glycosidase and/or α-arabinofuranosidase activity has also been detected in certain xylosidases (Eriksson, 1990; Sunna and Antranikian, 1997; Viikari et al., 1994).

α-L-arabinofuranosidase

α-L-arabinofuranosidase, α-glucuronidase as well as the acetyl and feruloyl esterases are known as accessory enzymes required for the debranching of substituted xylan. α-L-Arabinofuranosidase removes non-reducing arabinose residues linked to the C2 or C3 positions of xylan. Arabinosidases have been purified from bacteria, most notably *Bacillus* and *Streptomyces* species and from fungi, including *Aspergillus* and *Trichoderma* strains. Both bacterial and fungal arabinosidases are heterogeneous in terms of molecular weight (ranging between 53 kDa and 495 kDa) and isolelectric point (ranging between 3 and 9). The pH optima of bacterial and fungal arabinosidases are around 6 and 4.5 respectively (Sunna and Antranikian, 1997). Kaji (1984)

proposed the classification of arabinosidases into two groups. The first group shows similarity with an *Aspergillus niger* enzyme that utilizes the full spectrum of substrates, including polymeric arabinans, arabinoxylan and arabinogalactan as well as small arabinose containing oligosaccharides and *p*-nitrophenyl arabinoside. The second group is similar to a *Streptomyces purpurascens* enzyme that only utilizes arabinose containing oligosaccharides and *p*-nitrophenyl arabinoside. Most arabinosidases however, show a preference for oligomeric rather than polymeric substrates. Synergy between xylanases and arabinosidases in the breakdown of xylan has also been reported (Viikari et al., 1994).

α-D-glucuronidases

The cleavage of 4-*O*-methylglucuronic acid residues from the native xylan appears to be an essential step for its complete degradation. The presence of 4-*O*-methylglucuronic acid residues on the xylan polymer not only limits the degree and rate of acid hydrolysis (Roy and Timell, 1968), but also reduces the accessibility of the xylanases and β-xylosidases to the xylosidic bonds (Puls et al., 1991). Most xylanases produce oligosaccharides of a higher degree of polymerisation when 4-*O*-methylglucuronic acid substituents are present on the xylan chain and leave the 4-*O*-methylglucuronic acid moieties on the non-reducing ends of the chains, which inhibit the action of β-xylosidase (Puls et al., 1991). The importance of 4-*O*-methylglucuronic acid residues in the breakdown of lignocellulosic material is further emphasized by the fact that a high degree of ester bonding between lignin and 4-*O*-methylglucuronic acid moieties present in xylan has been reported (Das et al., 1984). Covalent bonding between xylan and lignin creates a barrier that

reduces the accessibility of microbial hemicellulolytic enzymes to the cell wall and is a major obstacle in the bleaching of paper pulp (Gübitz et al., 1998; Tenkanen et al., 1999) and in the digestion of lignocellulosic feed by rumen microorganisms (Chesson et al., 1999). Fungal α-D-glucuronidases are monomeric proteins with molecular weights between 91 kDa and 150 kDa, acidic isoelectric points (5 – 6) as well as acidic pH optima (3 and 5). In contrast, bacterial α-glucuronidases are usually dimeric proteins with subunits of lower molecular weight (~70 kDa). The pH optima of the bacterial α-glucuronidases, although acidic, are generally higher than the fungal enzyme (5 – 7), while the isoelectric points of the bacterial α-glucuronidases are more acidic than the fungal α-glucuronidases (4 – 5) (Sunna and Antranikian, 1997).

Acetyl xylan esterases

The presence of organic acids on the xylan polymer markedly decreases the accessibility of enzymes to the glycosidic bonds, thereby offering the plant cell wall some degree of protection from enzymatic attack. Acetyl groups attached to the xylose moieties act as a steric hindrance to xylanases. Acetyl xylan esterases have evolved to cleave the acid ester bonds allowing the glycolytic enzymes to degrade the polymer. They cleave the acetic acid groups esterified to the C2 and/or C3 position on the xylan backbone. Strong synergy in the degradation of xylan has been noted between the esterases and xylanases (Biely et al., 1996). The cleavage of acetyl esters leads to a greatly increased rate of xylan hydrolysis by generating new sites for xylanase action, while the generation of xylo-oligosaccharides by xylanases markedly enhances the rate of de-esterification. Acetyl xylan esterases from different

organisms vary in their preference for polymeric xylan or xylo-oligosaccharides and in their specificity towards the position of the acetyl group on the xylose moiety. The purified acetyl xylan esterases appear to be small monomeric proteins, with molecular weights ranging between 20 kDa and 50 kDa and acidic isoelectric points. Acetyl xylan esterases are secreted enzymes and the induction of enzyme activity occurs during growth of microorganisms on hemicellulosic substrates. Monosaccharides such as xylose and glucose do not act as inducers of acetyl esterase activity (Christov and Prior, 1993; Williamson, 1998b; Bornscheuer, 2002).

Feruloyl esterases

Production

The presence of feruloyl esterase activity was first detected in culture filtrates of *S. olivochromogenes* grown on oats spelt xylan and wheat bran (MacKenzie et al., 1987). Subsequently most researchers have found that feruloyl esterase is inducible and the producing microorganism requires a lignocellulosic substrate for induction. Repression occurs when the growth substrate contains glucose, therefore assuming, that like most enzymes involved in lignocellulose degradation, feruloyl esterases are catabolically repressed. The mechanism of induction of feruloyl esterase activity is still unclear and also appears to vary between different microorganisms. The production rate depends on the type of lignocellulosic substrate, above all the content of esterified phenolic acid, and supplemented free phenolic acid. But a high amount does not always guarantee high activity levels. Feruloyl esterase activity found in culture supernatants from *S. olivochromogenes* grown on oat spelt xylan (MacKenzie et al., 1987) and *Schizophyllum commune* grown on cellulose (MacKenzie and Bilous, 1988), both of which are substrates containing negligible amounts of esterified hydroxycinnamic acids was higher compared to destarched wheat bran that contains 1% esterified ferulic acid. However, *A. niger* produced a 2.3-fold increase in feruloyl esterase (FAE-III) activity in the supernatant when oat spelt xylan was supplemented with free ferulic acid compared to oat spelt xylan alone (Faulds et al., 1997). This level of FAE-III activity was comparable to the amount of

enzyme activity produced by growth on destarched wheat bran. Similarly, de-esterification of the wheat bran prior to incubation led to a 2.4-fold decrease in FAE-III activity in the supernatant. Additionally, Smith et al. (1991) reported that growth of several *A. niger*, *A. phoenicis* and *Trichoderma reesei* strains on meadow fescue grass, containing considerable esterified hydroxycinnamic acids, led to the induction of feruloyl esterase activity, whereas oat spelt xylan did not induce significant amounts of either activity. The growth substrate determines also the type of feruloyl esterase induced. Feruloyl esterases with different substrate specificities have also been reported to be induced by growth of *A. niger* on oats spelt xylan (Faulds and Williamson, 1994) and sugar beet pulp (Kroon et al., 1996).

It seems likely that different bound or unbound phenolic acids act as inducers of feruloyl esterase activity in different organisms. Molecular genetic studies on the expression of a *Butyrivibrio fibrisolvens* feruloyl esterase have revealed that regulation is mediated by feruloylated oligosaccharides (Dalrymple and Swadling, 1997). Cloning of the *cinB* gene, encoding the feruloyl esterase enzyme revealed the presence of another open reading frame *cinR*, coding for a protein with high homology to bacterial repressor proteins. Expression of the *cinB* gene was repressed by binding of the product of the *cinR* gene to a specific tandem repeat sequence occurring in the 170bp region between the two open reading frames. Repression of *cinB* expression was abolished by the presence of FAXX and 5-*O*-(*trans*-feruloyl)-arabinofuranose, both probable xylan degradation products, but not free ferulic or *p*-coumaric acid, xylose, xylobiose or any other monomeric hemicellulose component.

Kroon and co-workers (2000) identified two putative binding motifs on the *faeB* promotor of *Penicillium funiculosum.* Northern analysis showed that transcription of *faeB* was tightly regulated, being stimulated by growth of the fungus on sugar beet pulp but inhibited by free glucose. The *faeB* promoter sequence contains putative motifs for binding an activator protein, XlnR, and a carbon catabolite repressor protein, CreA.

A study (de Vries et al., 2002) on expression profiling of 26 pectinolytic genes of the fungus *A. niger*, amongst them *faeA* and *faeB*, both encoding feruloyl esterases, revealed that the expression of these genes respond to the presence of ferulic acid. Neither *faeA* nor *faeB* are expressed in response to the presence of D-galacturonic acid. However, monosaccharides can also act as inducers. For example, *faeA*, expression was also observed in the presence of D-xylose and this observation was attributed to a xylanolytic activation factor XylR (van Peij et al., 1998).

The presence of feruloyl esterase activity has also been reported in the culture supernatants of several bacteria (Table 2.2) such as *Streptomyces* spp. (Donelly and Crawford, 1988; Faulds and Williamson, 1991; Garcia et al., 1998 a, b; Donaghy et al., 2000), *Fibrobacter succinogenes* (McDermid et al., 1990), *B. fibrisolvens* (Dalrymple et al., 1996; Dalrymple and Swadling, 1997), *Bacillus* spp. and lactobacilli (Donaghy et al., 1998), *Ruminococcus* spp. (McSweeney et al., 1998) and *C. thermocellum* (Blum et al., 2000). Fungi producing feruloyl esterases include *S. commune* (MacKenzie and Bilous, 1988), *Aspergillus* spp. (Tenkanen et al., 1991; McCrae et al., 1994; Faulds and Williamson, 1994; Kroon et al., 1996;), *Trichoderma reesei* (Smith et al., 1991), *Neocallimastix* MC-2 (Borneman et al., 1992), *Penicillium* spp.

(Castanares et al., 1992; Kroon et al., 2000), *Piromyces equi* (Fillingham et al., 1999) and *A. pullulans* (Rumbold et al., 2003).

Enzyme assays

The determination of feruloyl esterase activity is based on the quantification of hydroxycinnamic acid released from the substrate. A variety of different activity assays have been developed. Separation and quantification of reaction products is achieved by chromatographic methods such as high performance liquid chromatography (MacKenzie et al., 1987; Borneman et al., 1990; O'Neill et al., 1996), thin layer chromatography (MacKenzie and Bilous, 1988) or gas chromatography of the silylated derivatives of the reaction products (Borneman et al., 1990). Additionally, hydroxycinnamic acids can be quantified in a photometric assay exploring the difference in their molar absorbance at 340 nm to the substrate (ferulic acid esters) (Ralet et al., 1994; Biely et al., 2002). The assay of feruloyl esterase activity was initially performed using native cell wall material in the form of starch-free wheat bran as a substrate (MacKenzie et al., 1987). Alternatively, the methyl and/or ethyl esters of ferulic, caffeic, *p*-coumaric and sinapic acids have been used as substrates (Faulds and Williamson, 1994; Kroon et al., 1996). Carbohydrate oligomers containing esterified hydroxycinnamic acids have been isolated from enzymatic hydrolysates of plant cell wall material and successfully employed as substrates in the assay of feruloyl esterases. Borneman et al. (1990) isolated *O*-[5-*O*-(*trans*-*p*-coumaryl)-*α*-L-arabinofuranosyl]-(1,3)-*O*-*β*-D-xylopyranosyl-(1,4)-D-xylopyranose (PAXX) from Coastal Bermuda grass shoots treated with Driselase, a fungal enzyme preparation containing a mixture of polysaccharide hydrolases. The feruloylated equivalent (FAXX) of

the above mentioned oligosaccharide could also be prepared from the same hydrolysate. An assay in order to differentiate feruloyl esterases exhibiting affinity for 5-O and 2-O-feruloylated α-L-arabinofuranosyl residues was developed by Biely et al. (2002). The assay uses synthetic *p*-nitrophenyl 5-O-*trans*-feruloyl-α-L-arabinofuranoside and *p*-nitrophenyl 2-O-*trans*-feruloyl-α-L-arabinofuranoside and is based on coupling the feruloyl esterase-catalysed formation of *p*-nitrophenyl α-L-arabinofuranoside with its efficient hydrolysis by α-L-arabinofuranosidase to release *p*-nitrophenol. The new substrates represent convenient tools to differentiate feruloyl esterases on the basis of substrate specificity and avoid the tedious isolation of natural substrate from plant material. A plate screening method for the detection of feruloyl esterase activity by microorganisms has also been developed (Donaghy and McKay, 1994). Colonies are grown on agar plates containing the ethyl esters of ferulic or *p*-coumaric acid that are subsequently flooded with a pH indicator, bromocresol green. A yellow zone against a blue background is observed around the colonies that release the hydroxycinnamic acids from their alkyl esters.

Substrate specificity

Several feruloyl esterases have been purified and characterised (Table 2.2), however, the range of substrates used to characterise these enzymes is diverse as both natural and synthetic and assays are not uniform. They range in size and complexity from small, soluble esters such as feruloylated oligosaccharides isolated from plant cell walls and phenolic acid methyl esters or synthetic feruloylated arabinosides to larger, more complex and often less soluble substrates such as feruloylated, polymeric plant cell wall fractions

(Williamson et al., 1998b). The only criterion in all cases is the release of free ferulic acid or other hydroxycinnamic acid by hydrolysis of an ester bond and furthermore it is extremely common for esterases to be active on a broad range of substrates. Specificity, as defined by the rate of catalysis divided by the Michaelis constant

$$\left(\frac{k_{kat}}{K_m}\right)$$

gives the best indication of "preferred" substrates. However, hydrolysis of polymeric substrates is more complicated since not all of the esterified substituents are chemically equal, and effects such as decreased solubility and steric hindrance further complicate any results obtained; these data should not be extrapolated to obtain kinetic constants.

The use of small, soluble substrates allows the determination of kinetic constants, giving some information on the affinity (from K_m values) and catalytic efficiency (V_{max} / K_m). Substrates in question are effectively two components joined by an ester bond: the phenolic component and the sugar moiety. Specificity for both of these components defines the overall catalytic rate of the reaction. The selectivity for each component gives important information for the classification on feruloyl esterases (Williamson et al., 1998a).

Table 2.2. Purified and characterised fungal and bacterial feruloyl esterases.

Organism	Enzyme	M_r (kDa)	pH optimum	pI	MFA	Ara_5F K_m (mM)	Ara_2F	Reference
Fungi								
Aspergillus awamori	FAEA	112	5	3.7	0.93	ND	ND	McCrae et al., 1994
Aspergillus niger	FAE-III	29.7	5	3.3	0.7	0.006	-	de Vries et al., 1997
Aspergillus niger	FAE-I (CinnAE)	145	6	4.8	1.3	0.41	0.29	Kroon et al., 1996
Aspergillus oryzae	FAE	29	ND	ND	ND	ND*	-	Tenkanen et al., 1991
Aureobasidium pullulans	FeE	210	6.7	6.5	0.05	0.268	0.23	Rumbold et al., 2003
Neocallimastix MC-2	FAE-I	69	6.2	4.2	ND	0.032	ND	Borneman et al., 1992
Neocallimastix MC-2	FAE-II	24	7	5.7	ND	0.01	ND	Borneman et al., 1992
Penicillium expansum	FAE	57	5.6	ND	2.6	ND	ND	Donaghy and McKay, 1997
Penicillium funiculosum	FAEB	53	ND	6	0.047	0.024	0.14	Kroon et al., 2000
Penicillium pinophilum	p-CAE/FAE	57	6	4.6	0.14	ND	ND	Castanares et al., 1992
Piromyces equi	FAEB (EstA)	37	6	ND	ND	0.004	0.075	Fillingham et al., 1999
Bacteria								
Clostridium stercorarium	FeE	33	8	ND	0.04	ND	ND	Donaghy et al., 2000
Clostridium thermocellum	$FAECDB_{XYNZ}$	45	6	5.8	ND	5	ND	Blum et al., 2000
Butyrivibrio fibrisolvens	CinI (CinA)	30	ND	ND	ND	ND	ND	Dalrymple et al., 1996
Butyrivibrio fibrisolvens	CinII (CinB)	35	ND	ND	ND	ND	ND	Dalrymple and Swadling, 1997
Streptomyces avermitilis CECT 3339	FAE	ND	6	ND	ND	0.06	ND	Garcia et al., 1998a
Streptomyces avermitilis UAH 30	FeE	ND	6	ND	ND	ND	ND	Garcia et al., 1998b
Streptomyces olivochromogenes	FeE	29	5.5	7.9 –8.5	1.9	0.24	ND	Faulds and Williamson, 1991
Streptomyces viridosporus	CinnAE	ND	9	ND	ND	ND	ND	Donelly and Crawford, 1988

ND=not determined; ND*=K_m value not determined, but substrate is catalysed; - =no activity; MFA=methyl ferulate; Ara_5F=feruloyl group on the C5 of arabinose; Ara_2F= feruloyl group on the C2 of arabinose;

Phenolic acids esterified to methyl or ethyl groups instead of the natural occurring sugar have been used for the characterisation of most feruloyl esterases. They are relatively easy to synthesize, a variety of phenolic acids can be used to examine the enzymes specificity and the assay is reliable and fast, especially useful during purification.

Using methyl ferulate, methyl caffeate, methyl *p*-coumarate and methyl sinapate, the specificities of several fungal feruloyl esterases were compared. From the relative activities shown in Figure 2.15 it can be distinguished between enzymes that have a broad substrate range, like *A. pullulans, P. funiculosum and P. expansum* feruloyl esterases and *A. niger* enzymes, which are more selective for one or the other substrate. When examining the preference of feruloyl esterases for individual substrates, methyl sinapate, for example, is a very poor substrate for *A. niger* CinnAE and *P. expansum* and *P. funiculosum* feruloyl esterases, whereas the *A. awamori* enzyme shows no activity on methyl *p*-coumarate and *A. niger* FAE III shows no activity on methyl caffeate and methyl *p*-coumarate (Donaghy and McKay, 1997; Kroon and Williamson, 1998; Kroon et al., 2000). A similar activity pattern, methyl *p*-coumarate > methyl caffeate > methyl ferulate > methyl sinapate can be observed for *A. pullulans* FeE, as well as for *A. niger* CinnAE, *P. funiculosum* and *P. pinophilum* feruloyl esterase. Methyl ferulate is preferably catalysed by *P. expansum* and *P. pinophilum*. The complimentary activities of the examined enzymes demonstrate that the active centres of individual enzymes must be very different, at least for recognition of the phenolic moiety. However, from activity patterns similar catalytic properties can be revealed,

even across fungal genera (Kroon and Williamson, 1998; Williamson et al., 1998b).

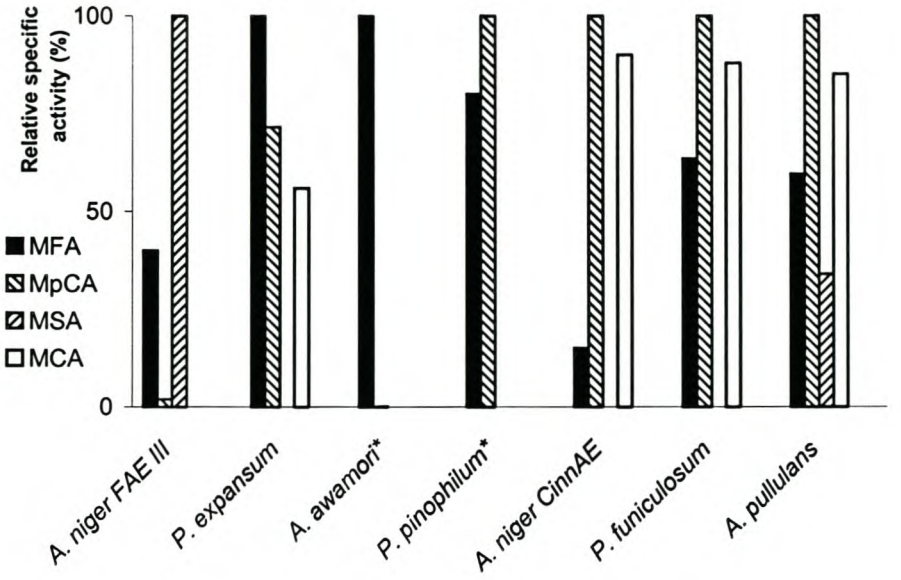


Figure 2.15. Comparison of fungal feruloyl esterases (Rumbold et al., 2003). MFA: methyl ferulate; MpCA: methyl *p*-coumarate; MSA: methyl sinapate; MCA: methyl caffeate. *MSA and MCA activity have not been determined.

The specificity of feruloyl esterases for the sugar moiety of the substrate is more difficult to examine systematically, mostly because a much more limited number of substrates are available. Substrates are usually obtained by limited hydrolysis of plant cell walls, or by organic synthesis. Comparison of catalysis properties gives information about the possible existence of sugar sites on feruloyl esterases, the importance of the position of attachment of the feruloyl group to the sugar, and the type of sugar (Williamson et al., 1998b). A feruloyl group can be attached to the C2 or C5 position of arabinose or xylose,

depending on the source of the starting material. Only few feruloyl esterases have been examined from this point of view; however, Table 2.2 shows a summary of fungal and bacterial enzymes that exhibit preferences for both or one or the other substrate. *A. pullulans* feruloyl esterase and *A. niger* CinnAE catalyse 2-feruloylated and 5-feruloylated substrates equally well whereas *P. equi* and *P. funiculosum* feruloyl esterases show a clear preference for 2-feruloylated substrates. *A. niger* FAE III and *A. oryzae* feruloyl esterase show activity exclusively on 5-feruloylated substrates. The reasons for this specificity remain to be elucidated, but differences in the active centre might be a valid explanation. A lack of uniform data does not allow clear prediction on the affiliation of individual enzymes to specific groups. Kinetic data of bacterial feruloyl esterases is even less available than of fungi. Therefore, an examination of all available feruloyl esterases with the same substrates would enable a more comprehensive classification of these enzymes.

Sequence structure and relationship

Until the end of 2002, sequences of nine fungal and seven bacterial feruloyl esterases (EC 3.1.1.73) have been published (Table 2.3) but the derived amino acid sequences do not appear to be highly conserved. Similar sequences have been discovered from genomic databases by BLAST search (www.ncbi.nlm.nih.gov/BLAST). Those are from *B. halodurans, Mesorhizobium loti* and *Agrobacterium tumefaciens*. However, feruloyl esterase activity has not been confirmed experimentally. Therefore, conclusions on feruloyl esterase classification based on amino acid sequences alone should be done with care. All feruloyl esterases, however share one conserved motif (GXSXG) sequence, which is a universal identifier

common to many esterases and lipases. Coutinho and Henrissat (1999) classified cell wall-degrading enzymes into families of carbohydrate esterases and their database shows that some feruloyl esterases are in family 1 (including *P. funiculosum* FAEA and FAEB, *Orpinomyces sp.*, *P. equi*, *C. thermocellum* XYNY and XYNZ and *A. tumefaciens*) whereas the remaining enzymes are not represented in any family. However, the alignment of amino acid sequences and using modern phylogenetic analysis tool suggests that distant relationships can be established (Figure 2.16).

Amongst fungal feruloyl esterases, *A. awamori* FAEA, *A. tubingensis* FAEA and *A. niger* FAE-III are most closely related, exhibiting more than 90% average identity over the entire amino acid sequence. This group also contains a conserved domain that is found in several lipases of class 3 (triglyceride lipases EC 3.1.1.3), including those of *Thermomyces lanuginosus* (37% identity), *A. oryzae* (34% identity) and *P. camembertii* (34% identity). The most conserved region in all these proteins is centred around a serine residue. Aliwan et al. (1999) showed, that despite sequence identity (32 %). *A. niger* FAE-III does not exhibit lipase activity. Andersen and co-workers (2002) tested amongst others *T. lanuginosus* lipase for ferulic acid esterase activity. The wild type had no significant ferulic acid esterase activity, but rationally designed structural variants showed significant activity on ferulic acid esters while their lipase activity decreased.

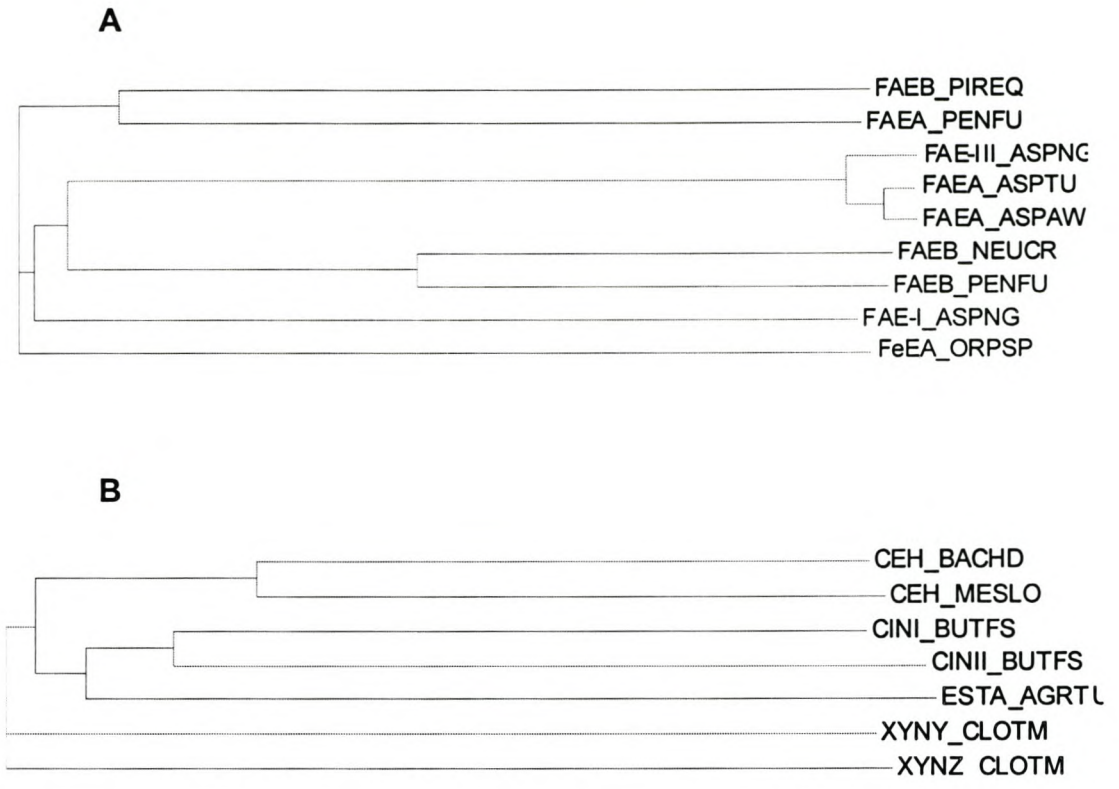


Figure 2.16. Phylogenetic tree of fungal (A) and bacterial (B) feruloyl esterases. For multiple sequence alignment BLOSUM62 was used as score matrix, gap penalty existence was 10, extension 0.1. FAEB_PIREQ: *P. equi*; FAEA_PENFU: *P. funiculosum* FAEA; FAE-III_ASPNG: *A. niger* FAE-III; FAEA FAEA_ASPTU: *A. tubingensis*; FAEA_ASPAW: *A. awamori*; FAEB_NEUCR: *N. crassa*; FAEB_PENFU: *P. funiculosum* FAEB; FAE-I_ASPNG: *A. niger* FAE-I; FeEA_ORPSP: *Orpinomyces sp.*; CEH_BACHD: *B. halodurans*; CEH_MESLO: *M. loti*; CINI_BUTFS: *B. fibrisolvens* CINI; CINII_BUTFS: *B. fibrisolvens* CINII; ESTA_AGRTU: *A. tumefaciens*; XYNY_CLOTM: *C. thermocellum* XYNY; XYNZ_CLOTM: *C. thermocellum* XYNZ.

P. funiculosum FAEB and *Neurospora crassa* FAEB show 50% identity and both are closely related to acetyl xylan esterases from *A. awamori* (44% identity; McCrae et al., 1994), *A. ficuum* (44%; Chung et al., 2002) and *P. purpurogenum* (39%; Gutierrez et al., 1998). *A. niger* FAE-I is very remotely related to this group (<10%). On the other hand, *P. funiculosum* FAEA shows 30% identity to *P. equi* FAEB that belongs to the family of *Neocallimasticaceae*, which is not even in the same order than the family of *Trichocomaceae*. Both share a conserved domain that is common in enzymes of glycosyl hydrolase family 62. Therefore it is apparent that the feruloyl esterases are highly diverse enzymes with little conservation amongst closely related species. *Orpinomyces* sp. FAEA shows 31 % homologies to the bacterial XYNZ of *C. thermocellum*, an endoxylanase from *Ruminococcus flavefaciens* (28%) and a tributyrin esterase from *S. pneumoniae* (24%). *A. niger* FAE-I exhibits significant identity to tannases from *Xanthomonas campestris* (26%) and *A. oryzae* (24%).

Homologies between different bacterial feruloyl esterases are generally lower than those found in fungi. Highest identity values can be found between *B. halodurans* CEH and *M. loti* CEH (39%) and 27% between CINI and CINII of *B. fibrisolvens*. *A. tumefaciens* ESTA is slightly related to the latter (15% identity). XYNY and XYNZ of *C. thermocellum* are not related to other bacterial enzymes but *C. thermocellum* XYNZ is homologous (31% identity) to the fungal *Orpinomyces* sp. FAEA.

Table 2.3. Fungal and bacterial feruloyl esterases with available sequence. Active serine is underlined.

Gene	Enzyme	Organism	Putative active site sequence	Reference
FaeB	FAE-I	*A. niger*	GC<u>S</u>TG	de Vries et al., 2002
FaeA	FAE-III	*A. niger*	GH<u>S</u>LG	de Vries et al., 1997
FaeA	FAEA	*A. tubingensis*	GH<u>S</u>LG	de Vries et al., 1997
FerA	FAEA	*A. awamori*	GH<u>S</u>LG	Koseki et al., unpublished
FaeB	FAEB	*N. crassa*	GD<u>S</u>LG	Crepin and Connerton, unpublished
FaeB	FAEB	*P. funiculosum*	G<u>S</u>SSG	Kroon et al., 2000
FaeA	FAEA	*P. funiculosum*	GC<u>S</u>PG	Furniss et al., unpublished
FaeA	FAEA	*Orpinomyces sp.*	GL<u>S</u>MG	Blum et al., 2000
EstA	FAEB	*P. equi*	GF<u>S</u>MG	Fillingham et al., 1999
cinA	CEH	*B. halodurans*	GC<u>S</u>EG	Takami et al., 2000
cinA	CEH	*M. loti*	not identified	Kaneko et al., 2000
xynY	XYNY	*C. thermocellum*	GF<u>S</u>MG	Fontes et al., 1995
xynZ	XYNZ	*C. thermocellum*	GL<u>S</u>MG	Grepinet et al., 1988
cinA	CINI	*B. fibrisolvens*	GH<u>S</u>QG	Dalrymple et al., 1996
cinB	CINII	*B. fibrisolvens*	GG<u>S</u>QG	Dalrymple and Swadling, 1997
estA	CEH	*A. tumefaciens*	GY<u>S</u>GG	Goodner et al., 2001

Crystal structures of feruloyl esterases from *C. thermocellum* XYNY and XYNZ have been published (Figure 2.17 and 2.18). The enzymes display the *α/β*-hydrolase fold and possess a classical Ser-His-Asp catalytic triad. The active centre reveals the binding site for ferulic acid and related compounds. Ferulate binds in a small surface depression that possesses specificity determinants for both the methoxy- and hydroxy-ring substituents of the substrate. There appears to be a lack of specificity for the xylan backbone, which may reflect the intrinsic chemical heterogeneity of the natural substrate (Prates et al., 2001).

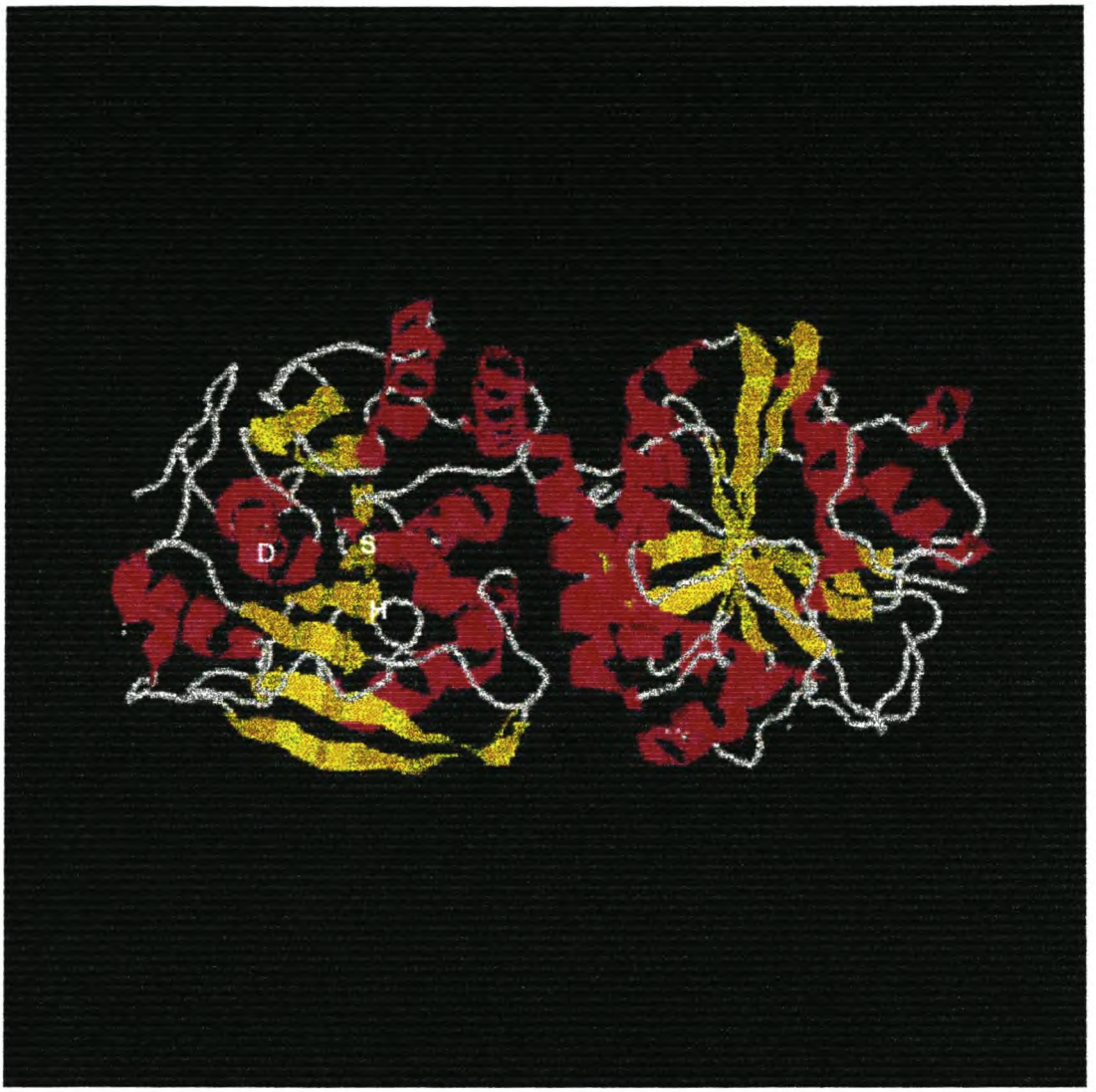


Figure 2.17. Three-dimensional structure of *C. thermocellum* XYNY homodimer. Pink: α-helix; yellow: β-sheet. The catalytic triad (Ser-His-Asp) is indicated in white letters (Prates et al., 2001).

Similar sequence similarities can be found in serine esterases, acetyl xylan esterases, lipases, tannases, glycosyl hydrolases and xylanases (Aliwan et al., 1999; Kroon et al., 2000; Andersen et al., 2002). When sequence data is compared to kinetic data it is even more difficult to establish relationships between individual and groups of enzymes. This points to the need for more data on properties of these enzymes before the functional properties can be

established and before the feruloyl esterases can be organized into discrete families based on ancestral origins.

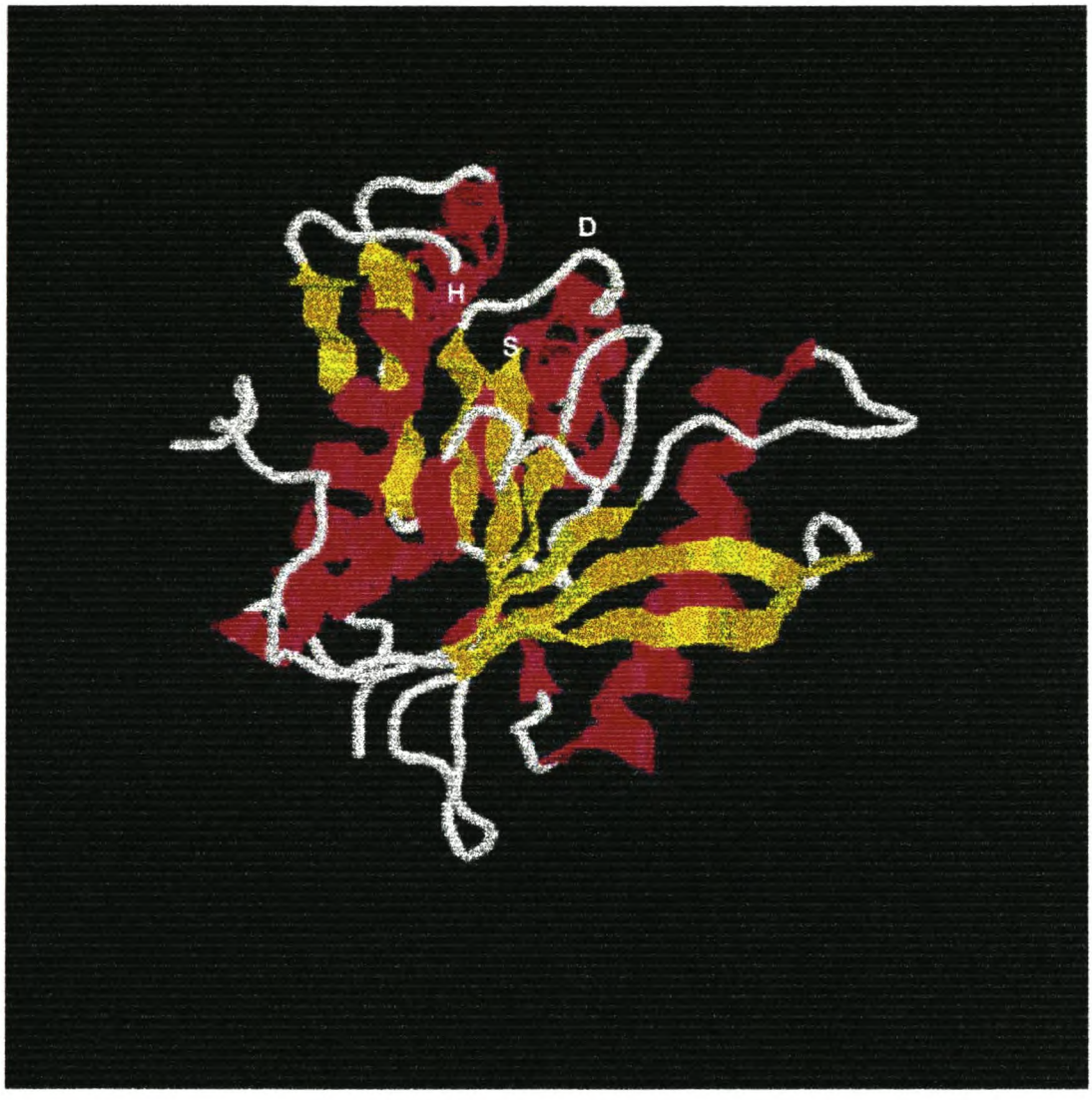


Figure 2.18. Three-dimensional structure of *C. thermocellum* XYNZ monomer. Pink: α-helix; yellow: β-sheet. The catalytic triad (Ser-His-Asp) is indicated in white letters (Prates et al., 2001).

Application

Feruloyl esterases show intriguing differences in substrate specificity and sequence structure. The main reason for the recent increase in interest in these enzymes is their potential application. Ferulic acid is an important precursor in the flavour industry (Bonnin et al., 2001), and has multiple uses as an ultraviolet light protection agent in sun creams and cosmetics (Saija et al., 1999). It suppresses inflammatory responses and skin tumor promotion (Murakami et al., 2002). Feruloyl esterases can be used to selectively remove ferulic acid from agro-industrial waste products on an industrial scale (Thibault et al., 1998). Many food processes involve ferulic acid, although the associated contributions are far from clear. For example, baking is thought to involve an important but yet unknown role for ferulic acid in the formation of cross-links in the dough (Schooneveld-Bergmans et al., 1999). Furthermore, ferulic acid acts as an antioxidant and is released and taken up in the intestines with the help of feruloyl esterases distributed along the intestines of mammals (Andreasen et al., 1999; Chesson et al., 1999). There is no doubt that applications for feruloyl esterases, together with a better understanding of the mechanism of action, will further grow in the future.

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CHAPTER 3

Current techniques for the analysis of enzymatic hydrolysis of lignocellulose: Microdialysis sampling, high pressure anion exchange chromatography / integrated pulsed amperometrical detection, on-line desalting and mass spectrometry

Introduction

Lignocellulose consists of cellulose, hemicellulose and lignin as well as other constituents. The distribution of these constituents is dependent upon the source of lignocellulose, whether it is from softwood, hardwood or grasses and cereals (Coughlan and Hazlewood, 1993). The enzymatic degradation of lignocellulose is of considerable interest to a number of industries such as pulp and paper, food, animal feed and textile industries. There is also an increasing interest in lignocellulose as a source of energy, especially fuel ethanol (South et al., 1995). A fundamental understanding of the degradation process can be especially useful in the identification of new applications of these enzymes. Furthermore, these enzymes are used to hydrolyse lignocellulose in order to make the process environmentally friendly and sustainable (South et al., 1995; Martin et al., 2002).

The kinetics of enzymatic hydrolysis of lignocellulose is an important part of each biodegradation study. Whether lignocellulose is broken down by means of lignocellulolytic microbial populations (Gutierrez-Correa et al., 1999; Tengerdy and Szakacs, 2003), cell-free extracts (Christov et al., 1997) or purified and characterised lignocellulolytic enzymes (Tenkanen et al., 1999; Rumbold et al., 2002), the experimental set-up is usually planned around the sampling of the bioprocess; separation, sometimes derivatisation and subsequent detection of the reaction products, most importantly carbohydrates. Various protocols have been established to separate and detect saccharides resulting from enzymatic hydrolysis of lignocellulose, including thin layer chromatography (Christov et al., 1999), gas

chromatography (Hernandez et al., 2001), column liquid chromatography techniques, commonly ion exchange (Hanko and Rohrer, 2000), gel filtration (Churms, 1996) and reverse phase (El Rassi, 1996), and also gel electrophoresis (Paulus and Klockow, 1996). These techniques are combined with chemical (Christov et al., 1999), electrochemical detection, mass spectrometry and nuclear magnetic resonance or combinations thereof (Torto et al., 1998 a, b; Syverud et al., 2003).

This chapter addresses issues concerning the monitoring of the enzymatic hydrolysis of lignocellulose by microdialysis sampling micro-high performance anion exchange chromatography with integrated pulsed electrochemical detection/mass spectrometry (Chapter 7 in this thesis). The following describes the advantages of the combinatorial method for total carbohydrate analysis in more detail.

Microdialysis sampling of lignocellulosic hydrolysates

Microdialysis presents most of the desired qualities for a sampling technique as it has recently been demonstrated to be appropriate for the sampling of enzymatic bioprocesses (Torto et al., 1998a, b; Nilsson et al., 2001). Because of permeable-selective membrane units, it is able to selectively sample for products of hydrolysis without significantly perturbing the reaction by depleting the bioreactor or by taking up the substrate and enzyme. The solute passes the membrane by diffusion that takes place from higher to lower concentration. Selectivity of the membranes is achieved by using membranes that exhibit different molecular weight cut-offs.

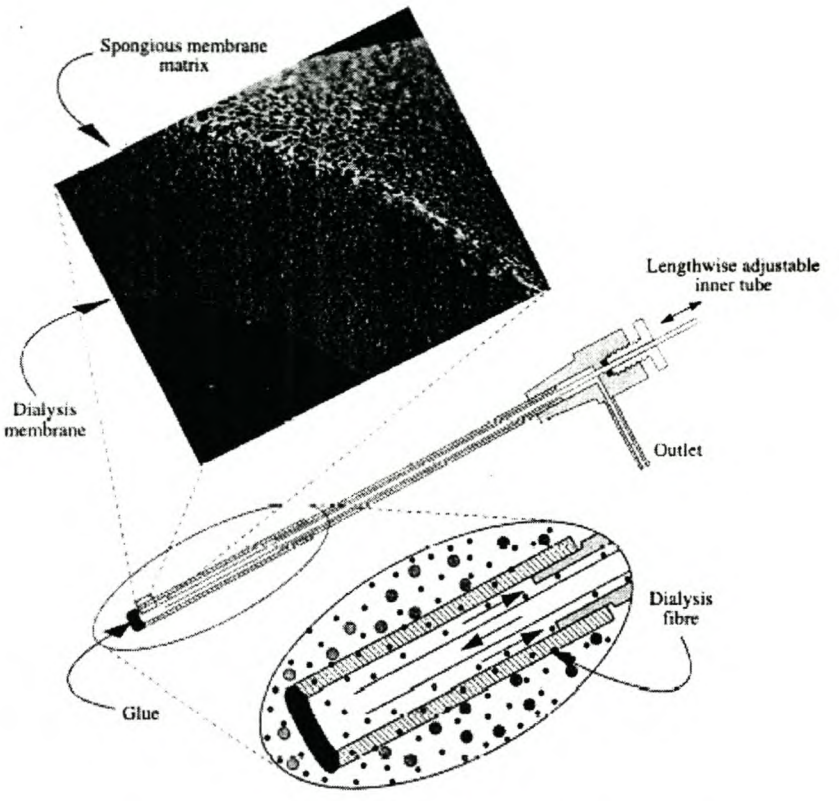

Figure 3.1. Schematic representation of the in situ tuneable microdialysis probe, showing the tortuous structure of the membrane in its dehydrated state as seen under a scanning electron microscope (Torto, 1999).

Different types of microdialysis probes are available depending on the nature of application (Torto, 1999). The concentric probe (Figure 3.1) has been proven to be ideal for *in vitro* sampling of enzymatic hydrolysis reactions (Torto et al., 1998 a, b; Rumbold et al., 2002). A proper choice of a microdialysis membrane with a known molecular weight cut-off ensures that a clean dialysate is available for chromatography. Microdialysis membranes are available with molecular weight cut-offs between 5 and 150 kDa. The use of microdialysis eliminates the tedious sample clean-up procedures and also allows in situ sampling that improves the reproducibility of the analysis and avoids the depletion of the enzymatic reaction. The reaction volume can

therefore be very low, which is an advantage when using expensive enzymes or substrates. However, microdialysis inherently results in the dilution of the sample and therefore demands a very sensitive detection system (Torto, 1999).

Separation of carbohydrates

The method of choice for the separation of carbohydrates when monitoring enzymatic hydrolysis of lignocellulose should involve minimum sample preparation and enable direct detection, thereby eliminating the need for derivatisation. These requirements are difficult to achieve regarding monosaccharides where the chemical structure only differs by the position of the OH-group. The structural complexity of oligo- and polysaccharides, which differ in their degree of linkage, substitution as well as branching, also poses a formidable challenge for chromatographic analysis (Torto et al., 1998 b).

Several approaches have been described to optimise and improve the existing chromatographic techniques for the separation of carbohydrates (Hanko and Rohrer, 2000). More notably, the use of anion exchange chromatography combined with pulsed amperometric detection (provided by Dionex Co) meets the above requirements and is widely used. Among other emerging techniques, capillary electrophoresis has not found much use for separation of carbohydrates from complex bioprocesses compared to anion exchange chromatography combined with pulsed amperometric detection (Torto et al., 1998 b).

The anion exchange chromatography system of carbohydrate analysis is based on pellicular resins. Carbohydrates are separated on the basis of their

pK_a values (Table 3.1). The difference in pK_a values results in selective interaction with the anion exchange resin leading to different retention times. Retention times are inversely proportional to pK_a values and increase with the degree of polymerisation, although they can be significantly decreased if acetate ions are added to the mobile phase (Torto et al., 1997). Carbohydrates can be routinely separated by high pressure anion exchange chromatography over a wide pH range and at a flow rate of 1 ml/min. This is attributed to the characteristics of the latex-based anion exchangers, as well as the rapid anomeric equilibration at high pH. The exchange resin consist of a surface sulphonated polystyrene divinyl benzene substrate and fully aminated porous beads of latex particles (high capacity). The particles are 0.1 µm in diameter and are agglomerated to the surface by electrostatic and van der Waals interactions (Weiss, 1994). The anion exchange process is very versatile as carbohydrates are separated in their enolate form on the basis of pH, temperature, size, composition, and degree of branching, linkage isomerism as well as anomerism without derivatisation (Hanko and Rohrer, 2000). The alkaline conditions enable dissolution of larger oligosaccharides, which would otherwise not dissolve in water and also facilitates anomeric equilibration. The chromatographic work in this thesis utilised an anion exchange column (CarboPac PA1 from Dionex) achieves good separation of mono- and oligosaccharides enabling detection by integrated pulsed amperometric detection.

Table 3.1. pK_a values for selected monosaccharides in water. Adapted from Torto (1999).

	pK_a value	
compound	*10 °C*	*25 °C*
D-Glucose	12.72	12.35
D-Galactose	12.82	12.35
D-Mannose	12.45	12.48
D-Arabinose		12.43
D-Ribose		12.21
D-Xylose		12.29
D-Fructose	12.53	12.03

Electrochemical detection of carbohydrates

Electrochemical detection is the most promising technique to detect and measure mono-, di-, and oligosaccharides. Alternative traditional detection techniques such as refractive index and photometry are limited by their poor sensitivity to underivatised carbohydrates. Furthermore, refractive index detection is not compatible with the NaOH gradient used for the elution of oligo- as well as polysaccharides in anion exchange chromatography (Buttler et al., 1996).

Under optimal conditions, the detection of carbohydrates should be achieved on-line, without any sample preparation to enable monitoring of fast reactions where carbohydrate concentrations change very rapidly such as during kinetic reactions. Therefore, their direct or catalytic electrochemical detection is preferred as it is inherently selective and sensitive.

A typical electrochemical set-up consists of a working electrode where the reaction takes place, a counter electrode which takes up the current from the reference electrode, an electrolyte, a recording device as well as a potentiostat which maintains current flow between the working and counter electrodes to allow the desired potential on the working electrode, relative to the reference electrode. Detection is carried out with various electrode materials such as Hg, Pt, Au, Ag, Ni and Cu (Buchberger et al., 1996).

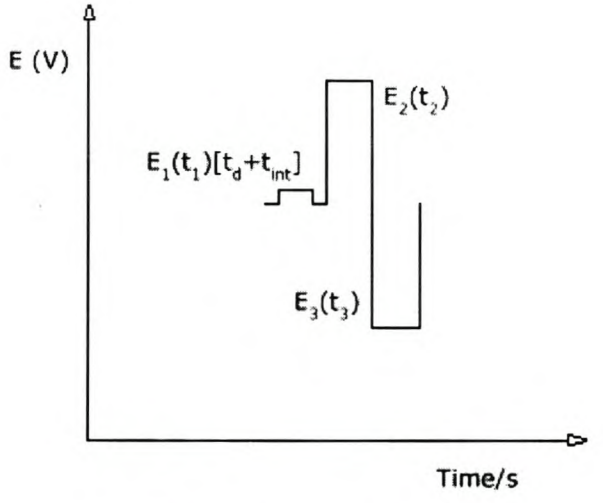

Figure 3.2. The waveform for integrated pulsed amperometric detection. E_1 represents the detection potential, E_2 the oxidative cleaning potential and E_3 the reductive regeneration potential. t_1, t_2, and t_3 are the time duration for potential applied. t_d and t_{int} are the delay and integration time, respectively. The elevated section on E_1 represents the current sampling time (adapted from Torto, 1999).

Some detector systems such as the Dionex system use glassy carbon, Pt or Au electrodes for the detection of carbohydrates. However, only gold

electrodes are commonly used because of their higher sensitivity and lower susceptibility to interference (Elliott et al., 1996). The mechanism of integrated pulsed amperometrical detection is detecting carbohydrates at a gold electrode with minimum contribution from background currents. The electrode surface is continuously cleaned and regenerated to suppress passivation. This is achieved by pulsed detection, which consists of three pulsed potentials as shown in Figure 3.2.

Mass spectrometry of carbohydrates

Mass spectrometry has proven to be a very sensitive and accurate technique to analyse carbohydrates when combined with column liquid chromatography or gas chromatography (Torto et al., 1998 a, b; Hernandez et al., 2001). In order to obtain structural or molecular weight information, labile carbohydrate molecules have to be transformed into gaseous phase, ionised and directed into the mass analyser. In previous studies, the molecules were fragmented by either electron ionisation (Watson, 1990) but recently techniques using matrix assisted laser desorption/ionisation and atmospheric pressure ionisation have become more common (Fenn et al., 1990; Harvey, 2003). These new ionisation techniques that can cope with labile molecules, have added a new impetus to carbohydrate research and are currently the most important and dominant ionisation tools available. However, in the process of coupling column liquid chromatography to mass spectrometry, only field desorption techniques like electrospray ionisation fulfils the requirement of an on-line method, although the effluent from the column needs to be desalted in order not to contaminate the ion source. This can be achieved by an in-line

cation exchange desalting device (Figure 3.3) using pure water for regeneration.

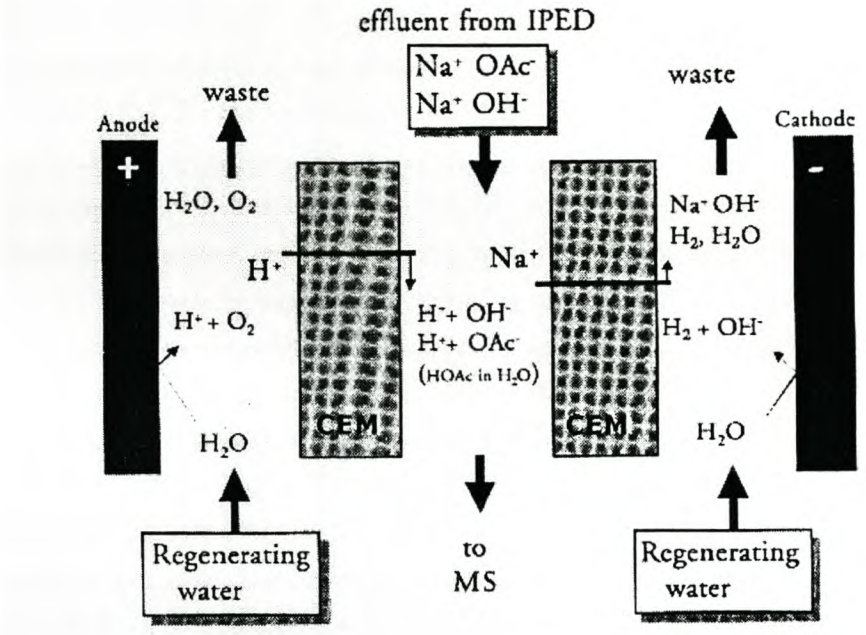

Figure 3.3. Schematic representation of how the cation exchange desalting device exchanges sodium ions with protons from the regenerating water, where CEM represents the cation exchange membrane (Torto, 1999). IPED = Integrated pulsed amperometrical detection.

In electrospray ionisation, carbohydrates produce the multiple charged ions of the form $[M+nH]^{n+}$ or $[M+nH]^{n-}$ which appear down field on the m/z scale by a factor equivalent to the charge of the ion (Ashcroft, 1997). This property of electrospray ionisation is important as large molecules can be analysed without the need to extend the mass range of the mass spectrometer (Ashcroft, 1997). The most common solvents for electrospray ionisation are water, methanol, acetonitrile, isopropanol and mixtures of water/acetonitrile or

water/methanol. For the cationisation of carbohydrates $[M+nH]^{n+}$, 10^{-4} M sodium acetate is often added (Rumbold et al., 2002).

Conclusions

A combination of microdialysis, mass spectrometry (MS) with HPAEC-IPED is a versatile tool for on-line monitoring of the enzymatic hydrolysis of lignocellulosic polymers (Torto et al., 1997). Unlike the conventional assay methods where the identity of hydrolysates is confirmed only based on retention times, this combination of techniques enables their unequivocal identification. The use of MS for analysis of labile biological molecules, especially carbohydrates has increased with the advent of atmospheric pressure ionisation techniques (Niessen, 1999) as well as efficient mechanisms to desalt chromatographic effluents before introduction into MS (Torto et al., 1998 a, b).

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CHAPTER 4

Influence of growth substrate and free ferulic acid on the production of feruloyl esterase by *Aureobasidium pullulans*

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Abstract

The effect of the carbon source and supplemented free ferulic acid on the production of extracellular feruloyl esterase, β-xylanase and α-arabinofuranosidase by *A. pullulans* was examined. *A. pullulans* was cultivated on birchwood xylan, oat spelt xylan, glucose, xylose, sugarcane bagasse and dissolving pulp as carbon source without and with the addition of ferulic acid. Production of feruloyl esterase activity was highest on birchwood xylan (1.6 U/mg protein). Ferulic acid (0.01% w/v) supplementation led to growth inhibition on all the carbon sources. Feruloyl esterase is also produced with glucose as carbon source, which is a repressor for xylanase production. It is therefore suggested that feruloyl esterase is regulated independently from xylanase. *A. pullulans* feruloyl esterase was found to possess activity against α- and β-naphthylacetate and naphthol-AS-D-chloroacetate but not against longer chain naphthyl substrates and others as determined in a screening with 14 esterase substrates.

Introduction

Hydroxycinnamic acids like ferulic acid and *p*-coumaric acid are bound as substituents on hemicellulose polymers in plant cell walls (Carnachan, 2000). Esterified to α-arabinofuranosyl side chains in arabinoxylans (Saulnier and Thibault, 1999), ferulic acid substituents can occur as single residues, or as dehydrodimers coupling two polysaccharide chains (Hatfield et al., 1999; Hernanz et al., 2001) or can be etherified to lignin (Iiyama et al., 1990; Lam et al., 2001). Feruloyl esterases catalyse the release of ferulic acid from plant material (Faulds and Williamson, 1995; Bartolome et al., 1996; Kroon et al., 1996). They play a key role in the cooperative decomposition of plant cell wall material together with other major hemicellulolytic enzymes (Williamson et al., 1998).

The production of feruloyl esterases is apparently regulated independently from other hemicellulolytic enzymes. *A. niger* feruloyl esterase production is stimulated by supplementing free ferulic acid to oat spelt xylan as the only carbon source, while xylanase levels are repressed (Faulds et al., 1997). In contrast, *S. avermitilis* feruloyl esterase production is independent from the amount of ferulic acid present in the carbon source, but is also induced under different conditions than xylanase (Garcia et al., 1998).

The black yeast *A. pullulans* produces a whole range of hemicellulolytic enzymes, with xylanase being produced at high level with high specific activity (Leathers, 1986) but is repressed when glucose is present in the growth medium (Leathers et al., 1984). Previous work indicated that the synthesis of β-xylosidase and α-arabinofuranosidase is dependent on arabinoxylan as

inducer, whereas xylanase and acetyl esterase are also produced when xylose is used as the carbon source (Myburgh et al., 1991).

Recently, we discovered that *A. pullulans* also produces extracellular feruloyl esterase activity (Chapter 5). The purpose of this study was to investigate the influence of the composition of the growth medium on the production of feruloyl esterase in comparison to the production of β-xylanase and α-arabinofuranosidase. Crude feruloyl esterase was also subjected to an activity screening on various commercially available esterase substrates. This could give valuable information on the specificity range of the enzyme and possibly identify novel substrates that could potentially be used for further characterisation of *A. pullulans* feruloyl esterase.

Materials and methods

Strain and culture conditions

A. pullulans NRRL Y2311-1 was cultivated in 50 ml yeast nitrogen base (0.7% w/v), L-asparagine (0.2% w/v) and KH_2PO_4 (0.5% w/v) with 1% (w/v) of the respective carbon source, birchwood xylan (Sigma), oat spelt xylan (Sigma) dissolving pulp from *Eucalyptus grandis* (SAPPI Saiccor, Umkomaas, South Africa), sugarcane bagasse (Tongaat-Huletts, Mt Edgecombe, South Africa), glucose and xylose. In a second set, the respective cultures were supplemented with 0.01 % (w/v) ferulic acid (Sigma). The cultures were incubated on a rotary shaker (120 rpm) at 30°C for 84 hours and one ml samples taken every 12 h for assay. Growth was followed by microscopic enumeration of yeast cells.

Enzyme assays

Culture supernatant recovered after centrifugation (10 min, 20000*g*) was assayed for feruloyl esterase activity using 4-nitrophenyl-5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (Sigma) as substrate (Biely et al., 2002), for α-arabinofuranosidase activity using 4-nitrophenyl-α-L-arabinofuranoside as substrate (Biely et al., 2002) and for β-xylanase activity using birchwood xylan as substrate (Bailey et al., 1992). One unit (U) of activity was defined as the amount of enzyme releasing 1 µmol product per min. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories) based on the Coomassie Blue method (Bradford, 1976) with bovine serum albumin as standard.

Screening of esterase substrates

α-naphthyl acetate, β-naphthyl acetate, β-naphthyl propionate, α-naphthyl butyrate, β-naphthyl valerate, β-naphthyl laurate, α-naphthyl myristate, α-naphthyl palmiate, naphthol AS β-Cl-propionate, naphthol AS benzoate, naphthol AS phenylacatate, naphthol AS acetate, naphthol AS-D acetate and naphthol AS-D chloroacetate as well as Fast Blue BB salt (4-Amino-2,5-diethoxybenzanilide diazotated zinc double salt) and carboxyl esterase from porcine liver were purchased from Sigma. Esterase activity was determined qualitatively by adding 50 µl culture supernatant of *A. pullulans* cultivated on birchwood xylan for 60 h to 1 ml of 0.1 M sodium phosphate buffer (pH 7) containing 25 µl Fast Blue BB solution (100 mg in 5 ml double distilled H_2O). The reaction was started by adding 50 µl of the respective substrate solution

(1 mM) and the formation of a purple colour pigment indicated enzyme activity.

Results and discussion

Production of feruloyl esterase on various substrates

A. pullulans was grown on six different carbon sources at a concentration of 1% w/v, without and with the addition of 0.01 % w/v FA, over a 84 hours period until late stationary phase was attained. The time course profiles of feruloyl esterase activity are presented in Figure 4.1. The highest xylanase levels were produced when *A. pullulans* was grown on birchwood xylan and oat spelt xylan whereas no xylanase activity was found in glucose and dissolving pulp grown cultures. The highest arabinofuranosidase activity was also found in cultures grown on birchwood xylan and oat spelt xylan providing further evidence that the regulation of these two hemicellulases may be linked. Feruloyl esterase activity was also highest in cultures grown on birchwood xylan and oat spelt xylan but also significant levels were found in cultures grown on glucose and xylose. On birchwood xylan, highest feruloyl acid esterase activity was reached with 1.6 U/mg protein after 60 hours. On oat spelt xylan, activity also showed a maximum after 60 hours (0.9 U/mg protein). Both substrates are equally well suited for the production of feruloyl esterase, although the activity is 1.8 fold higher when grown on birchwood xylan.

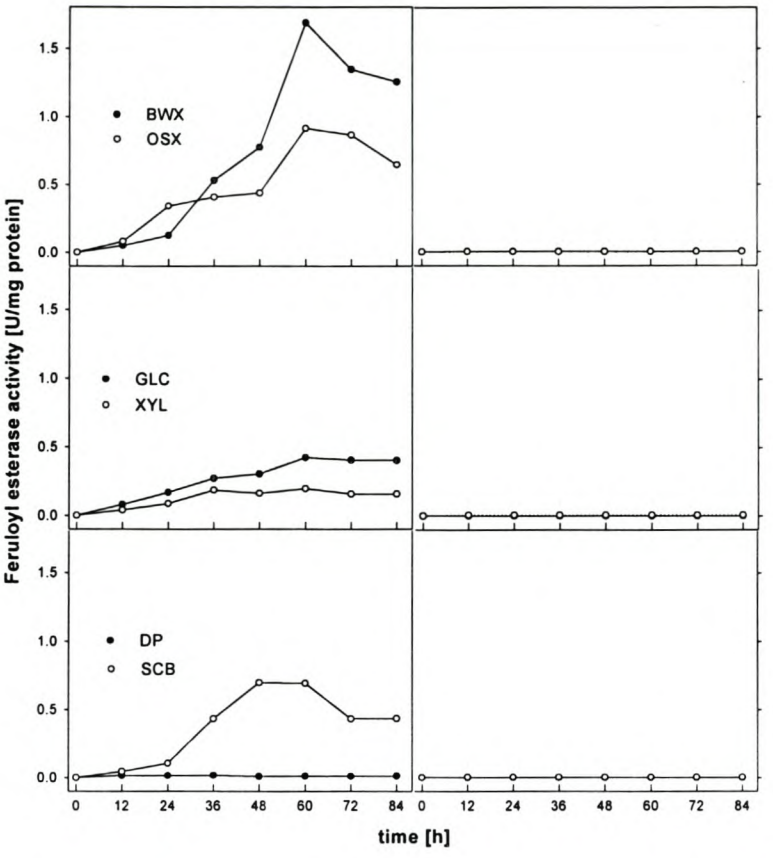

Figure 4.1. Effect of various carbon sources and free ferulic acid on the production of feruloyl esterase from *A. pullulans*. The upper row of graphs show feruloyl esterase activity produced on birchwood xylan (BWX) and oat spelt xylan (OSX), the middle row of graphs show activity on glucose (GLC) and xylose (XYL) and the lower row shows activity on dissolving pulp (DP) and sugarcane bagasse (SCB). The left column of graphs show activity without, the right column with a supplement of 0.01% free ferulic acid.

No feruloyl esterase activity was produced on dissolving pulp as a result of poor or no growth (not shown), which is probably because dissolving pulp consists to 97% from cellulose (Puls et al., 1985) and *A. pullulans* lacks cellulase activity (Leathers, 1986). When grown on sugar cane bagasse,

feruloyl esterase activity reached 0.7 U/mg protein after 48 hours. This is noteworthy, because sugar cane bagasse is more difficult to degrade and to metabolise as carbon source than birchwood xylan or oat spelt xylan, thereby linking growth and enzyme production.

Surprisingly, feruloyl esterase activity was also present when *A. pullulans* was grown on monosaccharides as carbon source, reaching maxima of 0.4 U/mg protein after 60 hours growth on glucose and 0.2 U/mg protein after 36 hours growth on xylose. This is particularly interesting, since the production of other hemicellulases like xylanase is suppressed in presence of glucose, as shown in Table 4.1. These findings also indicate that the production of feruloyl esterase from *A. pullulans* may also be regulated independently to xylanase production. This conclusion is further supported by the observation that xylose induces xylanase and xylosidase synthesis (Table 4.1) but that feruloyl esterase activity of *A. pullulans* grown on xylose was 50% lower than with cells grown on glucose (Figure 4.1).

When the cultures are supplemented with 0.01% ferulic acid, no feruloyl esterase activity was detected in the cultures (Figure 4.1), because of almost no growth of *A. pullulans* (data not shown). At a 10 times lower concentration of free ferulic acid (0.001%) the growth and the production of feruloyl esterase was still only half of the amount of that observed without the addition of ferulic acid (data not shown), suggesting that free ferulic acid does not only negatively affect the production of feruloyl esterase, but moreover inhibits growth of *A. pullulans*. These results are in contrast to data reported previously for *A. niger*, where 0.03% (w/v) ferulic acid was found to stimulate feruloyl esterase production (Faulds an Williamson, 1995; Faulds et al., 1997).

Table 4.1. Specific activities (U/mg protein) of feruloyl esterase, xylanase and arabinofuranosidase produced by *A. pullulans* on various carbon sources after growth for 60 h.

Carbon source	Feruloyl esterase	Xylanase	Arabinofuranosidase
Birchwood xylan	1.6 ± 0.01[a]	1310 ± 5	1.6 ± 0.02
Oat spelt xylan	0.9 ± 0.01	1250 ± 3	1.4 ± 0.06
Glucose	0.4 ± 0.01	0	0.2 ± 0.05
Xylose	0.2 ± 0.01	420 ± 5	0.2 ± 0.06
Dissolving pulp	0	0	0
Sugarcane bagasse	0.7 ± 0.01	390 ± 5	1.2 ± 0.05

[a]Mean ± standard deviation of triplicate determinations.

The induction of xylanase production when grown on xylose and repression when grown on glucose has been shown to be regulated by a Cre-mediated glucose repression mechanism in *A. pullulans* (Vanden Wymelenberg et al., 1999). Cre-mediated glucose repression has been implicated in the regulation of a variety of fungal genes including xylanases of *Aspergillus* (Mach et al., 1996; Zeilinger et al., 1996) and *Trichoderma* (de Graff et al., 1994; Pinaga et al., 1994). Arabinofuranosidase was produced at very low level on both xylose and glucose, whereas feruloyl esterase levels are higher when grown on glucose than on xylose. Feruloyl esterase could possibly be involved in the exopolysaccharide metabolism of *A. pullulans,* which produces pullulan preferably when grown on glucose (Vijayendra et al., 2001). However, there is no direct evidence to support this. This might be an indication for the relative high feruloyl esterase levels on glucose in comparison to growth on xylose. Cultivation on sugar cane bagasse resulted in relative lower levels of feruloyl

esterase and xylanase, possibly due to poor growth (data not shown) caused by the fibrous nature of the material.

Substrate range of crude feruloyl esterase

The substrate range of *A. pullulans* feruloyl esterase was determined by screening the culture supernatant produced on birchwood xylan with 14 commercially available α-naphthyl and naphthol-AS esters (Table 4.2). *A. pullulans* culture supernatant showed activity on α- and β-naphthyl acetate, but no activity on aliphatic esters of a chain length from C-3 to C-16. Activity was also shown for naphthol AS-D acetate and naphthol AS-D chloroacetate. Pig liver esterase showed activity on α-naphthyl acetate, β-naphthyl acetate, β-naphthyl propionate, α-naphthyl butyrate and β-naphthyl valerate, but no activity on naphthol AS or naphthol AS-D substrates. α- and β-naphthol substrates with varying aliphatic chain-lengths are routinely used to determine the substrate spectrum of esterases (Figure 4.2). Carboxyl esterases accept substrates with a chain length from C-2 to C-5, whereas lipases show higher activtity on substrates above C-10 (Bornscheuer, 2002).

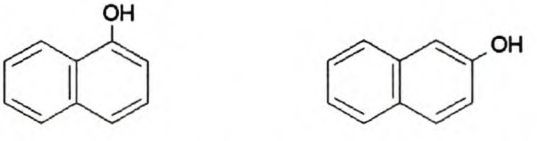

Figure 4.2. Structure of α-naphthol (1-naphthol, left) and β-naphthol (2-naphthol, right). Acyl esters of various chain length are bound to the α- and β-hydroxyl, respectively.

Naphthol AS and naphthol AS-D substrates (Figure 4.3) are used to detect specific esterase activity. Naphthol AS acetate, naphthol AS-D acetate and naphthol AS-D chloroacetate are used for the histochemical detection of the leucocyte specific esterase (Burstone, 1962), whereas naphthol AS phenylacetate is a histochemical substrate for chymotrypsin-like proteolytic enzymes (Zhao et al., 1994).

In our experiments, pig liver esterase behaved like a classical carboxyl esterase (Bornscheuer, 2002). In comparison, the crude feruloyl esterase preparation from *A. pullulans* did not accept naphthyl substrates with a chain length of more than C-2. Similar substrate range has been reported for feruloyl esterases from other organisms (Williamson et al., 1998).

Table 4.2. Substrate range of *A. pullulans* feruloyl esterase (AP) grown on birch wood xylan. Porcine liver esterase (PLE) was used as a reference. + activity, - no activity.

Substrate	Chain length	AP	PLE
α -naphthyl acetate	C-2	+	+
β -naphthyl acetate	C-2	+	+
β -naphthyl propionate	C-3	-	+
α -naphthyl butyrate	C-4	-	+
β -naphthyl valerate	C-5	-	+
β -naphthyl laurate	C-12	-	-
α -naphthyl myristate	C-14	-	-
α -naphthyl palmiate	C-16	-	-
naphthol AS β-Cl-propionate		-	-
naphthol AS benzoate		-	-
naphthol AS phenylacetate		-	-
naphthol AS acetate		-	-
naphthol AS-D acetate		+	-
naphthol AS-D chloroacetate		+	-

Interestingly, the crude feruloyl esterase preparation from *A. pullulans* showed activity on both naphthol AS-D acetate and naphthol AS-D chloroacetate. This has not previously been reported for a feruloyl esterase. Apparently, the additional phenolic moiety (3-o-toluidine) is essential for the enzymatic reaction. The phenolic moiety has been reported to contribute to feruloyl esterase catalytic specificity in general (Williamson et al., 1998, Prates et al., 2001). However, activity on naphthol AS-D acetate could not be detected, possibly because of the missing methyl group in the 3-naphthol bound aniline.

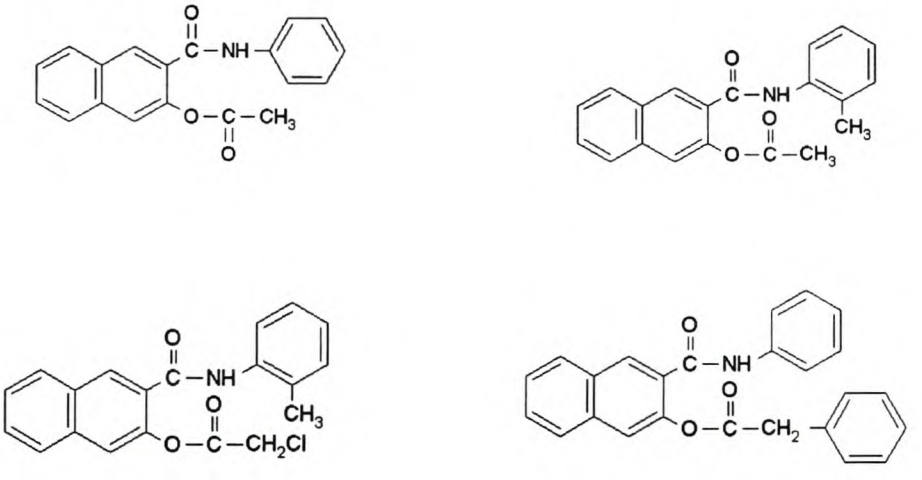

Figure 4.3. Structures of specific esterase substrates. Top left: naphthol AS acetate (3-acetoxy-2-naphthoic acid anilide). Top right: naphthol AS-D acetate (3-acetoxy-2-naphthoic acid toluidide). Lower left: naphthol AS-D chloroacetate (3-chloro-acetoxy-2-naphthoic acid toluidide). Lower right: naphthol AS phenylacetate (benzosalicylanilide phenylacetate).

In conclusion, it is apparent that xylanase, arabinofuranosidase and feruloyl esterase production by *A. pullulans* is elevated when grown on extracted lignocellulosic substrates (birchwood xylan and oat spelt xylan) in comparison

to raw natural substrates (sugar cane bagasse). This could be due to the architecture of the material, assuming that fibrous material is a poorer substrate than extracted material. The expression of feruloyl esterase in *A. pullulans* is apparently independent from the expression of xylanase, which is has been shown when the fungus was cultivated on glucose and xylose. However, under the same conditions the expression levels of feruloyl esterase and arabinofuranosidase are similar, which indicates an apparent link in the regulation of the two accessory enzymes. These results, however, would require transcriptional or promotor studies in order to be verified.

From a biochemical point of view it is remarkable that feruloyl esterase from *A. pullulans* accepts both naphthol AS-D acetate and naphthol AS-D chloroacetate as substrates, because they are both substrates for esterases with high specificity. It needs to be verified whether naphthol AS-D acetate or naphthol AS-D chloroacetate are substrates for feruloyl esterases in general. If they are, those substrates could be used when screening various microorganism on their ability to produce feruloyl esterase.

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CHAPTER 5

Purification and properties of a feruloyl esterase involved in lignocellulose degradation by *Aureobasidium pullulans*

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Abstract

The lignocellulolytic fungus *A. pullulans* NRRL Y 2311-1 produces feruloyl esterase activity when grown on birchwood xylan. Feruloyl esterase was purified from culture supernatant using ultrafiltration, anion exchange, hydrophobic interaction and gel filtration chromatography. The pure enzyme is a monomer with an estimated molecular mass of 210 kDa in both native and denatured forms and has an apparent degree of glycosylation of 48 %. The enzyme has a pI of 6.5, an optimum pH of 6.7 and a temperature optimum of 60°C. Specific activities for methyl ferulate, methyl *p*-coumarate, methyl sinapate and methyl caffeate are 21.6, 35.3, 12.9 and 30.4 µmol/min/mg, respectively. The pure feruloyl esterase transforms both 2-*O* and 5-*O* arabinofuranosidase-linked ferulate equally well and also shows high activity on the substrates 4-*O*-*trans*-feruloyl-xylopyranoside, *O*-{5-*O*-[(E)-feruloyl]-*α*-L-arabinofuranosyl}-(1,3)-*O*-*β*-D-xylopyranosyl-(1,4)-D-xylopyranose and *p*-nitrophenyl acetate but reveals only low activity on *p*-nitrophenyl butyrate. The catalytic efficiency (k_{kat}/K_m) of the enzyme was highest on methyl *p*-coumarate of all the substrates tested. Sequencing revealed the following eight N-terminal amino acids: AVYTLDGD.

Introduction

Plant cell wall topology is determined by the heterogeneous arrangement of carbohydrate and phenolic polymers, namely cellulose, hemicellulose and lignin (Boudet, 2000, Reid, 2000). Covalent linkages that connect these polymers are responsible for the three-dimensional microstructure and therefore accountable for the wide variety of plant cell walls properties. Hydroxycinnamic acids like 4-hydroxy-3-methoxycinnamic (ferulic) acid and 4-hydroxycinnamic (*p*-coumaric) acid occur in relative high frequency as substituents on hemicellulose but also on lignin polymers and are major components in forming covalent linkages between plant cell wall polymers. Ferulic acid is usually esterified at position C-5 to α-L-arabinofuranosyl side chains in arabinoxylans, at position C-2 to α-L-arabinofuranosyl residues in arabinans, at position C-6 to β-galactopyranosyl residues in pectic substances and galactans (Saulnier and Thibault, 1999) and at position C-4 to α-D-xylopyranosyl residues in xyloglucans (Ishii et al., 1990). Ferulic acid esterified to carbohydrates is found either as a monomer or linked to another esterified ferulic acid to form several types of dehydrodimers interlinking two polysaccharide chains (Fry, 1986; Iiyama et al., 1990; Ishii et al., 1990; Ralph et al., 1992). Carbohydrate esters of ferulic acid can also be involved in ester and/or ether linkages with lignin components enabling the formation of lignin-carbohydrate complexes (Lam et al., 2001). The presence of these complexes reduces the rate and extent of cell wall decomposition by microbial enzymes, not only in nature but also in industrial processes. Hydroxycinnamates can therefore be seen as important structural components, providing cell wall

integrity, hence protecting plants against digestion by plant-invading microorganisms and challenging enzyme technologies involving isolation and utilization of carbohydrate polymers (Ishii et al., 1990; Jeffries, 1990; Cornu et al., 1994; Gübitz et al, 1998).

Several hydroxycinnamic ester-hydrolysing enzymes have been isolated and characterized (Williamson et al., 1998 a, b), some have been cloned (Dalrymple et al., 1996; de Vries et al., 1997; Fillingham et al., 1999; Blum et al., 2000) and three-dimensional structures are now available (Prates et al., 2001). Recently, feruloyl esterase has been agreed on as the official name (EC 3.1.1.73) for this enzyme, but alternative names are ferulic acid esterase, hydroxycinnamoyl esterase or cinnamoyl ester hydrolase. Extracellular feruloyl esterases are produced by plant-invading microorganisms in addition to other lignocellulose-degrading enzymes but have also been reported to be endogenous to plants, such as in germinating barley (Sancho et al., 1999), where they probably participate in the cell wall extension process. Microbial feruloyl esterases commonly hydrolyse the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose. A whole range of different substrates, both natural and synthesized, have been used to characterize feruloyl esterases, such as *O*-{5-*O*-[(E)-feruloyl]-*α*-L-arabinofuranosyl}-(1,3)-O-*β*-D-xylanopyranosyl-(1,4)-D-xylanopyranose (FAXX) (Faulds and Williamson, 1991), methyl-4-hydroxy-3-methoxycinnamate (MFA) (Kroon et al.,1996), or 4-nitrophenyl 5-*O*-*trans*-feruloyl-*α*-L-arabinofuranoside (Biely et al., 2002). Feruloyl esterases have been classified according to sequence homologies (Coutinho & Henrissat, 1999) and into types according to their specificity for particular substrates

(Williamson et al., 1998 b). The ratios between K_m values of methyl ferulate and *p*-nitrophenyl acetate, 4-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (NPh-5-Fe-Ara*f*) and 4-nitrophenyl 2-*O*-*trans*-feruloyl-α-L-arabinofuranoside (NPh-2-Fe-Ara*f*), can be used to characterize and type feruloyl esterases (Faulds & Williamson, 1994; Williamson et al., 1998 a, b).

The black yeast *A. pullulans* is an ubiquitous saprophyte found on leaves of crop plants. The fungus is well established in applied biotechnology (Deshpande et al., 1992) and is particularly effective in sulphite pulp bleaching (Christov and Prior, 1996). However, only xylanase (Leathers, 1986), β-xylosidase (Dobberstein and Emeis, 1991), β-glucosidase (Saha et al., 1994) and α-L-arabinofuranosidase (Saha and Bothast, 1998) have been purified and characterized from the lignocellulose-degrading enzyme system of this organism. In this article we report the purification and characterization of a feruloyl esterase. Recently, we have shown that this enzyme contributes substantially to the lignocellulose-degrading potential of *A. pullulans* (Rumbold et al., 2002). Using synthetic and natural substrates, we demonstrate the broad substrate spectrum of this high molecular mass enzyme.

Materials and methods

Strain and fermentation conditions

A. pullulans NRRL Y 2311-1 was maintained on potato dextrose agar (Christov and Prior, 1996). Feruloyl esterase activity was produced by growing *A. pullulans* in a benchtop fermenter containing yeast nitrogen base (0.67% w/v), L-asparagine (0.2% w/v) and KH_2PO_4 (0.5% w/v) with 1% (w/v)

birchwood xylan (Sigma) in a total volume of 2 l. After 60 h cultivation at 30°C, at an agitation speed of 400 rpm, and at an aeration rate of 1000 ml/min, the medium was separated from the cells by centrifugation (5000*g*, 5 min) and the supernatant was stored at 4°C after 10 fold concentration in an Amicon stirred ultrafiltration cell (Millipore) fitted with a 30 kDa membrane.

Anion exchange chromatography

Total protein (200 mg) from the concentrated supernatant were loaded onto a column containing Q Sepharose Fast Flow (5 ml bed volume) (Amersham Biosciences) per run at a flow rate of 5 ml/min. Unbound material was eluted with 50 mM phosphate buffer (pH 7.0) whereas bound proteins were eluted by gradual addition (0 → 100%) of 50 mM phosphate buffer (pH 7.0) containing 1 M NaCl. Five ml fractions were collected and assayed for feruloyl esterase activity as described below.

Hydrophobic interaction chromatography

Fractions containing feruloyl esterase activity collected by anion exchange chromatography were pooled and concentrated using a 10 ml stirred ultrafiltration unit with a 10 kDa molecular mass cut-off (Pall Filtron Corp). Samples containing 20 mg protein were separated on a 1 ml HiTrap Phenyl FF column (Amersham Biosciences) at a flow rate of 4 ml/min. Unbound samples were eluted with 50 mM Tris pH 7.0 containing 1.2 M $(NH_4)_2SO_4$. Bound samples were eluted by a linear decreasing gradient of 50 mM Tris pH 7.0 containing 1.2 M $(NH_4)_2SO_4$ (100 → 0 %). Fractions of 1.5 ml were collected and assayed for feruloyl esterase activity. Active fractions were pooled, desalted and concentrated using ultrafiltration.

Gel filtration chromatography

Pooled samples (100 μl) from the previous purification step were applied on a Superdex 75 HR 10/30 column (Amersham Biosciences) and separated by isocratic elution using 50 mM phosphate buffer, pH 7.0 at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and assayed for feruloyl esterase activity. For molecular weight determination of the purified feruloyl esterase, the column was calibrated with myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa) and ovalbumin (45 kDa) (Sigma).

Feruloyl esterase activity and protein determination

Total protein in crude and purified samples was determined using the Coomassie Blue method (Bradford, 1976; BIORAD) using bovine serum albumin as standard. Fractions generated during the purification of the enzyme from *A. pullulans* culture supernatant were routinely assayed for feruloyl esterase activity by a spectrophotometric method (Biely et al., 2002) using 0.6 mM 4-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside as substrate.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7, 10 or 12 % SDS-PAGE gels with the Mini - PROTEAN II (BIORAD). Denatured protein molecular mass was estimated using high molecular weight standards consisting of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa) (Sigma).

Isoelectric focusing (IEF) was performed using Ready-gels (pH 3 – 10; BIORAD) according to the manufacturer's instructions. IEF standards (pI 4.6-9.6; BIORAD) were used for the accurate pI calibration of IEF gels. All gels were stained with Coomassie R-250 (Sigma). Esterase activity was assayed *in situ* on IEF and SDS-polyacrylamide gels. After electrophoresis, the gels were incubated in 20 ml 100 mM phosphate buffer (pH 7.0) containing 2 ml substrate solution (1 % [w/v] naphthol-AS-D-chloroacetate [Sigma] in acetone) and 500 µl Fast Blue BB (Sigma) solution (2 % [w/v] in water). Ester hydrolysis was detected as purple bands in the gel.

Protein deglycosylation

The degree of Asp – linked glycosylation of the purified feruloyl esterase was determined by digesting the protein using a N-glucosidase F deglycosylation kit (Roche Diagnostics) according to manufacturer's instructions and separated as described above using 12 % SDS-PAGE.

Enzyme Assays

Fractions generated during the purification of the enzyme from *A. pullulans* culture supernatant were routinely assayed for feruloyl esterase activity by a spectrophotometric method (Biely et al., 2002) using 0.6 mM 4-nitrophenyl 5-O-*trans*-feruloyl-α-L-arabinofuranoside as substrate in 100 mM sodium phosphate buffer (pH 7). The same method was used to determine the temperature optimum. For pH optimum experiments the following buffers were used: citrate from pH 4.5 to pH 6, phosphate buffer from pH 6.5 to pH 7 and Tris buffer for pH 7.5 and pH 8. For the investigation of substrate specificity, synthesized methyl esterified phenolic acids (Faulds and Williamson, 1994)

and isolated water-soluble feruloylated oligosaccharide (FAXX) from wheat bran (MacKenzie and Bilous, 1988) were used. Methyl ferulate (MFA), methyl caffeate (MCA), methyl *p*-coumarate (MpCA), methyl sinapate (MSA) (Apin chemicals, a generous gift from Dr. Craig Faulds, Institute for Food Research, Norwich, UK) and *O*-{5-*O*-[(E)-feruloyl]-*α*-L-arabinofuranosyl}-(1,3)-O-*β*-D-xylanopyranosyl-(1,4)-D xylanopyranose (FAXX) were incubated at various concentrations together with the purified enzyme in MOPS buffer pH 7.0 at 37°C with a final volume of 0.6 ml. Reactions were terminated after 15 min by adding 0.2 ml methanol. Samples were assayed for phenolic acids by reversed-phase HPLC (Waters). Ferulic, caffeic, *p*-coumaric and sinapic acid (Sigma) were used for calibration. Acetyl esterase and butyryl esterase activity was respectively determined by measuring an increase in the absorbance at 405 nm using 4 mM 4-nitrophenyl acetate and 4 mM 4-nitrophenyl butyrate as substrates (Sigma) in 100 mM phosphate buffer (pH 7.0) at room temperature. Activity is expressed as µmol of 4-nitrophenol released per min per mg of enzyme. Another spectrophotometric assay (Biely et al. 2002) was used to determine enzymatic activity on 4-nitrophenyl 5-O-*trans*-feruloyl-α-L-arabinofuranoside (NPh-5-Fe-Ara*f*), 4-nitrophenyl 2-O-*trans*-feruloyl-α-L-arabinofuranoside (NPh-2-Fe-Ara*f*) and 4-nitrophenyl 4-O-*trans*-feruloyl-xylanopyranoside (NPh-4-Fe-Xyl*p*). The substrates were incubated with the purified enzyme at 30°C in a 1 mm quartz cuvette and a decrease of the absorbance at 340 nm was recorded over 10 min. The difference in molar absorbance values between the substrate and free ferulic acid at 340 nm was used to calculate the amount of reacted substrate. The values were 9000, 8000, 9100 per M per cm for 4-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-

arabinofuranoside, for 4-nitrophenyl 2-*O*-*trans*-feruloyl-α-L-arabinofuranoside and for 4-nitrophenyl 4-*O*-*trans*-feruloyl-xylanopyranoside, respectively (Figure 5.1). *Aspergillus oryzae* feruloyl esterase (Tenkanen et al, 1991) was a generous gift from Dr. Maija Tenkanen (VTT, Espoo, Finland) and was used to compare substrate specificities.

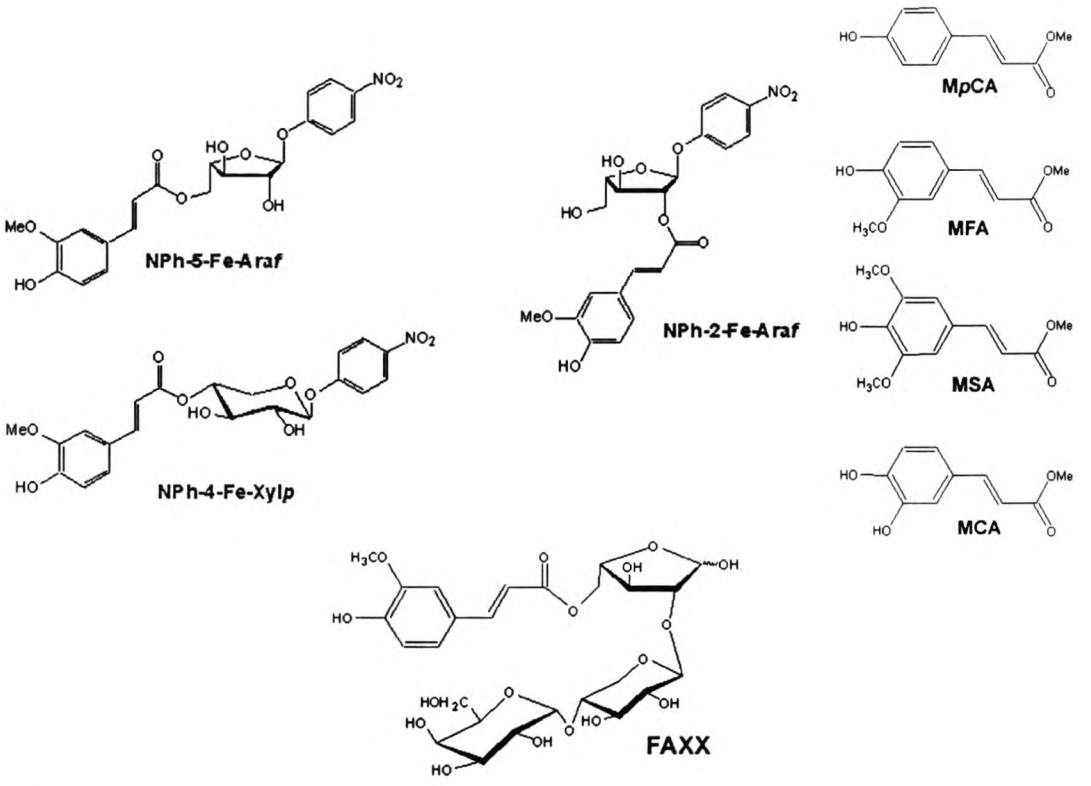

Figure 5.1. Structures of feruloyl esterase substrates. 4-nitrophenyl 5-O-*trans*-feruloyl-α-L-arabinofuranoside (NPh-5-Fe-Ara*f*), 4-nitrophenyl 2-O-*trans*-feruloyl-α-L-arabinofuranoside (NPh-2-Fe-Ara*f*) and 4-nitrophenyl 4-O-*trans*-feruloyl-xylanopyranoside (NPh-4-Fe-Xyl*p*), methyl ferulate (MFA), methyl *p*-coumarate (MpCA), methyl caffeate (MCA), methyl sinapate (MSA), *O*-{5-*O*-[(E)-feruloyl]-α-L-arabinofuranosyl}-(1,3)-O-β-D-xylanopyranosyl-(1,4)-D-xylanopyranose (FAXX).

Protein sequencing

Prior to sequencing, purified feruloyl esterase was concentrated by passing the protein through a ProSorb cartridge (Applied Biosystems, Foster City, CA). The N-terminal amino acid sequence was determined by Edman degradation of the purified feruloyl esterase using a ABI Procise 491 sequencer (Applied Biosystems).

Peptide mass mapping

Peptide mapping was performed by Professor W. Brandt at the University of Cape Town. Feruloyl esterase from *A. pullulans* was digested with trypsin and peptide masses were determined on a Maldi-TOF Voyager-DE Pro, Biospectrometry workstation (Applied Biosystems). Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) at a concentration of 10 mg/ml was used as matrix, 50 % acetonitrile and 3 % trifluoroacetic acid were used as solvents. The resulting peptide masses were submitted to PeptideSearch (EMBL, Heidelberg) using the following internet address:

www.mann.embl-heidelberg.de/GroupPages/PageLink/peptidesearchpage.html

The peptide match threshold was 5, the protein mass range was set to 180-220 kDa and the peptide mass accuracy was 2 Da.

Results

Production and purification of feruloyl esterase

A. pullulans feruloyl esterase was produced by cultivation on 1 % birchwood xylan reaching a maximum specific activity after 60 h of 1.6 U/mg (Chapter 4, Rumbold et al., 2003). The enzyme was isolated from the culture supernatant by means of ultrafiltration and purified by protein liquid chromatography (Table 5.1).

Table 5.1. Purification of *A. pullulans* feruloyl esterase.

Purification Step	Volume (ml)	Specific activity (U/mg protein)	Total activity (units)	Purification (fold)	Yield (%)
Crude culture supernatant	2000	1.6	695	1	100
Ultrafiltration	200	10.8	472	6.8	68
Anion-exchange chromatography	25	32.8	151	20.5	22
Hydrophobic interaction chromatography	20	41.5	123	25.9	18
Size exclusion chromatography	20	68.8	93	43	13

The ultrafiltration of crude culture supernatant using a 30 kDa membrane was a major purification step (6.8-fold) and 68 % of the feruloyl esterase activity was recovered in the concentrate.

After anion exchange chromatography, feruloyl esterase activity was assigned to the unbound fraction (Figure 5.2).

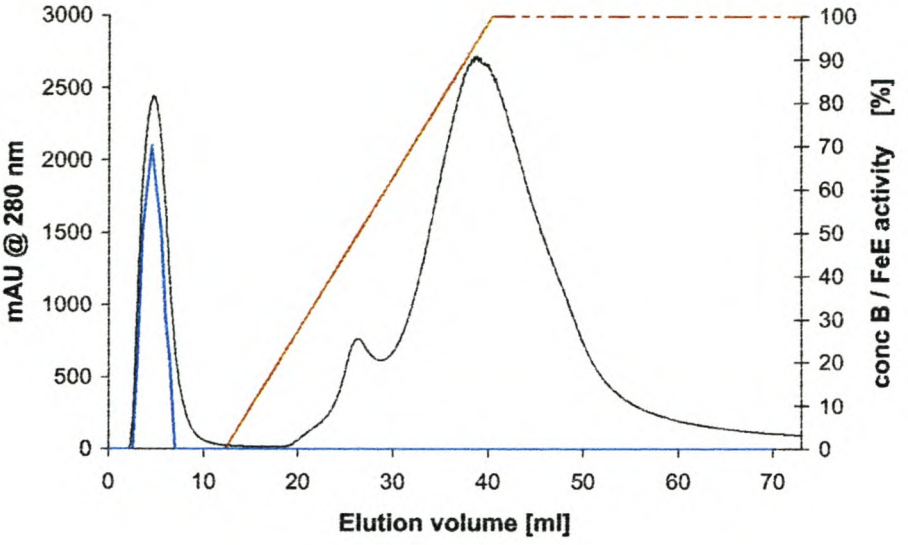

Figure 5.2. Anion-exchange chromatography of concentrated culture supernatant. Feruloyl esterase activity (FeE; blue line) was found in the unbound fraction. Absorbance at 280 nm, black line. Conc B (red line) represents the gradient of 50 mM phosphate buffer, pH 7.0 (0 %) and 50 mM phosphate buffer, pH 7.0 containing 1 M NaCl (100 %). 100% activity is equivalent to 46 U/mg protein from all tubes containing feruloyl esterase activity.

None of the fractions obtained during NaCl gradient elution contained feruloyl esterase activity and therefore overloading of the column was not assumed. At a pH of 9.5 the enzyme was bound to the column matrix but the purification efficiency at pH 7 was higher and therefore the unbound fraction represented a better starting material for subsequent purification. After hydrophobic

interaction chromatography, feruloyl esterase activity could only be detected in the unbound fraction (Figure 5.3).

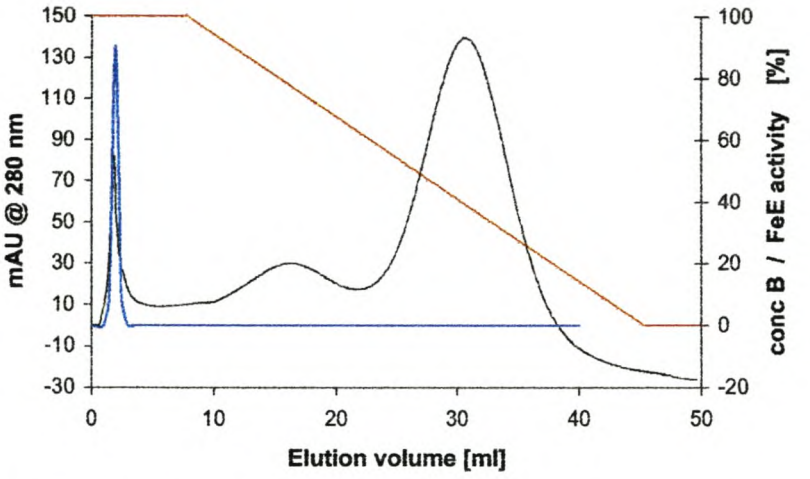

Figure 5.3. Hydrophobic interaction chromatography of pooled fractions from anion exchange chromatography containing feruloyl esterase activity. Feruloyl esterase activity was found in the unbound fraction. Conc B = concentration of buffer B (50 mM Tris pH 7.0 containing 1.2 M $(NH_4)_2SO_4$). FeE = feruloyl esterase. 100% activity is equivalent to 60 U/mg protein from all tubes containing feruloyl esterase activity.

An increase of ammonium sulphate concentration in order to bind the enzyme to the column matrix was not considered because other proteins were successfully removed during this purification step. Finally, size exclusion chromatography yielded a single active peak (Figure 5.4) that contained feruloyl esterase purified 43-fold with a 13 % yield compared to the crude culture supernatant (Table 5.1).

SDS-PAGE analysis was conducted and shows that a single protein representing feruloyl esterase remained after the final purification step (Figure 5.5).

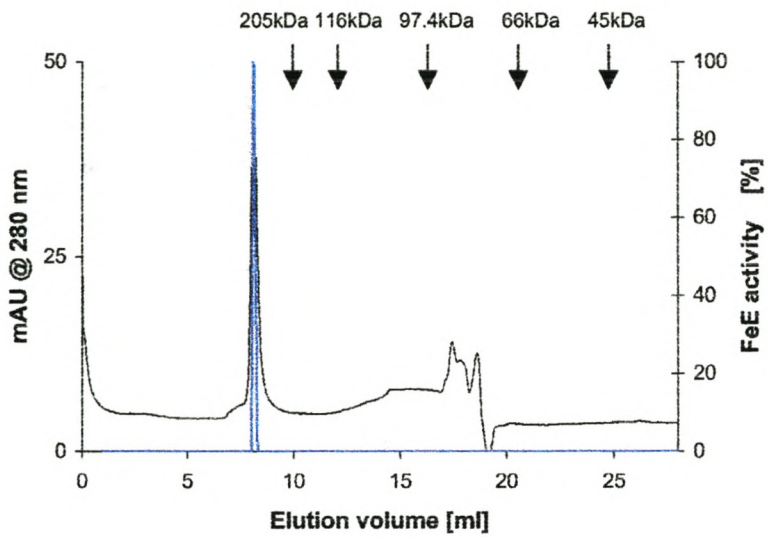

Figure 5.4. Gel filtration chromatography. Feruloyl esterase activity elutes as a single peak after 8 ml. Standards of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa) and ovalbumin (45 kDa) elute at positions marked with arrows. 100% activity is equivalent to 68.8 U/mg protein.

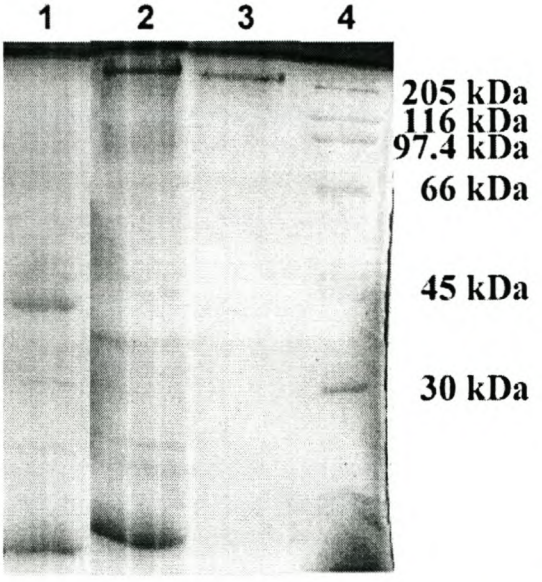

Figure 5.5. SDS-PAGE of *A. pullulans* feruloyl esterase purification steps. **1** after anion exchange chromatography (100 µg total protein). **2** after hydrophobic interaction chromatography (150 µg total protein). **3** after gel filtration chromatography (50 µg total protein). **4** high range molecular weight standard markers, myosin (205 kDa), *β*-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30kDa).

Properties of feruloyl esterase

SDS-PAGE analysis of the *A. pullulans* feruloyl esterase gave a single band with an estimated molecular mass of 210 kDa after both Coomassie blue and activity staining (Figure 5.6).

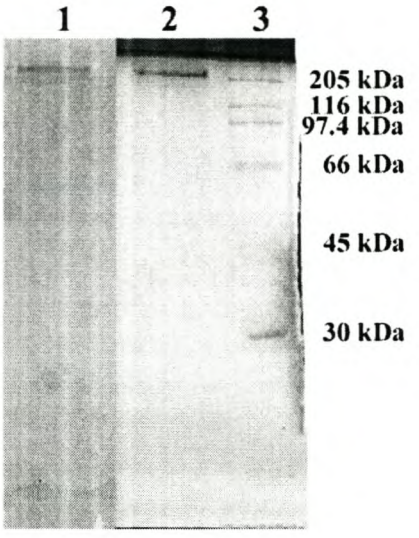

Figure 5.6. SDS-PAGE of *A. pullulans* feruloyl esterase. **1** Activity stain using naphthol AS-D chloroacetate as substrate (50 µg total protein). **2** Coomassie blue stain (50 µg total protein). **3** high range molecular weight standard markers, myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30kDa).

The same molecular mass was determined by gel filtration chromatography suggesting that the enzyme consists of a single monomer with a high M_r (Figure 5.4). The purified enzyme also showed a single band after isoelectric focusing which corresponded to a pI of 6.5 (Figure 5.7).

Enzymatic deglycosylation of the feruloyl esterase and subsequent SDS-PAGE (Figure 5.8) and gel filtration (data not shown) showed a protein with an apparent molecular mass of 110 kDa suggesting that the *A. pullulans* feruloyl esterase is 48 % glycosylated.

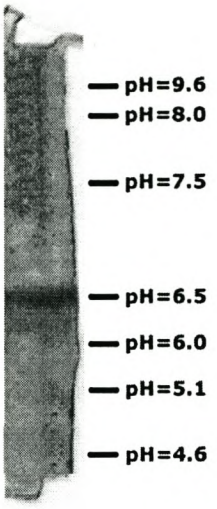

Figure 5.7. Isoelectric focusing polyacrylamide gel. 50 µg of feruloyl esterase from *A. pullulans* NRRL Y 2311-1 was loaded.

The enzyme proved to be relatively stable particularly in conjunction with other proteins. For instance, the crude culture filtrate preserved 80 % of its initial activity after 6 month at 4°C, whereas the half-life time of feruloyl esterase after anion exchange chromatography or pure feruloyl esterase was only one month at 4°C.

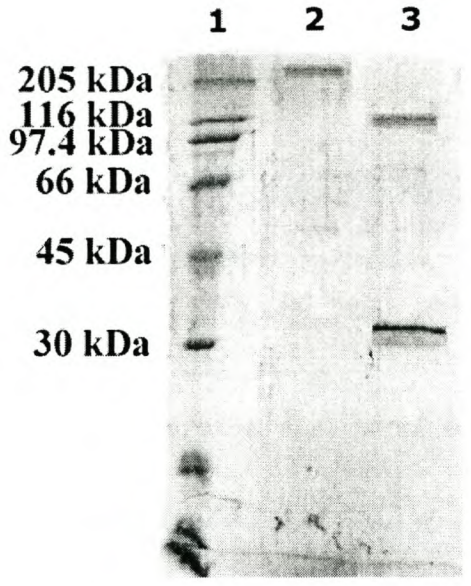

Figure 5.8. SDS-PAGE analysis of purified feruloyl esterase (50 µg, lane **2**) and deglycosylated feruloyl esterase from *A. pullulans* NRRL Y 2311-1 (110kDa) and N-glucosidase F (30 kD, lane **3**). Lane **1** represents markers myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30kDa).

Pure feruloyl esterase had a temperature optimum between 60 and 65°C using NPh-5-Fe-Ara*f* or NPh-2-Fe-Ara*f* as substrates (Figure 5.9) and at the standard assay temperature of 30°C it showed 79 % of optimum activity. With the same substrates, feruloyl esterase exhibited 100 % activity at a pH of 6.7 and only 50 % at pH 4 and 8.5, respectively (Figure 5.10).

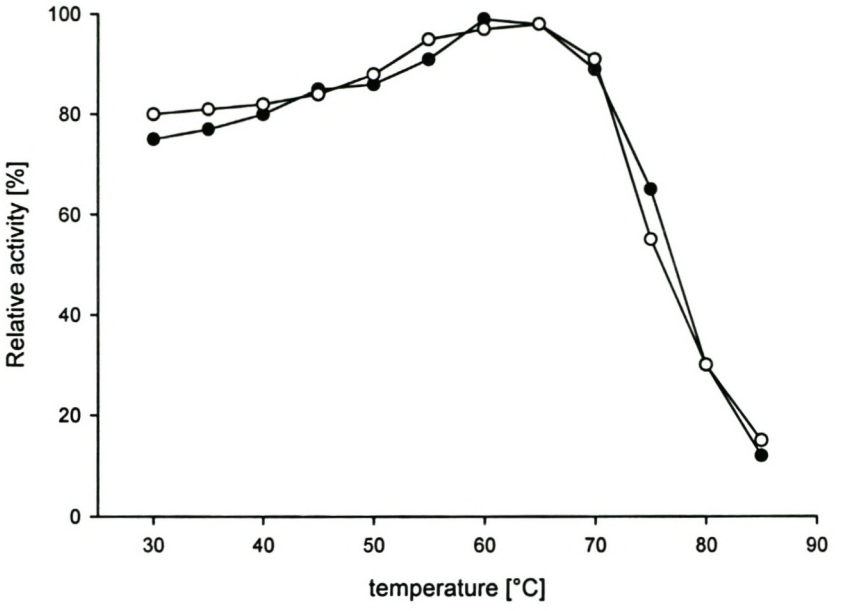

Figure 5.9. Temperature optimum of feruloyl esterase from *A. pullulans* NRRL Y 2311-1. □ NPh-5-Fe-Ara*f*, □ NPh-2-Fe-Ara*f*. 100 % activity is equivalent to 87 U/mg.

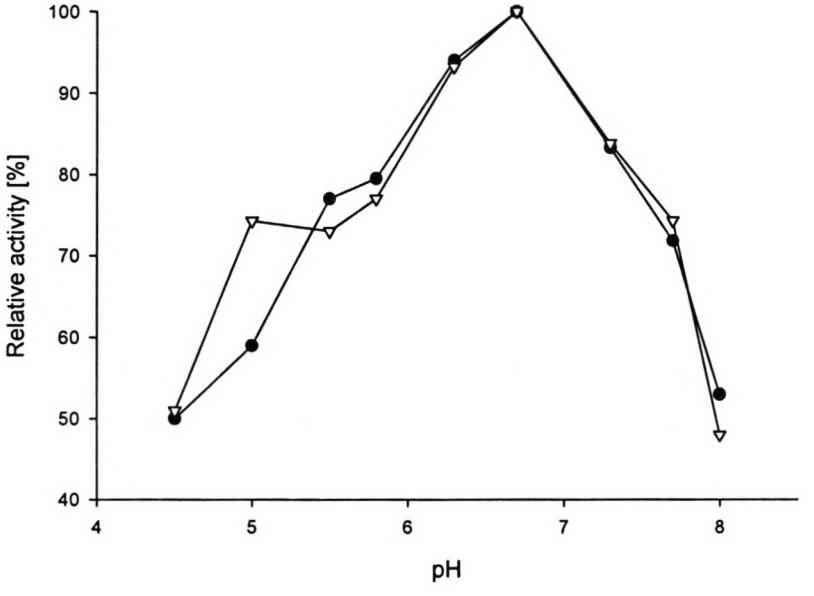

Figure 5.10. pH optimum of feruloyl esterase from *A. pullulans* NRRL Y 2311-1. □ NPh-5-Fe-Ara*f*, ▽ NPh-2-Fe-Ara*f*. 100 % activity is equivalent to 68.6 U/mg.

Substrate specificity of *A. pullulans* feruloyl esterase compared to *A. oryzae* feruloyl esterase

A. oryzae feruloyl esterase hydrolyses NPh-5-Fe-Ara*f*, but NPh-2-Fe-Ara*f* and NPh-4-Fe-Xyl*p* are hardly hydrolysed whereas the feruloyl esterase from *A. pullulans* hydrolyses all three substrates at about the same rate (Figure 5.11). The absorbance decrease with feruloyl esterase from *A. oryzae* was almost linear indicating a lower K_m values than for the feruloyl esterase from *A. pullulans*.

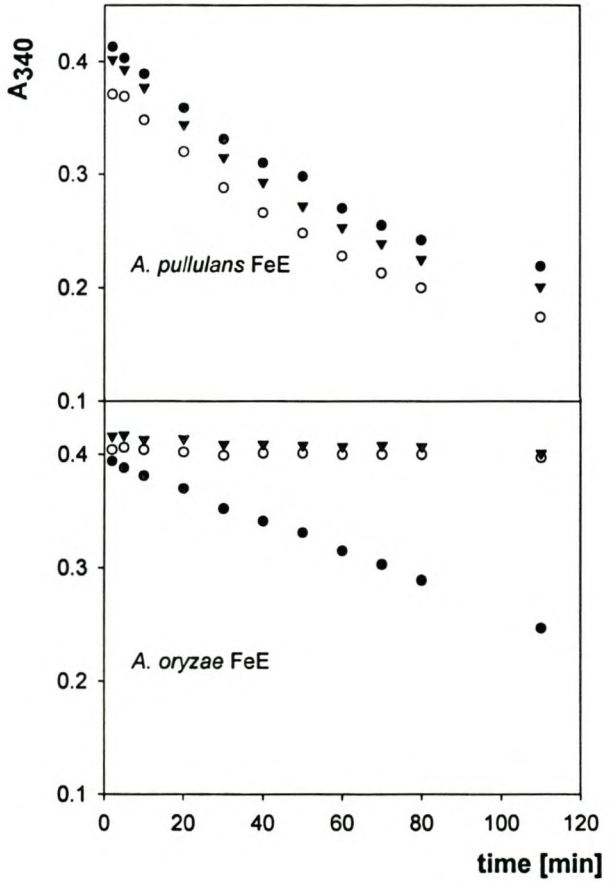

Figure 5.11. Comparison of hydrolysis of different substrates by *A. pullulans* NRRL Y 2311-1 feruloyl esterase and *A. oryzae* feruloyl esterase. □ NPh-5-Fe-Ara*f*, □ NPh-4-Fe-Xyl*p*, □ NPh-2-Fe-Ara*f*

A summary of kinetic data for *A. pullulans* feruloyl esterase on a variety of substrates is presented in Table 5.2.

Table 5.2. Substrate specificities for *A. pullulans* feruloyl esterase.

Substrate	Specific activity µmol/min/mg	K_m (µM)	k_{kat} (s^{-1})	k_{kat}/K_m ($M^{-1}s^{-1}$)
Methyl ferulate	21.6 (0.7)[e]	50.2 (0.9)	15.3 (0.5)	0.3×10^6
Methyl *p*-coumarate	35.3 (0.5)	10.6 (0.2)	31.1 (0.3)	2.9×10^6
Methyl sinapate	12.9 (0.9)	137 (11)	23.7 (0.5)	0.17×10^6
Methyl caffeate	30.4 (0.5)	98 (8)	30 (0.9)	0.3×10^6
NPh-5-Fe-Ara*f*[a]	74.6 (1)	268 (13)	65.5 (1)	0.24×10^6
NPh-2-Fe-Ara*f*[b]	71.1 (0.9)	230 (13)	70.2 (0.9)	0.3×10^6
NPh-4-Fe-Xyl*p*[c]	73.2 (0.9)	ND[f]	ND	ND
FAXX[d]	35.9 (1.2)	ND	ND	ND
4-Nitrophenyl-Acetate	31.8 (0.2)	ND	ND	ND
4-Nitrophenyl-Butyrate	0.02 (0.01)	ND	ND	ND

[a]4-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside

[b]4-nitrophenyl 2-*O*-*trans*-feruloyl-α-L-arabinofuranoside

[c]4-nitrophenyl 4-*O*-*trans*-feruloyl-xylanopyranoside

[d]*O*-{5-*O*-[(E)-feruloyl]-*α*-L-arabinofuranosyl}-(1,3)-O-*β*-D-xylanopyranosyl-(1,4)-D-xylanopyranose

[e]Figures in parenthesis give an estimate of the standard error

[f]ND, not determined

The K_m and V_{max} values were calculated from the reciprocal rate (1/v) as a function of the substrate concentration (1/[S]), shown in Figure 5.12, which gives

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]}$$

The turnover number of the enzyme is given by

$$k_{kat} = \frac{V_{max}}{[E_t]}$$

where E_t is the total concentration of the enzyme. The enzyme catalysed the hydrolysis of all tested methyl cinnamates with the activity for methyl *p*-coumarate highest followed by methyl caffeate, methyl ferulate and methyl sinapate. Similarly, catalytic efficiency (k_{kat}/K_m) calculations revealed a value ten fold greater for MpCA, than the other methyl cinnamate substrates.

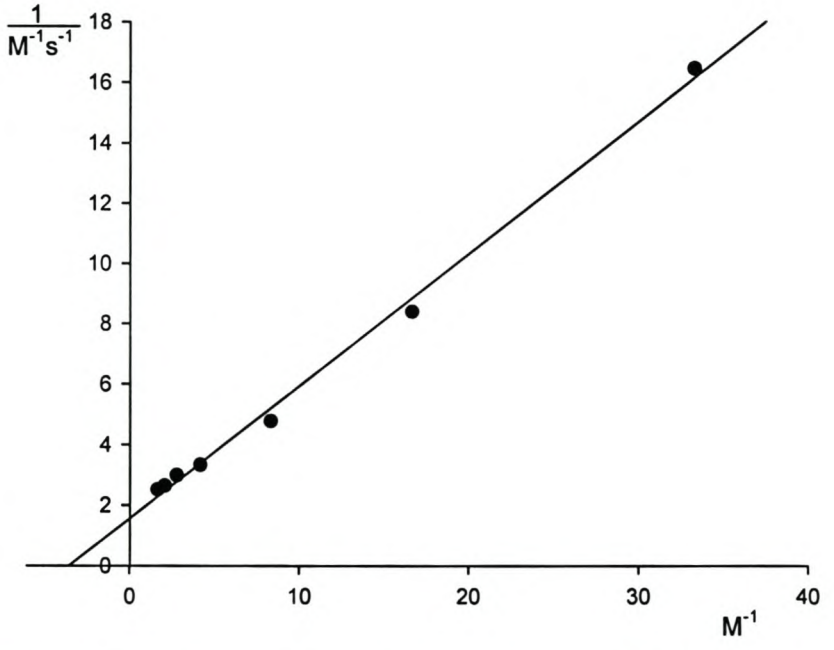

Figure 5.12. Double-reciprocal plot of *A. pullulans* NRRL Y 2311-1 feruloyl esterase activity on NPh-5-Fe-Ara*f*. Substrate concentration is given in M and s represents seconds.

Compared to methyl cinnamates, feruloyl esterase showed greater activity with NPh-5-Fe-Ara*f*, NPh-2-Fe-Ara*f* and NPh-4-Fe-Xyl*p* as substrates. Furthermore the substrate turnover rate (k_{kat}) was greater with NPh-5-Fe-Ara*f* and NPh-2-Fe-Ara*f* although the substrate affinity (K_m) was lower. Feruloyl

esterase from *A. pullulans* also hydrolyses FAXX isolated from wheat bran and shows high activity for 4-nitrophenol acetate but almost no activity for 4-nitrophenol butyrate. The k_{kat}/K_m catalytic efficiency values were similar for most substrates although the constant was much greater for methyl *p*-coumarate suggesting that the chemical structure of the phenolic moiety of the substrate is essential for the catalysis by feruloyl esterase.

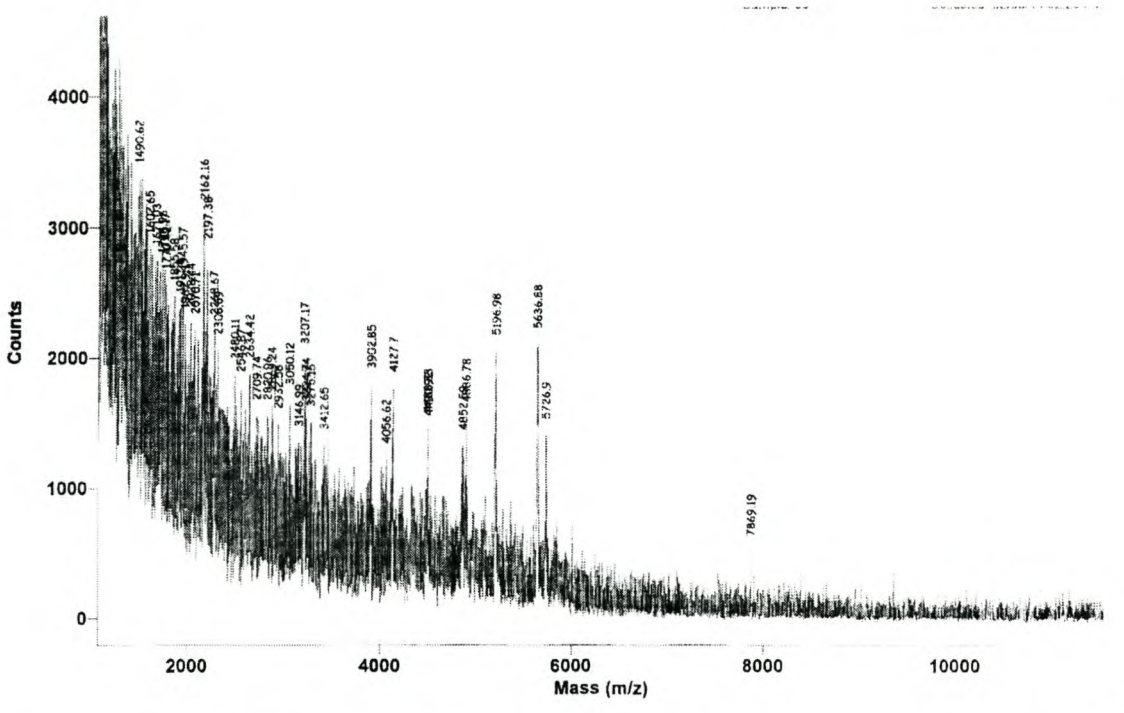

Figure 5.13. Maldi-TOF mass spectrum of tryptic digested feruloyl esterase of *A. pullulans* NRRL Y 2311-1. Detected peptide masses are indicated in the figure.

Protein sequencing

The first eight residues of the N-terminal amino acid (AVYTLDGD) sequence of the *A. pullulans* feruloyl esterase were successfully determined after which the sequencing reaction was terminated. A rerun of the sequencing reaction resulted in the same sequence. A BLAST search using the N-terminal

sequence of *A. pullulans* feruloyl esterase revealed no similarities to other known N-terminal regions of feruloyl esterases.

Table 5.3. PeptideSearch results. Molecular mass, name and number of peptides matched as a result from the submission of tryptic digested feruloyl esterase from *A. pullulans* NRRL Y 2311-1.

Peptides matched	Mass [kDa]	Protein name
6	194.89	L23972|PPNRNA_2 product: "195K
6	113.14	O43352 Probable dynein protein
6	194.67	Q94HT9 Putative retroelement.//
6	108.71	Q9HTS8 Adenylate cyclase.//:tre
5	106.5	Q92DN5 Hypothetical protein lin
5	145.11	Q8YRE3 Hypothetical protein Alr
5	120.02	Q9RWC9 Drug transport protein,
5	125.35	O37061 RNA-dependent RNA polyme
5	115.14	D86068|HIMCK1_2 gene: "pol"; pr
5	114.16	Q994G3 Pol polyprotein (Fragmen
5	138.99	O41516 Structural protein.//:tr
5	183.96	Q8RGB7 Fusobacterium outer memb
5	161.82	Q99BU5 ORF1b polyprotein (Fragm
5	122.94	AC023628|AC023628_3 gene: "F6F3
5	116.06	AC023673|AC023673_21 product: "
5	199.29	Q9ZT67 Resistance protein candi
5	120.77	U68408|ZMU68408_2 gene: "pol";
5	172.17	Q9C6U0 Clathrin heavy chain, pu
5	150.63	Q8Z320 DNA-directed RNA polymer
5	165.14	Q93EP6 Toxin complex protein.//
5	175.72	O85156 Insecticidal toxin compl
5	189.91	T41628 probable transcription r
5	198.6	S69949 TyB protein
5	150.56	STRPOB_1 S. typhimurium rpoB ge
5	126.15	AX359707_1 unnamed ORF; Sequen
5	150.54	MMRONRTK_1 gene: "STK"; product
5	129.6	MMCOL1A2_1 gene: "COL1A2"; prod
5	190.19	SCD9954_20 gene: "GCN2"; produc

Peptide mass mapping

Feruloyl esterase was digested using trypsin, the resulting peptides were analysed with Maldi-TOF mass spectrometry. The undigested enzyme The mass spectrum is shown in Figure 5.13. The following masses were used in the internet-based PeptideSearch similarity search: 2194.172, 2196.807,

2276.1, 3209.646, 4492.432, 4856.34, 5201.851, 5643.841, 5734.592. The best 30 search results are shown in Table 5.3. No significant similarities to other feruloyl esterases or carboxyl esterases were found. A mass-spectrum for the undigested enzyme could not be obtained.

Discussion

The production, purification and characterization of a novel, high M_r feruloyl esterase from *A. pullulans* is presented. The extracellular enzyme, produced by cultivation on birchwood xylan was purified by ultrafiltration, followed by three steps of column chromatography, where the enzyme was always detected in the unbound fractions. However, the total yield of pure protein was equal or even higher when compared to strategies, where the protein was eluted from the column (Faulds et al., 1991; Kroon et al., 1996).

With an apparent M_r of 210 kDa, feruloyl esterase from *A. pullulans* is the largest, single subunit enzyme of its kind. Taking into account 48 % of Asp – linked glycosylation, the size of the deglycosylated enzyme is in the range of *Aspergillus niger* (Kroon et al., 1996) and *Aspergillus awamori* (McCrae et al., 1994) feruloyl esterases. The reason for the high degree of glycosylation is unclear, although *A. pullulans* produces significant amounts of the exopolysaccharide (pullulan) and other hemicellulolytic enzymes (xylanase, xylosidase, α-glucuronidase) from this organism have also been found to be heavily glycosylated when estimated by SDS-PAGE (BJM de Wet, J Myburgh, unpublished data). Using activity staining, the enzyme was functionally identified by SDS-PAGE. Despite the denaturing conditions, the remaining activity was sufficient to detect the enzyme in the gel, however, this has been reported for esterases before (Gudelj et al., 1998).

Physical and biochemical properties of fungal feruloyl esterases are compared in Table 5.4. The pI of 6.5 and the optimum pH of 6.7 indicate that these properties of *A. pullulans* feruloyl esterase are slightly above values

reported for other fungal feruloyl esterases, which typically show pI values between 3 and 4.5 and pH values between 5 and 6 (Williamson et al., 1998 b). The temperature optimum of feruloyl esterase of 60 °C is similar to the value of 65 °C for the β-glucosidase (Saha et al., 1994) and 70 °C previously reported for α-L-arabinofuranosidase (Saha and Bothast, 1998) of *A. pullulans*.

Various cinnamic acid methyl esters have been used to evaluate the specificity of feruloyl esterase for the phenolic moiety of its substrate. *A. pullulans* feruloyl esterase shows highest specific activity for methyl *p*-coumarate, however, the difference in selectivity for the four substrates is rather low compared to enzymes from other fungi (Figure 5.14). Methyl sinapate, for example, is a very poor substrate for *A. niger* CinnAE , *P. expansum* and *P. funiculosum* feruloyl esterases, whereas *A. niger* FAE III shows no activity on methyl caffeate. However, the order methyl *p*-coumarate > methyl caffeate > methyl ferulate > methyl sinapate observed for *A. pullulans* feruloyl esterase, is analogous to the order reported for *A. niger* CinnAE (Kroon et al., 1996), *P. funiculosum* (Kroon et al., 2000) and *P. pinophilum* (Castanares et al., 1992) feruloyl esterases.

Table 5.4. Comparison of fungal feruloyl esterases.

Fungus	Enzyme	M_r (kDa)	pH optimum	pI	MFA[a]	Ara_5F[b]	Ara_2F[c]	Reference
					Km (mM)			
Aureobasidium pullulans	FeE	210	6.7	6.5	0.05	0.268	0.23	this paper
Aspergillus awamori	FAE	112	5	3.7	0.93	NA[d]	NA	McCrae et al., 1994
Aspergillus niger	FAE-III	29.7	5	3.3	0.7	0.006	-	Faulds and Williamson, 1994
Aspergillus niger	CinnAE	145	6	4.8	1.3	0.41	0.29	Kroon et al., 1996
Neocallimastix MC-2	FAE-I	69	6.2	4.2 - 5.8	NA	0.032	NA	Borneman et al., 1992
Neocallimastix MC-2	FAE-II	24	7	4.2 - 5.8	NA	0.01	NA	Borneman et al., 1992
Penicillium pinophilum	*p*-CAE/FAE	57	6	4.6	0.14	NA	NA	Castanares et al., 1992
Aspergillus oryzae	FAE	29	NA	NA	NA	NA	-[e]	Tenkanen et al., 1991
Penicillium expansum	FAE	57	5.6	NA	2.6	2.9	NA	Donaghy and McKay, 1997
Penicillium funiculosum	FAEB	53	NA	6	0.047	0.024	0.14	Kroon et al., 2000

[a]methyl ferulate

[b]5-feruloulated arabinosides

[c]2-feruloulated arabinosides

[d]NA, data not available

[e]FAE from *A. oryzae* is active on 5-feruloulated arabinosides but not 2-feruloulated arabinosides (see Figure 5.11)

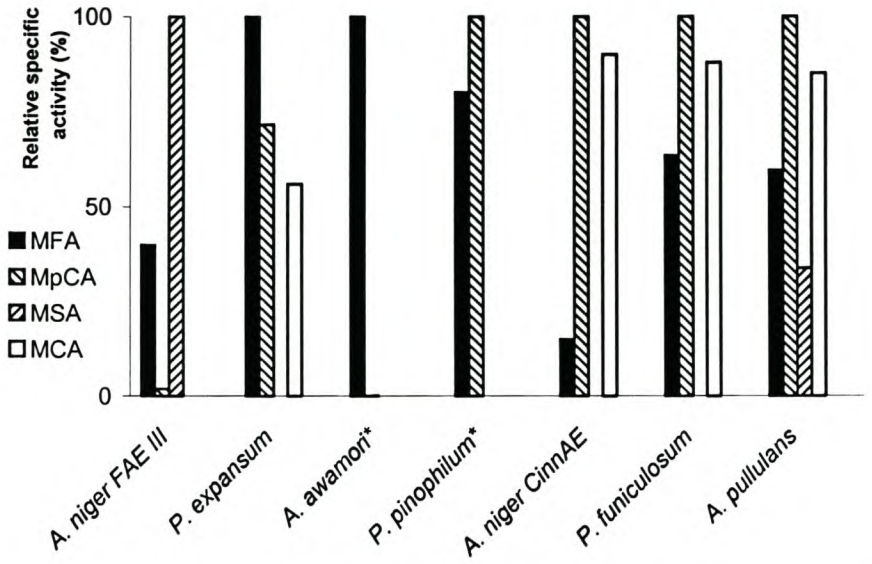

Figure 5.14. Comparison of fungal feruloyl esterases. MFA = methyl ferulate; MpCA = methyl *p*-coumarate; MSA = methyl sinapate; MCA = methyl caffeate. *MSA and MCA activity have not been determined.

The specificity of feruloyl esterases for the sugar moiety of the substrate was investigated using synthetic substrates that have a feruloyl group attached to either the C-2 or C-5 position of arabinofuranose, similar to natural occurring compounds. So far, feruloyl esterases can be divided into two classes based on their activity for either only C-5 or C-5 and C-2 ferulated substrates. Amongst fungal enzymes, that have been examined, *A. pullulans* feruloyl esterase belongs to the latter, together with *A. niger* CinnAE and *P. funiculosum* FAEB, whereas *A. niger* FAE III and *A. oryzae* feruloyl esterase are members of the first class (Table 5.4). Feruloyl esterases that catalyse exclusively C-2 ferulated arabinofuranosides have not been described to date.

The nature of feruloyl esterase specificity, both for the phenolic and the sugar moiety, remain to be elucidated. Still too few enzymes have been isolated and characterization data is mostly not uniform. However, *A. pullulans* feruloyl esterase could be classified according to available data and shows properties similar to other fungal feruloyl esterases, especially *A. niger* CinnAE (Kroon et al., 1996) and *P. funiculosum* FAEB (Kroon et al., 2000). The N-terminal sequence of *A. pullulans* feruloyl esterase was reliably identified as the protein was sequenced twice. However, the sequencing reaction was prematurely terminated possibly due the presence of a proline that is cleaved less efficiently (R. van Wyk, personal communication). A BLAST search (www.ncbi.nlm.nih.gov/BLAST) failed to reveal significant similarity to known feruloyl esterases or to carbohydrate esterases in general. However a search based on eight N-terminal amino-acids is generally too few to establish similarity and furthermore the N-terminal regions of feruloyl esterases are moreover poorly conserved (Williamson et al., 1998 b).

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CHAPTER 6

Monitoring on-line desalted lignocellulosic hydrolysates by microdialysis sampling micro-high performance anion exchange chromatography with integrated pulsed electrochemical detection/mass spectrometry

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Abstract

An on-line system based on microdialysis sampling (MD), micro-high performance anion exchange chromatography (micro-HPAEC), integrated pulsed electrochemical detection (IPED), and electrospray ionisation mass spectrometry (MS) for the monitoring of on-line desalted enzymatic hydrolysates is presented. Continuous monitoring of the enzymatic degradation of dissolving pulp from *E. grandis* as well as degradation of sugar cane bagasse in a 5 ml reaction vessel was achieved up to 24 h without any additional sample handling steps. Combining MD with micro-HPAEC-IPED/MS and on-line desalting of hydrolysates enabled injection (5 µl) of at least 23 samples in a study of the sequential action of hydrolytic enzymes in an unmodified environment where the enzymes and substrate were not depleted due to the perm-selectivity of the MD membrane (30 kDa cut-off). Xylanase, feruloyl esterase and a combination of endoglucanase (EG II) with cellobiohydrolase (CBH I) resulted in the production of DP 1 after the addition of esterase, DP 2 and DP 3 after the addition of EG II and CBH I, from the dissolving pulp substrate. Similar sequential enzyme addition to sugar cane bagasse resulted in DP 1 production after the addition of esterase and DP 1, DP 2 and DP 3 production after the addition of the EG II and CBH I mixture. Combining MS on-line with micro-HPAEC-IPED proved to be a versatile and necessary tool for such a study compared to conventional methods. The mass selectivity of MS revealed complementary information, including the co-elution of saccharides as well as the presence of more than one type of DP 2 in the case of dissolving pulp and several types of DP 2 and DP 3 for sugar cane

bagasse. This study demonstrates the limitation of the use of retention time alone for confirmation of the identity of saccharides especially when dealing with complex enzymatic hydrolysates. *In-situ* sampling and sample clean-up combined with on-line desalting of the chromatographic effluent, provides a generic approach to achieve real time monitoring of enzymatic hydrolysates when they are detected by a combination of IPED and MS.

Introduction

Lignocellulosic material is one of the most abundant sources of carbohydrates and thus has high industrial importance. Whenever there is shortage of resources, alternative raw materials as well as methods to access abundant natural sources are sought after extensively. Since the extraction of cellulose by means of pulping is as old as paper, recently there have been efforts to find new methodologies to make the process less energy consuming and more environmentally friendly (Christov and Prior, 1998; Breen and Singleton, 1999). This has facilitated an intensive search for new enzymes to be employed in this new area that has become an industry of its own. Two examples of lignocellulosic materials that have attracted interest are dissolving pulp and sugar cane bagasse.

Sugar cane bagasse is a waste product from the sugar cane industry that has been considered as a carbon source for biofuel fermentation (Gutierrez-Correa et al., 1999) or if enzymatically modified, as animal feed (Pandey et al., 2000). On the other hand, dissolving pulp is industrially manufactured from wood and is the raw material for numerous applications that include man-made fibres (Hinck et al., 1985). The enzymatic removal of hemicelluloses,

delignification and structure investigations of lignocellulosic substrates have been carried out by means of selective enzymatic treatment (Gübitz et al., 1998; Tenkanen et al., 1999). These studies have enabled the evaluation of the cooperative effect of enzyme combinations on complex, native as well as isolated material (de Jong et al., 1997).

Many studies have been carried out in order to understand the composition of complex substrates and/or the effect of polysaccharolytic enzymes. Such studies are usually carried out by analysing the soluble sugars as well as solubilised residual material using conventional assay methods or high performance anion exchange chromatography with integrated pulsed electrochemical detection (HPAEC-IPED) (Hanko and Rohrer, 2000).

The limitation of an off-line approach has been that 1.5 ml sample aliquots (or even higher volumes) have to be continuously taken from the reactor and the hydrolytic reaction is stopped by either boiling or freezing (Buchert et al., 1993; Ostergaard et al., 2001). The sample volume impacts on the size of the reactor, the number of samples that can be taken, the costs of highly purified enzymes as well as substrates, the hydrolytic environment for the enzymes, and the integrity of the information obtained from the reaction under study. Further, the results of the cooperative effect of the hydrolytic enzymes can only be seen at the end point because the samples have to be thawed, filtered or centrifuged prior toinjection into the HPAEC-IPED system. It is therefore essential to employ microassay methods with an on-line capability as this can limit sample volumes to a few microlitres in order to maintain the composition and environment of the reaction mixture.

For the total analysis of products during enzymatic decomposition of lignin-polysaccharide substrates it is necessary to achieve *in-situ* sampling and sample clean-up, on-line sample work-up, on-line injection, high resolution separation, selective and sensitive on-line detection as well as unequivocal characterisation of hydrolysates. Microdialysis presents most of the desired qualities for a sampling technique as it has recently been demonstrated to be well suited for sampling from enzymatic bioprocesses (Torto et al., 1998, Nilsson et al., 2001). Because of its perm-selective membrane units, it is able to selectively sample for products of hydrolysis without significantly perturbing the reaction, depleting the bioreactor or taking up the substrate and enzyme. Selectivity of the membranes is achieved by using membranes that exhibit different molecular weight cut-offs.

A combination of microdialysis, mass spectrometry (MS) with HPAEC-IPED is a versatile tool for on-line monitoring of the enzymatic hydrolysis of lignocellulosic polymers (Torto et al., 1997). Unlike the conventional assay methods where the identity of hydrolysates is confirmed only based on retention times, this combination of techniques enables their unequivocal identification. The use of MS for analysis of labile biological molecules, especially carbohydrates has increased with the advent of atmospheric pressure ionisation techniques (Niessen, 1999) as well as efficient mechanisms to desalt chromatographic effluents before introduction into MS (Torto et al., 1998).

Using microdialysis sampling, micro-HPAEC-IPED/MS, the decomposition of dissolving pulp and sugar cane bagasse by means of sequential treatment with xylanase, feruloyl esterase and both endoglucanase and

cellobiohydrolase was monitored. The use of microdialysis sampling allowed the monitoring to occur without intervention for 23 h. The impact of the combination of enzymes could be followed in real-time and the on-line desalted hydrolysates were confirmed by electrospray MS in the single ion monitoring mode, without contamination of the ion source.

Experimental

Reagents

Glucose, cellobiose and cellotriose were obtained from Sigma. Xylose, xylobiose and xylotriose were obtained from Megazyme (Wicklow, Ireland). Propan-2-ol, sodium hydroxide (50% w/w) and sodium acetate were obtained from J. T. Baker. All solutions were prepared using ultrapure water from a Milli-Q purification system (Millipore). For sequential decomposition of lignocellulosic material we used the following types of enzymes: Endoglucanase II and cellobiohydrolase I from *Trichoderma reesei* RUT C 30 purified as described by Pere (1995), xylanase from *A. pullulans*, purchased from Sigma, and a feruloyl esterase isolated from *A. pullulans*. The esterase has been purified to homogeneity, has a molecular weight of 210 kDa and a pI of 6.5 and showed activity on both esterified ferulic acid (Chapter 5) and *p*-coumaric acid (data not shown).

Substrates

Unbleached *E. grandis* dissolving pulp from Sappi Saiccor (Umkomaas, South Africa) contained 97% carbohydrate, determined by sulfuric acid hydrolysis

(Puls et al., 1985) and 3% lignin determined according to the Klason method (TAPPI method T249 cm-85). Sugar cane bagasse from Tongaat-Huletts (La Lucia, South Africa) contained 70% carbohydrate and 30% lignin using the above analysis methods. Prior to enzymatic treatment, sugar cane bagasse was milled in a hammer mill (0.5 mm mesh) and both dissolving pulp and sugar cane bagasse were washed twice to remove soluble sugars and autoclaved.

Hydrolysis conditions

Enzymatic hydrolysis of sugar cane bagasse and dissolving pulp was carried out at 40°C, in 10 ml reaction vessels with gas permeable seal, housed in a Pierce React-Therm (heating/stirring module no 18971: Pierce, Rockford, IL). 20 mg of either dissolving pulp or sugar cane bagasse were hydrolysed in 5 ml of a 50 mM sodium acetate buffer adjusted with sodium hydroxide to a pH of 5. The enzymes were added sequentially starting with 167 nkat xylanase followed by 167 nkat feruloyl esterase after 8 h. After 16 h hydrolysis, 500 nkat endoglucanase II and 500 nkat cellobiohydrolase I were added at the same time. The control sample was treated similarly except that no enzymes were added.

Microdialysis sampling

In-situ sampling and sample clean-up of the hydrolysates from the reaction vessels were achieved using a microdialysis probe equipped with a polysulfone membrane, A/G Technology (Needham, MA, USA) having a 30 kDa molecular weight cut-off and a 5 mm effective dialysis length. The probe was perfused with pure water delivered by a CMA/100 syringe pump,

CMA/Microdialysis (Stockholm, Sweden) at 2 µl/min. The sample was either collected using a CMA/142 fraction collector or injected directly to the chromatographic system via a CMA/160 on-line injector, all from CMA/Microdialysis.

Separation and detection of hydrolysates

After sampling, 5 µl of hydrolysates were injected into the metal-free Dionex 500, Chromatographic system (Dionex). The saccharide hydrolysates were separated on microbore (250 x 2 mm) CarboPac PA 1 pre- and analytical columns (from Dionex), using a sodium hydroxide/sodium acetate gradient at a flow rate of 0.25 ml/min. The gradient composition consisted of 10 %, 150 mM sodium hydroxide and 90 % water for 10 min. The composition was then changed between 10 and 20 min with 50 % 250 mM sodium acetate/150 mM sodium hydroxide, 10 % 150 mM sodium hydroxide and the rest was pure water. After 20 min, the gradient was gradually changed to reach 10 % 150 mM sodium hydroxide and 90 % water at the end of the run (30 min).

The separated saccharides were either detected directly on-line using the IPED on the ED 40 of the Dionex 500 or they were desalted using the cation exchange membrane desalting device (CMD™, also from Dionex) and introduced into the MS system. The CMD was connected to the waste outlet of the electrochemical detector cell as described previously (Torto et al., 1998), and a helium pressure of 25 kPa was applied to a pressurised container to maintain a water flow of 4 ml/min. Desalting of the analyte was carried out by the electrolysis of ultrapure water at 500 mA by means of the Dionex SRS-1 controller. The H_3O^+ ions produced at the anode were exchanged for Na^+ ions via two cation exchange membranes. The H_3O^+ ions

combined with the OH^- and the OAC^- ions from the analyte to form water and acetic acid, respectively. The Na^+ ions combined with OH^- ions from the cathode to form sodium hydroxide, which was collected as waste.

The desalted effluent was directed to the electrospray interface. Mass chromatograms were acquired using a Finningan LCQ Deca Ion Trap MS, Finnigan. The sheath liquid was propan-2-ol: water (80:20) containing 10^{-4} M sodium acetate, at a flow rate of 50 µl/min, together with nitrogen as sheath gas at a flow rate of 6 l/min. The MS conditions were optimized with respect to capillary temperature (300°C), spray voltage (4.7 kV) and the spectra were acquired in the single ion monitoring (SIM) mode.

Results and discussion

The application of enzyme hydrolysis of dissolving pulp and sugar cane bagasse have potential in processes such as prebleaching of paper or production of carbohydrates. Therefore more detailed knowledge about their structure and properties is important. A systematic approach to evaluation of their structure could be achieved through monitoring of enzymatic degradation products. Enzymes such as xylanases, feruloyl esterases and cellulases can be useful for such studies. Xylanases and glucanases cleave the backbone of xylan and cellulose, respectively. Feruloyl esterases cleave ester linked phenolic substituents like ferulic acid or *p*-coumaric acid from polysaccharide chains. Feruloyl esterases also cleave diester-bridges that are known to form interlinkages between individual polysaccharide chains as well as between polysacchaide chains and lignin forming lignin carbohydrate complexes (LCCs). Results of the on-line monitoring of hydrolysis products after the

sequential addition of hydrolytic enzymes to dissolving pulp as well as sugar cane bagasse are presented.

On-line hydrolysis of dissolving pulp

Figure 6.1 shows a profile of the production of saccharides during the enzymatic degradation of dissolving pulp over the monitored 24 h period using HPAEC-IPED. Throughout the first 8 h, the xylanase did not seem to have an impact on the substrate as no hydrolysis products were detected by the sensitive IPED.

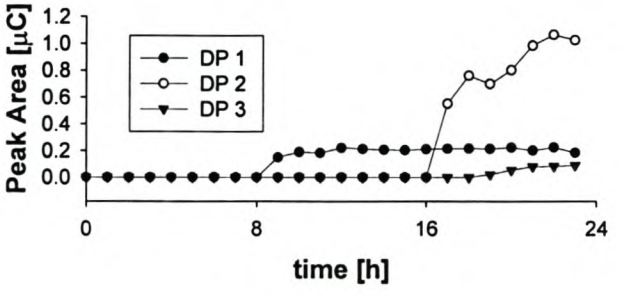

Figure 6.1. Hydrolysis profile during the enzymatic hydrolysis of dissolving pulp. Enzymes were added sequentially starting with xylanase after 0 h, feruloyl esterase after 8 h and endoglucanase and cellobiohydrolase after 16 h, respectively.

After the addition of the esterase the release of DP 1 could be observed. Simultaneous addition of endoglucanase and cellobiohydrolase resulted in the production of DP 2 and DP 3. The profile of the DP 1 shows no significant change after the addition of endoglucanase and cellobiohydrolase. There

seem to be no cooperative effect between the enzymes that leads to an increased production in DP 1.

The information presented in Figure 6.1 shows a typical enzymatic hydrolysis pattern irrespective of whether the analysis had been carried out to give real time data or just the end point result. In contrast, the true real time monitoring chromatographic profile is shown in Figure 6.2(i). However, for clarity the hydrolysis profile and the cooperative effect of the enzymes can also be presented as in Figure 6.2(ii) where only the chromatograms at particular sampling times are examined closely. The chromatographic profile in Figure 6.2 shows that saccharides that can be differentiated by their size were produced during the enzymatic degradation. The true identity of such saccharides can only be confirmed if authentic standards are used to spike the sample. Spiking of the sample with the authentic standards is the most preferred method to confirm identity of components separated by chromatography and is routinely used in hydrolytic/fermentation studies (Hanko and Rohrer, 2000).

Combining HPAEC with MS confirmed the presence of DP 1, DP 2 and DP 3 in the sample taken after 23 h of enzymatic degradation of dissolving pulp (see Figure 6.3). The MS chromatograms for the sodiated saccharides further show that there are two types of DP 2 (m/z= 365), the first of which elutes at the same time as DP 1 (m/z=203). The MS chromatograms also show the presence of one type of DP 1 as well as one type of DP 3.

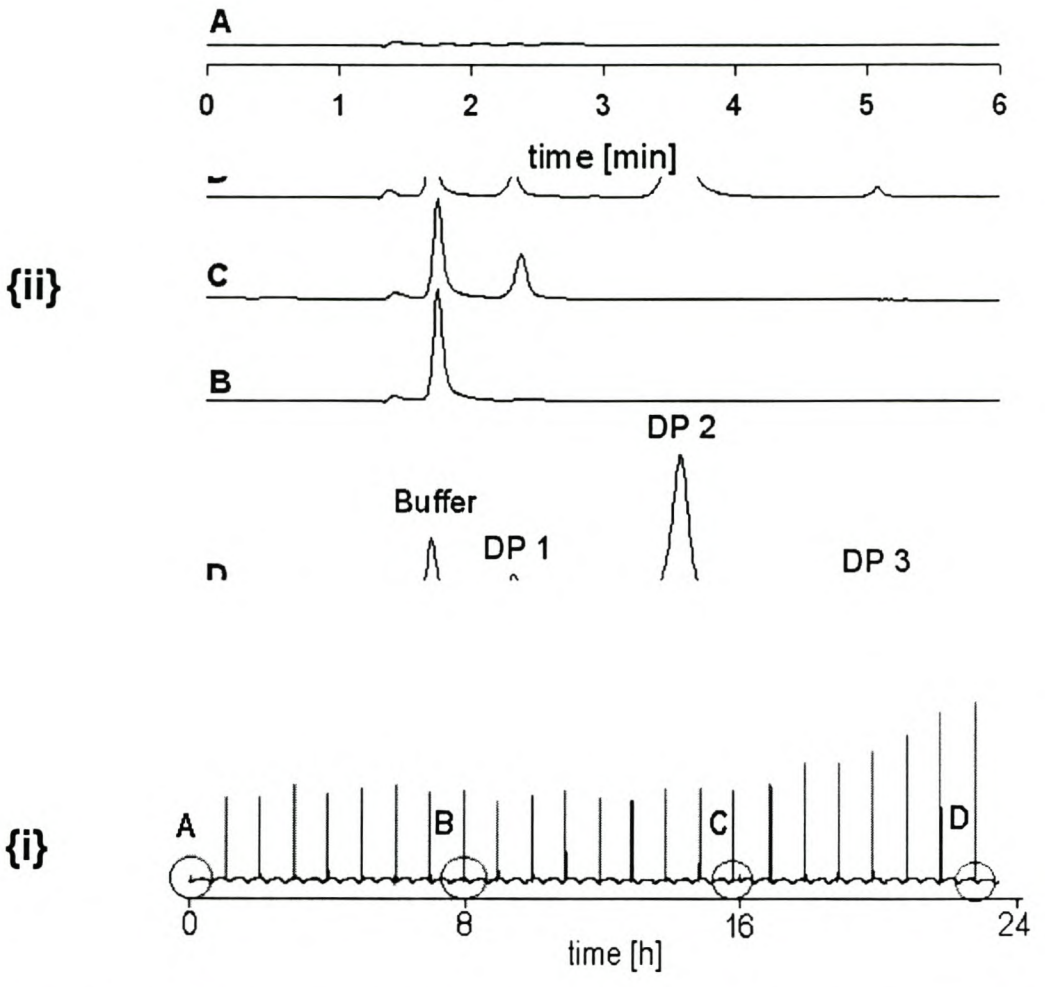

Figure 6.2. Chromatographic profile of the on-line sequential enzymatic hydrolysis of dissolving pulp acquired with HPAEC-IPED for 23 h {i}, where the selected parts {ii} represent **A** Substrate, no enzymes, 0 h, **B** after addition of xylanase, 8 h, **C** after addition of esterase, 16 h, **D** after addition of endoglucanase/cellobiohydrolase, 23 h.

Therefore, complementing the information derived from HPAEC-IPED and that from HPAEC-MS revealed much more detailed information about the hydrolysis products that would otherwise not been observed if only HPAEC-IPED was used for monitoring hydrolysis products. Use of authentic standards, as is often the practice, to unequivocally identify saccharides

would be very limited due to the complexity of the hydrolysate matrix because the retention and elution profile of the saccharides can not be easily predicted based only on their size. The subtle difference in pK_a values for saccharides (Rendleman, 1973) is easily affected by complex matrix conditions as has been observed previously (Torto et al., 2000). This is not surprising because the complexity of carbohydrates, especially the saccharides, is overwhelming. For example the multiplicity of a homologous series (aldoses, pentoses, ketoses, alditols, aldonic acids) and the subsequent epimers due to configuration differences (glucose, mannose and galactose), sequential variation and/or various positions of attachment of the glycosidic linkages in di- and oligosaccharides (1,1; 1,3; 1,4 or 1,6 linked glucose, mannose, xylose or galactose) (Honda, 1996). Use of MS especially the collusion induced fragmentation (MS/MS) of monosaccharides, disaccharides or oligosaccharides has been shown to be a versatile tool that can be used to confirm the identity of carbohydrates. However in the absence of authentic standards, MS chromatograms do not just show the presence of saccharides, but also clearly group them according to their mass and types of glycosidic linkage which can be confirmed after carrying out MS/MS analysis.

The identities of DP 1 and DP 3 were confirmed as glucose and cellotriose, respectively. The other DP 2 was confirmed as cellobiose (Figure 6.3B {ii}), however the coeluting DP 2's identity was not confirmed. Interestingly, the analysis did not reveal any xylan breakdown products probably due to the relative little portion (2%) of xylan present in dissolving pulp as determined by acid hydrolysis. Further the xylan is very likely to be trapped in recalcitrant lignin-hemicellulose complexes (Gübitz et al., 1998).

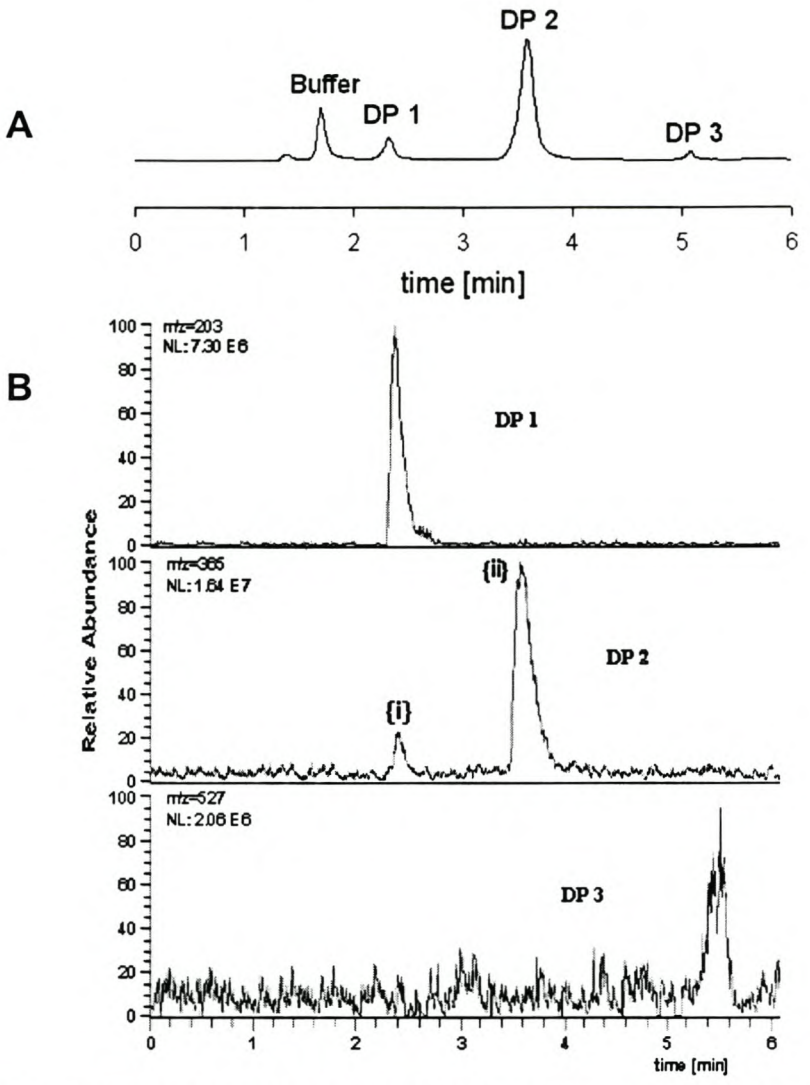

Figure 6.3. Chromatograms aquired using HPAEC-IPED (**A**) and HPAEC-MS (**B**) showing complementary information about the presence of two types of DP 2s (indicated as {i}, {ii} on the chromatogram) after the hydrolysis of dissolving pulp. The DP 2 marked {i} coelutes with DP 1.

On-line hydrolysis of sugar cane bagasse

The chromatographic profile of the hydrolysis of sugar cane bagasse is shown in Figure 6.4 for samples analysed at defined periods and the change in hydrolysis products is shown in Figure 6.5. From the chromatographic profile, addition of xylanase resulted in the appearance of a peak representing DP 1,

during the first 8 h of hydrolysis. After the addition of esterase, the production of DP 1 increased and there was also a production of another peak that was further identified as a pentoses. When the glucan degrading enzymes were added two additional peaks were observed, identified as DP 2 and DP 3 using standards. The production of DP 1s increased with the addition of the glucan degrading enzymes.

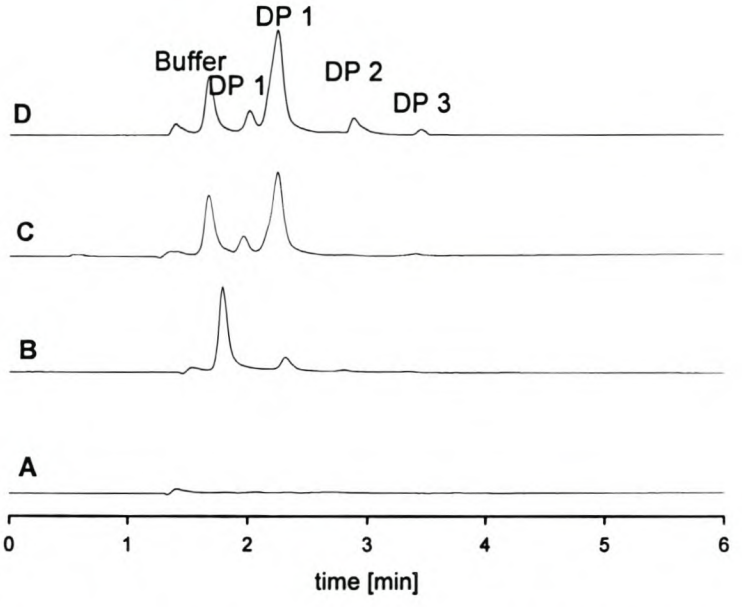

Figure 6.4. Chromatographic profile of sugar cane bagasse before the addition of enzyme, 0 h (**A**), after the addition of xylanase, 8h (**B**), after the addition of esterase, 16 h (**C**) and finally after the addition of endoglucanase and cellobiohydrolase, 23 h (**D**).

In Figure 6.6 however, when comparing the HPAEC-IPED (**A**) and HPAEC-MS (**B**) results one finds additional information. Monitoring of the hydrolysates in SIM showed the presence of three types of sodiated monosaccharides. They were two pentoses (m/z=173) and a hexoses (m/z=203). As shown in

the mass chromatogram (Figure 6.6 **B**) one of the pentoses eluted at the same time as the hexoses. The identity of the pentoses was not confirmed due to lack of authentic standards, but it is most likely that they would be arabinose and xylose. The mass chromatogram also showed the presence of four types of sodiated disaccharides (m/z=365) as well as two types of sodiated trisaccharides (m/z=527).

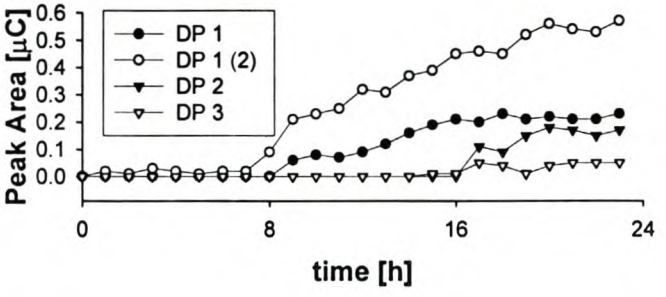

Figure 6.5. Hydrolysis profile during the enzymatic hydrolysis of sugar cane bagasse. Enzymes were added sequentially starting with xylanase after 0 h, feruloyl esterase after 8 h and endoglucanase and cellobiohydrolase after 16 h, respectively.

These results further demonstrated the versatility of combining chromatography, electrochemical detection and mass spectrometry. Clearly the chromatogram obtained using HPAEC-IPED does not give sufficient information relating to the type of saccharides as well as the variety that is eluted when monitoring enzymatic hydrolysates. These observations emphasize the essential role of MS as a complementary detector. The results also pose a question regarding the characterisation of enzyme hydrolytic

properties if the data does not elucidate the type of glycosidic linkage of the hydrolysis products. Such a difference in the type of saccharide, for those that co-elute, if not elucidated could affect the observed enzyme characteristics and the assumed activity.

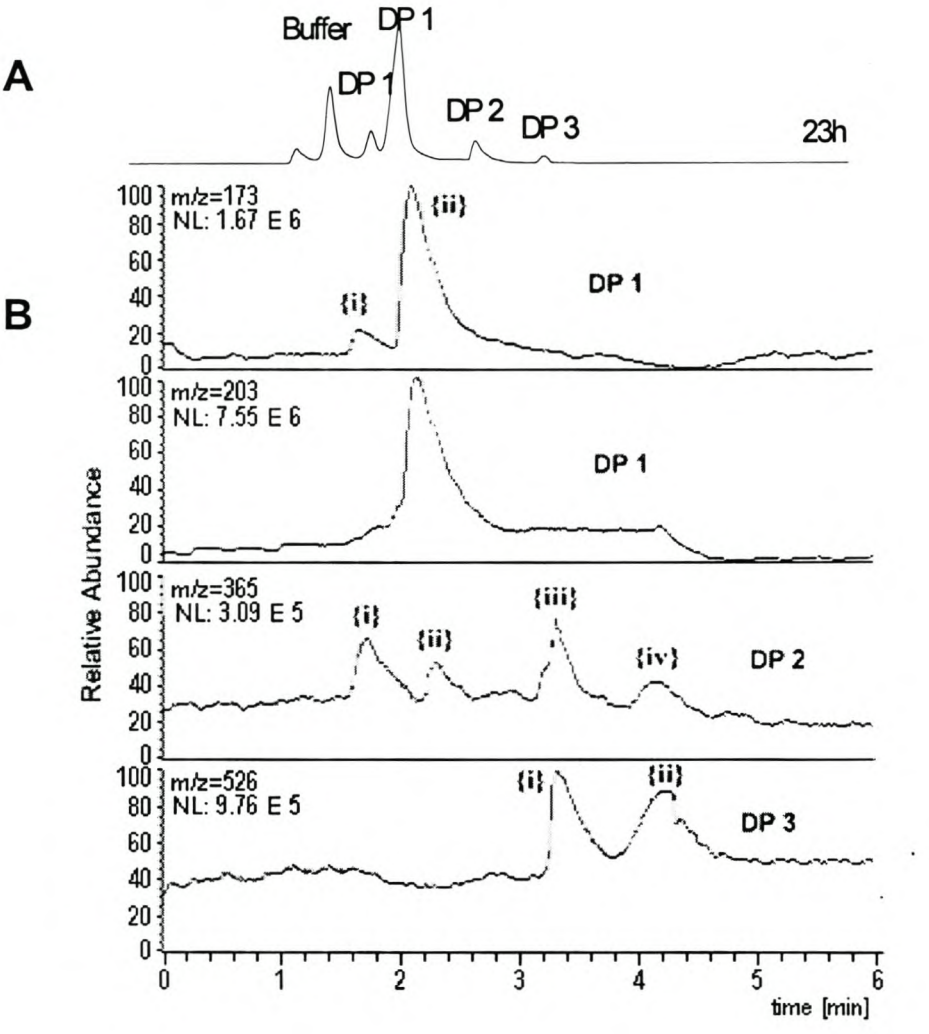

Figure 6.6. Chromatograms acquired using HPAEC-IPED (**A**) and HPAEC-MS (**B**) showing complementary information about the presence of three types of DP 1s (two pentoses, m/z=173, marked as (i) and (ii) and one hexoses, m/z=203). The mass chromatogram further shows four types of DP 2, m/z=365, (numbered (i), (ii), (iii), and (iv)) and two types of DP 3, m/z=526 (numbered as {i} and {ii}).

Conclusion

Microdialysis sampling, micro HPAEC-IPED/electrospray ionisation MS was used to monitor on-line desalted enzymatic hydrolysates of lignocellulosic substrates. This combination of techniques showed a generic approach to achieving real time monitoring as well as qualitative analysis of hydrolysis products. The mass selectivity of MS lead to the unequivocal characterisation of the hydrolysis products as it complemented HPAEC-IPED. Even if the chromatographic conditions seemed optimal, without obvious multiple component peaks, the SIM procedure based on aldopentoses and aldohexoses demonstrated the inadequacy of using retention time only for confirming the identity of enzymatic hydrolysates.

The results from this study further show that the action of endoenzymes (xylanases) does not necessarily result in larger breakdown products as this is dependent upon the structure of the substrate. Although sugar cane bagasse has a relative high portion of xylan the hydrolysis thereof was hindered by the architecture of the fibre of the material.

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CHAPTER 7

General discussion

The production, purification and functional characterisation of feruloyl esterase from *A. pullulans* were set as the primary objectives of this study. A further objective was to investigate a possible co-operative effect with other selected lignocellulolytic enzymes on substrates relevant to industry.

The production of feruloyl esterase by *A. pullulans* reached a maximum when the fungus was grown on birchwood xylan (Chapter 4). This observation is consistent to previous studies, where feruloyl esterase production was achieved under similar growth conditions (Christov and Prior, 1993). In contrast to reports from other fungi (de Vries and Visser, 1999), feruloyl esterase activity could be detected when *A. pullulans* was grown on glucose as only carbon source. This gave rise to the assumption, that the expression of feruloyl esterase is regulated independently from the latter xylanolytic enzymes. Free ferulic acid added to the medium did not increase feruloyl esterase production, whereas for *A. niger*, the contrary was observed (Faulds et al., 1997).

With a molecular weight of 210 kDa, the feruloyl esterase from *A. pullulans* is the largest single subunit protein in its class (Chapter 5). However, glycosylation accounts for 48 % of its weight determined by SDS-polyacrylamide gel electrophoresis. The optimal temperature and pH is in the range of the ones reported for other enzymes characterised from *A. pullulans* (Saha et al., 1994, Saha and Bothast, 1998).

A. pullulans feruloyl esterase has broad substrate specificity determined on various aliphatic esterase substrates of increasing chain length (Chapter 4) and on different phenolic acids substrates (Chapter 5). Analogous to feruloyl esterase from other organisms (Williamson et al., 1998), the activity of *A.*

pullulans feruloyl esterase is limited to acetyl esters. However, acetyl esters with an additional phenolic moiety as can be found in naphthol-AS-D acetate and naphthol AS-D chloroacetate are also accepted. For phenolic acid methyl esters, *A. pullulans* feruloyl esterase shows the activity pattern methyl *p*-coumarate > methyl caffeate > methyl ferulate> methyl sinapate, which can also be observed for *P. pinophilum* (Castarnes et al., 1992), *P. funiculosum* (Kroon et al., 2000) and *A. niger* CinnAE (Kroon and Williamson, 1998). Other feruloyl esterases are more selective for either methyl sinapate like *A. niger* FAEIII (Faulds and Williamson, 1994) or methyl ferulate like *A. awamori* (McCrae et al., 1994) and *P. expansum* (Donaghy and McKay, 1997).

Structural data on feruloyl esterase from *A. pullulans* could not be acquired. Neither N-terminal sequencing nor peptide mass mapping revealed enough information in order to classify the enzyme according to its primary structure (Chapter 5). In an effort to correlate data on activity with structural information of feruloyl esterases, amino acid sequences of all feruloyl esterases available in databases were compared in a multiple sequence alignment (Chapter 2). Some *Aspergillus* sp. feruloyl esterases show high homology and are closely related to the lipase 3 family motif. However, they do not possess lipase activity (Aliwan et al., 1999). *Penicillium* (Donaghy and McKay, 1997) and *Neurospora* (Crepin et al., 2003) feruloyl esterases show homologies to acetyl xylan esterases, *Piromyces* (Fillingham et al., 1999) feruloyl esterases show homologies to a conserved domain that is common in glycosyl hydrolases. Several other similarities were found to xylanases and tributyrin esterases for *Orpinomyces* feruloyl esterase (Blum et al., 2000) and tannases for *A. niger* CinnAE (Kroon and Williamson, 1998). However, additional glycosyl

hydrolase, xylanase and tannase activity, respectively, has not been reported for those enzymes. Bringing kinetic and structural information together does therefore not seem to be a feasible approach to establish relations between these enzymes (Chapter 2). Nor would be the comparison of molecular weight and other physicochemical properties. A forecast for the structure of *A. pullulans* feruloyl esterase has not been attempted.

In an effort to evaluate the significance of feruloyl esterase from *A. pullulans* in the degradation of lignocellulose, dissolving pulp and sugar cane bagasse was selectively treated using feruloyl esterase and hemicellulolytic enzymes (Chapter 6). In addition, selective treatment of lignocellulosic material with purified and characterised enzymes gives information on the nature of the substrate (Tenkanen et al., 1999). The hydrolysis reaction was monitored using microdialysis sampling, anion exchange chromatography, online desalting and mass spectrometry. This combination of techniques showed a generic approach to achieving real time monitoring as well as qualitative analysis of hydrolysis products (Torto et al., 1998). The mass selectivity of mass spectrometry lead to the unequivocal characterisation of the hydrolysis products as it complemented anion exchange chromatography with pulsed amperometric detection. It has been shown, that feruloyl esterase activity together with xylanase activity releases monosaccharides from both substrates. Sugars of higher degree of polymerisation were not released, giving evidence for the recalcitrance of the material. The architecture of the fibre of the used substrates seemed to be not accessible for enzymes therefore complete hydrolysis was hindered. Similar observations have been made before, especially with dissolving pulp (Gübitz et al., 1998). However,

there is no doubt that feruloyl esterase in combination with cellulases and hemicellulases contributes to the decomposition of plant cell wall material (de Vries et al., 2000),

During the past ten years, feruloyl esterases have been described in various plant-invading fungi and bacteria (Chapter 2). Research interest was primarily driven by the potential applicability in various industrial sectors involving the release of ferulic acid from plant material. Ferulic acid is an important precursor in the flavour industry (Bonnin et al., 2001), and has multiple uses as an ultraviolet light protection agent in sun creams and cosmetics (Saija et al., 1999). It suppresses inflammatory responses and skin tumor promotion (Murakami et al., 2002). Many food processes involve ferulic acid, although the associated contributions are far from clear. For example, baking is thought to involve an important but yet unknown role for ferulic acid in the formation of cross-links in the dough (Schooneveld-Bergmans et al., 1999). Very recently, ferulic acid was discovered to play a role as an antioxidant and is released and taken up in the intestines with the help of feruloyl esterases distributed along the intestines of mammals (Andreasen et al., 2001). There is no doubt that applications of feruloyl esterases, together with a better understanding of the mechanism of action, will further grow in future.

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