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Novel biotransformations of agro-industrial cereal waste by ferulic acid esterases

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Abstract

With the yearly accumulation of agro-industrial waste-material generated by the milling, brewing and sugar industries in Europe, the importance of extracting high value residues must be considered to offset the cost of treating and disposing of the residues. This work reviews the identification, purification and characterisation of novel microbial esterases capable of releasing the bioactive phenolic compound, ferulic acid, from cereal cell-walls and agro-industrial waste. These phenolic residues restrict the extent of hydrolysis of cell-wall carbohydrates. Potential applications for the esterases in the food and pharmaceutical industries are described. Enzymic removal of ferulic acid is very mild, allowing further treatment/processing of the residue and removes the need for environmentally-unfriendly chemical clean-up processes. We now report the hydrolysis, on a laboratory scale, of wheat bran (1 kg) by a *Trichoderma* xylanase preparation and an *Aspergillus niger* ferulic acid esterase (FAE-III) to produce free ferulic acid (5.7 g). © 1997 Elsevier Science B.V.

Keywords: Ferulic acid; Cereal waste; Wheat bran; Esterase; Biotransformation; *Aspergillus*

1. Introduction

Agro-industrial plant cell-wall waste material generated by the European Community member countries each year amounts to millions of tonnes. At present, most of the material is used as animal feed, but academic and industrial researchers are putting more and more effort into obtention of high-value compounds from this waste, which can be converted into novel consumer goods by the industries involved. A high proportion of this waste material is carbohydrate and phenolic in nature. Carbohydrases can release specific sugar residues from the cell-wall, but the presence of phenolic moieties, ester-linked to polysaccharides and forming structural cross-linking bridges between various polymers (Scalbert et al., 1985;

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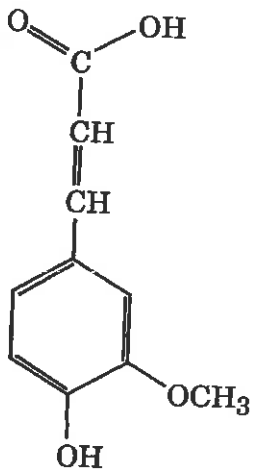
Watanabe et al., 1989; Iiyama et al., 1994) restrict complete hydrolysis of the polysaccharide.

Ferulic [3-(3-methoxy-4-hydroxy phenyl)-2-propanoic] acid (Fig. 1) is the major cinnamic acid found in a wide variety of plant cell-walls. Cereals contain ferulic acid at concentrations of: wheat bran (0.5–1% w/w dry matter; Ralet et al., 1990), maize bran (3%; Saulnier et al., 1995a), barley (0.14%; Nordkvist et al., 1984) and spent grain (0.32%; Bartolomé et al., 1996). Ferulic acid is synthesised via the shikimate pathway and is an intermediate in lignin biosynthesis (Higuchi, 1990). The free acid is an antioxidant (Graf, 1992).

Commercial utilisation of natural ferulic acid has been limited by its availability and cost. It can be used as a preservative due to its ability to inhibit peroxidation of fatty acids, and constitutes the active ingredient in many skin lotions and sunscreens. Some Japanese textile makers use it for the manufacture of golf wear with ultraviolet absorption properties and it can confer excellent photoprotection to ultraviolet-sensitive biological materials. Additionally, ferulic acid has been claimed to lessen side-effects of chemo- and radiotherapy, and to exhibit strong anti-inflammatory properties (Graf, 1992).

Certain microorganisms possess the ability to transform ferulic acid to vanillic acid and vanillin, the main flavour component of vanilla (Falconnier et al., 1994). Vanillin can either be extracted from the vanilla pod or chemically synthesised. The use of microbial enzymes to extract ferulic acid avoids unnecessary clean-up of chemical wastes from industrial processes, and will allow the systematic extraction of specific products, depending upon process technologies and specific applications. As the ferulic acid extraction process and further microbial bioconversions involve no chemical treatments, the released compounds can be classified as being 'natural flavours by fermentation'.

Over the last 5 years, we have concentrated on the isolation and purification of a number of novel microbial esterases which can cleave ferulic acid from sugar residues in agro-industrial wastes. Esterases from *Streptomyces* (Faulds and Williamson, 1991; 1993a) and *Aspergillus* (Faulds and Williamson, 1993b; 1994) have been purified to homogeneity, and the gene encoding an acetylesterase with feruloyl-hydrolysing activity from *Pseudomonas* (Ferreira et al., 1993; Faulds et al., 1995b) has been cloned. The physical properties of these enzymes are shown in Table 1. Other phenolic acid esterases have been purified by other laboratories (Borneman et al., 1991; Tenkanen et al., 1991; Castanares et al., 1992). Initially, a wide range of methyl esters of naturally occurring benzoic and cinnamic acids were synthesised (Faulds and Williamson, 1994). These compounds permitted us to purify *S. olivochromogenes* ferulic acid esterase (FAE; Faulds and Williamson, 1991), and to determine the substrate specificity of the esterases isolated since. Each of the enzymes was shown to have different affinities for these cinnamic acids, and we have been able to map out their active sites according to degree and type of substitution on the benzene nucleus. None of the esterases so far examined hydrolyse the methyl esters of the benzoic acids, syringic and vanillic. *A. niger* FAE-III was found to prefer the benzene nucleus of the cinnamic acid to be di-substituted at positions 3,4 and/or 5 with a methoxy group (Faulds and Williamson, 1994). Substitutions with various numbers of hydroxy groups at



Ferulic acid

Fig. 1. Structure of ferulic [3-(3-methoxy-4-hydroxy phenyl)-2-propanoic] acid.

Table 1
Properties of ferulic acid esterases

	Source	pI	Native M_r
FAE-I	*Aspergillus niger* (commercial pectinase preparation)[a]	3.0	132 000 (2)[1]
FAE-II	*Aspergillus niger* (commercial pectinase preparation)[a]	3.6	29 000 (1)
FAE-III	*Aspergillus niger* (cultures grown on oat spelt xylan)[b]	3.3	36 000 (1)
Cinnamic acid esterase (CinnAE)	*Aspergillus niger* (cultures grown on sugar beet pulp)[c]	4.8	145 000 (2)
Sinapic acid esterase (SAE)	*Aspergillus niger* (cultures grown on sugar beet pulp)[d]	4.0	ND[2]
Acetyl esterase (XYLD)	*Pseudomonas fluorescens*[e]	N.D.	58 500 (1)
FAE	*Streptomyces olivochromogenes*[f]	7.9 8.5	29 000 (1)

[1] Value in brackets is the number of subunits; [2] ND, not determined; [a] Faulds and Williamson (1993b); [b] Faulds and Williamson (1994); [c] Kroon et al. (1996); [d]Unpublished results; [e] Ferreira et al. (1993); [f] Faulds and Williamson (1991).

these positions reduced activity, and no hydrolytic activity was measured on the methyl ester of 3,4-dihydroxycinnamic (caffeic) acid. Another *A. niger* esterase, CinnAE (cinnamoyl esterase; Kroon et al., 1996), was found to have affinity for different cinnamic acids, preferring either hydroxy- or no substitutions on the benzene ring.

Feruloylated oligosaccharides were purified from ferulic acid esterase-free enzymic digestion of wheat bran, maize bran and sugar beet pulp (Colquhoun et al., 1994; Ralet et al., 1994a; Saulnier et al., 1995a). Ferulic acid was found to be ester-linked at different positions of the primary sugar, depending upon the source of feruloylated oligosaccharide (Fig. 2): 1,5 to arabinofuranoside in wheat and maize, and either 1,2 to arabinopyranoside or 1,6 to galactopyranoside in sugar beet pulp (Colquhoun et al., 1994; Saulnier et al., 1995a). Further differences in the position of the glycosidic linkage between the primary and the secondary sugars were also noticed between maize and wheat brans. *A. niger* FAE-I, III and CinnAE were all found to have a high affinity for feruloylated trisaccharides, but whereas FAE-III only hydrolysed the 1,5-linked ferulic acid, FAE-I and CinnAE hydrolysed the 1,5-linked compounds poorly and preferred 1,2-linked ferulic acid (Ralet et al., 1994b; Kroon et al., 1996). *P. fluorescens* acetylesterase was able to hydrolyse low molecular weight 1,2-linked feruloylated oligosaccharides, but had higher affinity for a 1,5-linked tetrasaccharide (Faulds et al., 1995b). Thus, we have found it important to select the right esterases for the material to be hydrolysed.

After investigating the specificities of the esterases, we studied the release of free ferulic acid from agricultural waste materials by the enzymes. Cereal brans contain a high proportion of phenolic residues, and we have examined the suitability of using brans from wheat and maize, spent grain from barley, and sugar beet pulp, as substrates for the enzymic extraction of free ferulic acid.

Wheat bran is a product of the milling industry, after the white flour has been removed from the coloured bran and husk of the wheat germ. The only esterase capable of releasing ferulic acid without further aid was *A. niger* FAE-III (Faulds and Williamson, 1994). The other isolated esterases required the presence of a second enzyme, usually an endo-β-1,4-xylanase, to break down the polysaccharide into lower molecular weight fragments suitable for the esterases to act upon. Even the specific activity of FAE-III increased at least 10-fold upon the addition of a *Trichoderma viride* xylanase, both enzymes exhibiting a reciprocal synergistic interaction (Faulds and Williamson, 1994; 1995). FAE-III was found to be the most effective of the esterases studied to date in the release of ferulic acid from wheat bran. A total of 95% of the extractable ferulic acid was released from wheat bran after a 5 h incubation in the presence of xylanase, which would represent a major advance in obtaining a source of otherwise unrecovered material for further utilisation (Faulds and Williamson, 1995).

As well as using the right esterase to release ferulic acid from wheat bran, we have also examined the influence of different xylanases on the

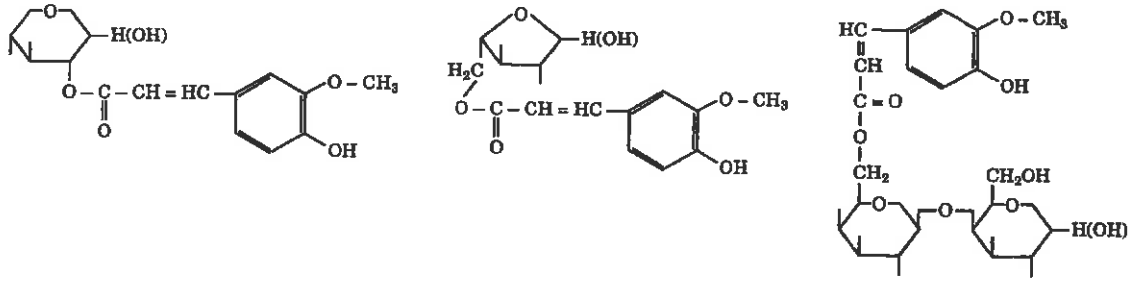


Fig. 2. Structures of three feruloylated oligosaccharides isolated from cell-walls of wheat bran and sugar beet showing the position of the ferulic acid-sugar ester linkage.

hydrolytic reaction. Increase in esterase activity was directly influenced by the rate of polysaccharide degradation, which was also dependent upon the specific activity of the xylanase on the complex substrate (Bartolomé et al., 1995). It was also shown that the most efficient xylanase produced products of higher molecular mass (trisaccharides and tetrasaccharides), which were previously shown to be more suitable to hydrolytic attack by the esterases of *P. fluorescens* and *A. niger* FAE-III (Ralet et al., 1994b; Faulds et al., 1995b). In both cases this has been *T. viride* xylanase. The synergistic interactions between ferulic acid esterase and xylanase was shown to be reciprocal since the hydrolysis of polysaccharides by the xylanase was more complete in the presence of the esterase (Bartolomé et al., 1995). This cooperation between a main chain and an accessory enzyme has been previously termed bi-product heterosynergy (Coughlan et al., 1993), and has been shown for other xylanolytic enzymes. This work thus contributes to the understanding of the cell wall degradation as a process carried out by systems of interactive enzymes.

Two *A. niger* esterases, FAE-III and CinnAE, were able to quickly degrade feruloylated oligosaccharides isolated from maize bran (Faulds et al., 1995a). However, upon de-starched maize bran itself, only small amounts of ferulic acid were released (0.3% w/w total alkali-extractable ferulic acid), even after addition of various hydrolytic enzyme preparations. We can conclude that physical hinderance affects the access of the esterase and other enzymes to the maize bran heteroxylan. On sugar beet pulp, it is the nature of the ester linkage between ferulic acid and arabinopyranoside (1,2) that prevents *A. niger* FAE-III from releasing free ferulic acid (Ralet et al., 1994b).

Spent grain is a brewing residue of malted barley grain which remains in the mash-kettle after the wort has been removed by filtration. *A. niger* FAE-III alone removed 3.3% of the ferulic acid from spent grain after 7 h. The addition of *T. viride* xylanase increased this percentage to 30%; again the action of the xylanase on spent grain solubilises low-molecular mass feruloylated material that is further degraded by the esterase to produce ferulic acid (Bartolomé et al., 1996). The use of spent grain as a source of enzymatically-released ferulic acid does not require any pre-treatment (such as starch removal for wheat bran), which represents an important advantage in relation to other cereal brans.

These differences in the percentages of total ferulic acid released from cereals brans are due to variations in composition and structure of feruloylated arabinoxylans. The lower the degree of branching of the xylan substrates (wheat bran < spent grain < maize bran), then the higher FAE-III activity upon it and consequently more ferulic acid is released, either in the absence and in the presence of xylanases (Bartolomé et al., 1996). Substituents may restrict physical accessibility of the enzymes to the feruloyl groups in the insoluble substrate, leading to a lower release of ferulic acid.

In this paper, we show that by treatment of wheat bran (1 kg) with a *Trichoderma* (100 000 U), followed by treatment of the solubilised material with *A. niger* FAE-III (17 U), we can obtain 5.7 g (1.11 mmoles) of free ferulic acid.

2. Materials and methods

2.1. Materials

De-starched wheat bran was obtained from Agro-industrie Researches et Développement (ARD), Pomacle, France. The *Trichoderma* preparation (SP-431) was obtained from NOVO Nordisk, Denmark. *A. niger* FAE-III was purified from oat spelt xylan grown culture supernatant as previously described (Faulds and Williamson, 1994). One unit (U) of activity is defined as the amount of enzyme required to release 1 μmol product per min under assay conditions (pH 6 and 37°C for FAE-III; pH 5.3 and 37°C for xylanase).

2.2. HPLC analysis

Free ferulic acid and soluble feruloylated oligosaccharides were analyzed by reversed phase HPLC on an Spherisorb ODS-2 C_{18} column (Phenonomex, UK) by the method of Kroon and Williamson (1996). Ferulic acid and feruloylated compounds were detected at 310 nm. Samples were prepared by first diluting 1000-fold in 100 mM MOPS buffer (pH 6) and filtered through a 0.22 μm membrane. Amount of ferulic acid was compared to an external standard curve of free acid.

2.3. Absorbance spectroscopy

The absorbance spectrum of soluble material was examined on a Beckman DU-70 spectrophotometer. Samples were diluted in 100 mM MOPS (pH 6), centrifuged to remove particulates and spectra obtained against a buffer blank.

2.4. Total sugar analysis

Total sugar composition of the solubilised wheat bran after xylanase treatment was determined on semi-dried material by the method of Blakeney et al. (1983).

2.5. Alkali-extractable ferulic acid content of wheat bran preparation

The ferulic acid content of de-starched wheat bran was determined by alkali-extraction (1 M NaOH, 24 h) and the amount released was neutralised and analysed by HPLC. The ferulic acid content was determined by this method to be 1% (w/w dry weight).

3. Results and discussion

De-starched wheat bran (1 kg) was incubated overnight with 100 000 U xylanase in 20 l water at 37°C (Fig. 3; pH of mixture was 4.5). The sample was continuously stirred with an overhead motorised stirrer. The soluble material was decanted through muslin cloth into a fresh container then

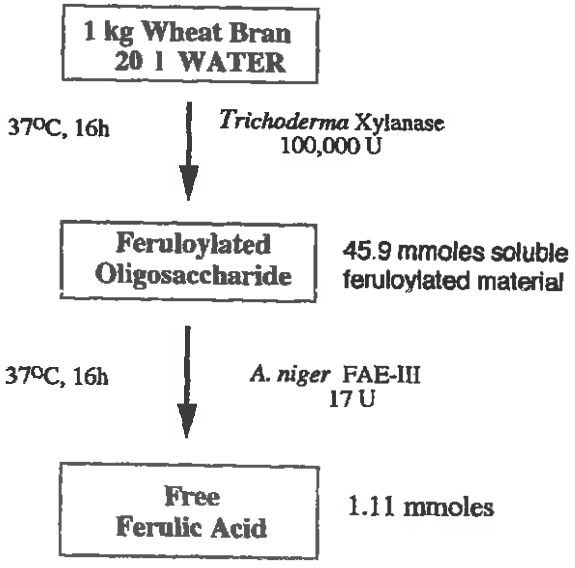


Fig. 3. Flow-chart of the hydrolysis of wheat bran to produce free ferulic acid.

Table 2
Sugar composition of the solubilised wheat bran after treatment with a *Trichoderma* xylanase preparation and semi-dried

Sugar	Contribution (%)
Arabinose	8.0
Xylose	32.7
Mannose	1.4
Galactose	0.5
Glucose	34.5
Unknown	19.9
Water	5.0

filtered through Whatman No. 1 filter paper in a Büchner funnel. The residual insoluble wheat bran was washed with a further 5 l of water and treated as before. The clear, light brown solution obtained was concentrated further by rotary evaporation down to 1.74 l at 40°C. The sample was further clarified by centrifugation for 60 mins at 15 000 × *g*. The concentration of feruloylated material released was calculated from an absorbance spectrum of a 1000-fold dilution in 100 mM MOPS (pH 6), using the extinction coefficient of linked ferulic acid (19 524 M^{-1} cm^{-1}, Ralet et al., 1994b). 45.9 mmoles of soluble feruloylated material was obtained from this hydrolysis, which corresponds to 29.6 g of FAXX (O-[5-O-(*trans*-feruloyl)-α-L-Ara*f*]-(1 → 3)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl*p*)-like equivalents (MW = 644, Colquhoun et al., 1994; Ralet et al., 1994a). There was thus, 8.9 g of ferulic acid equivalents available for further extraction. Sugar composition of this sample is shown in Table 2. High proportion of xylose or xylo-oligosaccharides, as expected with xylanase activity, and glucose in the total mixture was observed. The glucose is released mostly as the monosaccharide (data not shown).

A. niger FAE-III (9.8 U/ml) was added to the feruloylated material and incubated for 20 h at 37°C with gentle stirring. Analysis of the reaction product by HPLC (Fig. 4) and absorbance spectroscopy, showed that all the linked ferulic acid had been hydrolysed by the esterase, releasing a total of 5.7 g (1.11 mmole) free ferulic acid. This corresponds to 57% of the total alkali-extractable ferulic acid in the original wheat bran sample, and represents 64% of the soluble feruloylated material available to FAE-III after xylanase hydrolysis. However, the HPLC chromatogram (Fig. 4) suggests that all the material known to be feruloylated oligosaccharides from the retention time of external standards (Colquhoun et al., 1994; Ralet et al., 1994a) had been converted to the free acid. The reason for this discrepancy in values is not yet known. It may suggest that some of the alkali-extractable ferulic acid from the wheat bran may not be hydrolysed by FAE-III. The deficit of ferulic acid or feruloylated compounds remaining in the insoluble and soluble wheat bran components amounts to 43% of the total alkali-extractable ferulic acid. Part of this material was not solubilised by the action of the *Trichoderma* xylanase. Previously, we reported 95% release of the alkali-extractable ferulic acid from de-starched wheat bran using *A. niger* FAE-III and *T. viride* xylanase (Faulds and Williamson, 1995) and we should have achieved a similar conversion in this hydrolysis reaction. A difference in the source of wheat bran or the xylanase preparation used in these two experiments may explain why we get differences in the release of free acid, and further examination of wheat varieties and enzyme preparations is required in relation to ferulic acid extraction. A recent report has shown that there is a high natural variation in the arabinoxylan content of 22 varieties of wheat grown in France (Saulnier et al., 1995b). Differences in the degree of branching and location of the feruloyl groups may prevent solubilisation by xylanase, similar to the problems found with maize bran heteroxylans (Faulds et al., 1995a).

In summary, we have purified a number of enzymes that release ferulic acid from agro- industrial cereal waste material. We have achieved the recovery of 5.7 g free ferulic acid from 1 kg de-starched wheat bran using FAE-III from *A. niger* and a *Trichoderma* xylanase preparation. This ferulic acid can be utilised in industrial processes to make a variety of natural products by biotransformations. The enzymic process allows further saccharification of sugars, leaving the residue suitable for animal feed. We have studied the substrate specificity of these esterases and characterised their suitability for certain applica-

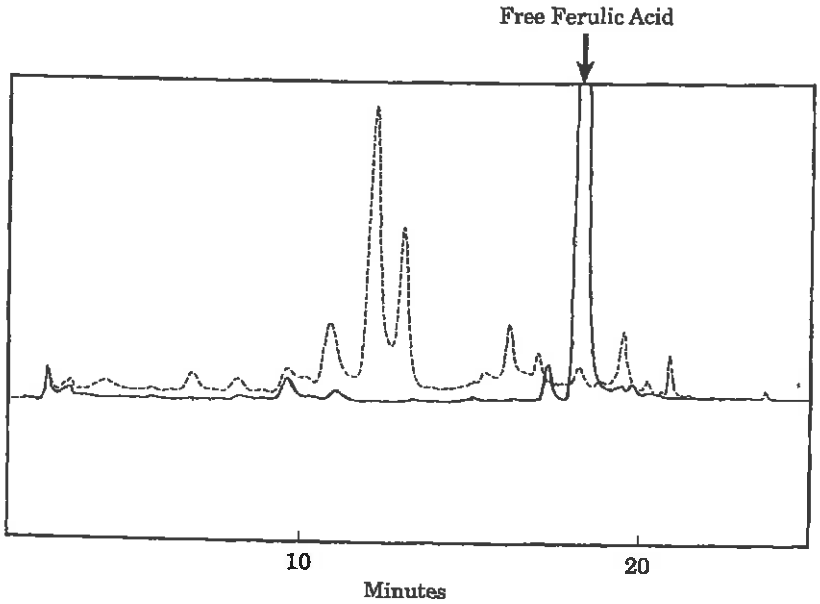


Fig. 4. HPLC chromatogram of the recovered linked (dashed line) and free ferulic acid (solid line) from the enzymic hydrolysis of wheat bran. Products were separated on a Spherisorb ODS_2 C-18 column as described in Section 2. Peaks were identified by comparison with known standards.

tions. A cheaper and more 'natural' source of ferulic acid may thus be obtained in the future.

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