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Thermal and enzymatic treatments for the release of free ferulic acid from maize bran

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Abstract

Different commercial enzyme preparations have been tested to release ferulic acid from maize bran. Best results were obtained with a preparation from *Humicola insolens*: Novozym 342. However, only 30% of the ferulic acid contained in the bran was released by Novozym 342; moreover, a part (1/3) of the solubilised ferulic acid was still esterified to sugars.

Thermal pretreatment (flash-explosion or autoclaving at 160°C) has been tested in order to improve ferulic acid solubilisation. About 80% of ferulic acid contained in the bran was solubilised by a sequential procedure using first a flash-explosion pretreatment of the bran (10% w/v in water) at 180°C (1 min) or 190°C (1 min) followed after cooling by Novozym 342 treatment (3% w/w protein/bran at 40°C for 24 h). Flash-explosion treatment solubilised feruloylated substrates and after Novozym 342 action about 1/3 of solubilised ferulic acid was still esterified to sugars.

A similar 80% extent of solubilisation of ferulic acid as feruloylated oligosaccharides was also obtained by autoclaving maize bran (10% w/v in water) at 160°C for 1 h. Subsequent treatment with feruloyl esterases was then necessary to get ferulic acid in its free form. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Enzymatic treatment; Ferulic acid; Maize bran

1. Introduction

Ferulic acid is a very important component for the structure and the biology of the cell-wall, as it can cross-link polysaccharide chains through dimerisation reaction (Ishii, 1997). Ferulic acid can be used as an antioxidant (Graf, 1992), or be transformed into vanillin. Actually, the microbial conversion of ferulic acid into vanillin may give "natural vanillin" provided that ferulic acid is of natural origin, e.g. obtained by enzymic release from plant material. Owing to the wide use of vanillin as flavour in food, cosmetic and pharmaceutical industries, the high cost of the vanilla aroma extracted from vanilla pods, and the request of consumers for natural products, the microbial conversion of ferulic acid into "natural vanillin" has been extensively studied (Thibault et al., 1998). Among agricultural by-products that are potential sources of ferulic acid, maize bran is probably one of the most promising, since it contains the highest amount of ferulic acid (~3% w/w, Saulnier & Thibault, 1999) as compared to wheat bran or sugar-beet pulp (~1% w/w, Bartolomé et al., 1997; Saulnier & Thibault, 1999)

In maize bran, ferulic acid is ester-linked to heteroxylans, which are the main components of the cell-wall (Saulnier & Thibault, 1999). Generally, feruloyl esterases are not able to release ferulic acid from polymeric material (Kroon, Garcia-Conesa, Fillingham, Hazlewood & Williamson, 1999), and it is therefore necessary to break down the xylan core using cell-wall degrading enzymes (xylanases, arabinofuranosidases, xylosidases,…) to provide feruloylated oligosaccharides which are substrates for feruloyl esterases. This paper investigates the effect of thermal treatment on the solubilisation of feruloylated oligosaccharides from maize bran and the subsequent release of ferulic acid by a commercial enzyme preparation from *Humicola insolens*.

2. Materials and methods

2.1. Materials

All enzymes were from Novo Nordisk (Denmark) except Driselase from Fluka. Feruloyl esterase, FAEA (*Aspergillus*

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Table 1
Solubilisation of feruloyl compounds (free acid + feruloylated oligosaccharides) from destarched maize bran by different enzyme mixture (1% w/w protein/bran 16 h at 40°C in water; results are expressed as % of ferulic acid present in the bran)

Enzyme preparation	Protein content determined by the Bradford method (mg/ml of enzyme preparation)	%Ferulic acid
Novozym 342	16	18.4
SP 249	65[a]	0.0
SP 431	17	0.0
SP 584	17	1.1
Glucanex	130[b]	0.0
Celluclast	55	0.0
Xylanase I	531[b]	1.6
Xylanase II	366[b]	0.0
Pentopan 500BG	54[b]	9.4
Driselase	125[a,b]	0.0

[a] Protein determined by the Lowry method.
[b] Measured in mg/g of enzyme preparation.

niger) was a gift of Dr Gary Williamson from IFR (Norwich, England). Protein content in the preparation was generally determined by the Bradford method (Bradford, 1976). For SP249 and Driselase, the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) was used.

Commercial micronised maize bran was provided by Ulice (Riom, France). Destarched maize bran was prepared as follows: 100 g of maize bran were suspended in 1000 ml of water, 10 ml of Termamyl 120L were added, and the mixture was stirred for 1.5 h in a boiling water bath. The suspension was centrifuged, the supernatant rejected, and the residue washed with hot water and centrifuged again twice. The final residue was dried for 24 h at <40°C. The yield was 82% (d.m.) destarched maize bran.

2.2. Enzymatic degradation

All enzyme degradations were carried out on destarched bran. An aqueous suspension (2% w/v) of maize bran was usually incubated for 16 h with 1 or 2% enzyme (w/w protein/substrate) at 40°C in rotating tubes or in stirred vessels. The pH of the bran/water suspension was ~4.5, attempts to buffer the media at 4.5 with an acetate buffer, or a universal buffer depressed the efficiency of ferulic acid release strongly. Samples were inactivated by boiling for 10 min.

2.3. Enzymes

Enzyme preparations were commercial culture supernatants of different microorganisms (Table 1). Enzymatic activity towards soluble linear xylan from *Palmaria palmata* (Bobin-Dubigeon et al., 1997) and carboxymethylcellulose (CMC) was calculated from the increase in reducing ends (Nelson, 1944), using xylose or glucose as standard. Reaction mixtures contained a substrate (0.01 g/l in acetate buffer 50 mM, pH 5.0) incubated with appropriately diluted enzymes at 40°C. Osidase activities were measured on *p*-nitrophenylglycosides (2 mM final concentration in acetate buffer 50 mM, pH 5.0) incubated with suitably diluted enzymes at 40°C (Rouau & Odier, 1985). They were calculated from the release of *p*-nitrophenol measured spectrophotometrically at 400 nm. Assays for feruloyl esterase activities were performed using 5-*O*-(*trans*-feruloyl)-L-Araf (FA) isolated from maize bran (Saulnier, Vigouroux & Thibault, 1995) at about 35 nmol l^{-1} in 3-(*N*-morpholino)-propane sulfonic (MOPS) buffer 100 mmol l^{-1} pH 6, incubated at 40°C with suitably diluted crude enzymes. The enzymic activity was determined spectrophotometrically at 286 (free ferulic acid) and 323 nm (esterified ferulic acid) (Ralet, Faulds, Williamson & Thibault, 1994). The following molar absorption coefficients were determined at pH 6 in a MOPS buffer ($\varepsilon_{286} = 14,176$ and $\varepsilon_{323} = 10,350$ l mol^{-1} cm^{-1} for free ferulic acid, and $\varepsilon'_{286} = 12,465$ and $\varepsilon'_{323} = 19,345$ l mol^{-1} cm^{-1} for esterified ferulic acid) and were used in the calculation.

2.4. Thermal treatments

An agitated laboratory autoclave (100 ml) was used for both flash-explosion or autoclaving treatments. In both cases 5 g of maize bran were suspended in 50 ml of water.

In case of flash-explosion a setting temperature (160–210°C) was maintained for 1 min, then the pressure was released and the autoclave quickly cooled to room temperature. It took 15–25 min to raise the setting temperature.

Autoclaving treatments were carried out in the same equipment. Samples were heated at 160°C for varying periods of time (0.5–4 h) and then cooled to room temperature (30 min) without any release of pressure.

In both cases, supernatants were recovered by centrifugation and residues were washed with water. Washings and supernatants were then pooled and freeze-dried while residues were washed with EtOH and acetone and then dried overnight in an oven at 40°C.

2.5. Determination of ferulic acid

Total ferulic and dehydrodiferulic acids in bran, residues recovered after pretreatments and the supernatant were determined after alkaline treatment with NaOH (2 M) at 35°C for 30 min. *o*-coumaric acid was added as an internal standard, the mixture was acidified to pH 2.0 with HCl, and then extracted with Et_2O. Et_2O-extracts were evaporated to dryness at 40°C. Samples were dissolved in MeOH/H_2O (50:50 v/v), and analysed by HPLC on a C18 column (Purospher, Merck Germany) as previously described (Saulnier et al., 1999). Free ferulic acid in supernatant was determined as follows: an internal standard (*o*-coumaric acid) was added to the supernatant, then NaCl solution was added to the supernatant (final NaCl concentration 2 M) and the

Table 2
Specific activities of enzymes from Novozym 342 preparation

Substrate	Specific activity (nKat/mg)[a]
Arabinan	16
Xylan	232.9
CMC	55.6
pNP-Gal	0
pNP-Ara	4.5
pNP-Xyl	3
FA	0.3

[a] mg of proteins determined by the Bradford method.

mixture was acidified to pH 2.0 with HCl, and then extracted with Et_2O. Et_2O-extracts were then treated and injected into the HPLC system as described above. Ester-linked ferulic acid was calculated as the difference between total and free ferulic acid.

2.6. Determination of neutral sugars

Total neutral sugars were determined in the supernatant after hydrolysis (1 M H_2SO_4, 100°C, 1.5 h) by GLC of their alditol acetates derivatives (Englyst & Cummings, 1988) on a BP-225 (SGE; 30 m × 0.32 mm i.d.) fused-silica capillary column. For insoluble samples (starting bran and residue of extraction) a prehydrolysis step with 72% H_2SO_4 for 30 min at 25°C was carried out before hydrolysis (1 M H_2SO_4, 100°C, 1.5 h). Monomers in supernatants were determined as follows: an internal standard (inositol) was added to the supernatant, free sugars in the supernatant were then converted into alditol acetates derivatives (Englyst & Cummings, 1988) and analysed by GLC.

3. Results and discussion

3.1. Enzymatic degradation of destarched maize bran

Destarched maize bran is mainly composed of neutral sugars coming from heteroxylans: xylose 27.6% (w/w; dry weight), arabinose 15.8%, and galactose 5.1%, but also of glucose 22.0%, coming from cellulose. The bran also contained 3.1% (w/w) of ferulic acid, 0.3% of *p*-coumaric acid and 0.3% of 5-5′-, 0.6% of 8-*O*-4′- and 0.5% of 8-5′-dimer of ferulic acid.

In a preliminary step, different enzyme mixtures (Table 1) were used in standard conditions (1% w/w protein/substrate, 16 h at 40°C) on a maize bran suspension in water (pH ~ 4.5) in order to test their ability to solubilise feruloylated oligosaccharides and to release free ferulic acid. Only Novozym 342 and Pentopan 500 BG were able to solubilise some ferulic acid from the bran (Table 1). Both commercial preparations are from *Humicola insolens.*

The degradation of the bran by Novozym 342 was further studied. Enzymic activities exhibited by N342 preparation are reported in Table 2 and the extent of solubilisation of neutral sugars and ferulic acid is reported in Table 3. The solubilisation of ferulic acid was increased by using an enzyme preparation/substrate ratio of 2% (w/w protein/bran) for 16 h at 40°C with the addition of a fresh preparation (1% w/w protein/bran) and an extra incubation time of 8 h. In these conditions, the solubilisation extent of ferulic acid increased up to ~30%, but further addition of N342 did not show any improvement. A similar extent of solubilisation for ferulic acid (33%) was obtained with a very high preparation concentration (5% w/w protein/substrate) for 16 h. In N342, xylanase and cellulase were the highest activities. Arabinose, xylose and galactose, which are components of heteroxylans, were released to an extent similar to ferulic acid from the bran, but glucose solubilisation was more efficient and up to 50% of cellulose of the bran was solubilised by N342 (2 + 1%). Feruloyl esterase activity was detected in N342 (Table 2) and about 70% of the ferulic acid solubilised from the bran was detected in a free form, e.g. not esterified to sugars. Dimers of ferulic acid were also solubilised but remained linked to sugars (90%) indicating that feruloyl esterases from N342 were less efficient towards ester linkages involving ferulic acid dimers.

The solubilisation of ferulic acid from maize bran by enzymic treatment was quite low. We have looked for some pretreatments which might help enzyme accessibility and improve the solubilisation of ferulic acid from bran cell-walls.

Table 3
Solubilisation of neutral sugars and phenolic acids from maize bran by Novozym 342 (results are expressed as % of total compound present in the starting material)

	N342 — 1%[a]	N342 — 2 + 1%[b]
Arabinose	16.7	29.1
Xylose	14.7	26.9
Galactose	13.5	26.3
Glucose	29	52.9
Ferulic acid	19.4	29.6
p-Coumaric acid	13.5	22.9
5-5′diFA	7.4	15.3
8-0-4′diFA	13.8	12.9
8-5′diFA	6.1	6

[a] N342 1% (w/w) 16 h at 40°C.
[b] N342 2% (w/w) 16 h + N342 1% 8 h, at 40°C.

3.2. Flash-explosion treatment of maize bran

Flash-explosion treatment is known to improve the accessibility of enzymes to lignocellulosic materials (Excoffier, Toussaint & Vignon, 1991). The autohydrolysis reaction generally occurs over a temperature of 180°C and the structure of the material is also broken down during decompression (Debzi, 1992) improving enzyme accessibility. We have therefore simulated flash-explosion treatments by heating for 1 min at various temperatures (160–210°C) a water suspension of bran in a laboratory autoclave, with a

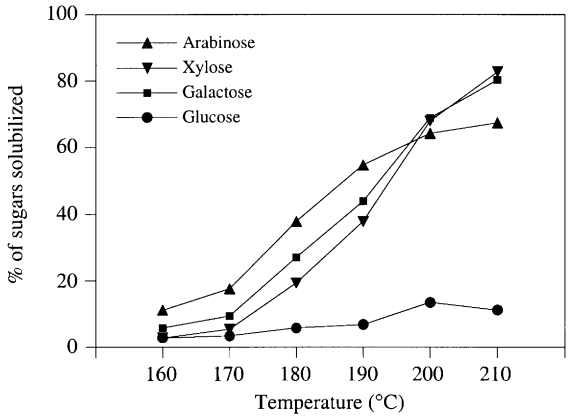

Fig. 1. Effect of the temperature of flash-explosion on the solubilisation of neutral sugars from the bran.

subsequent release of pressure and fast cooling. Depending on the final temperature, total heating time varied from 15 to 25 min. The conditions of temperature and pressure as well as the pH of the solution at the end of the treatment are reported in Table 4. The amount of solubilised material increased with the temperature and pressure applied to the bran, but significant solubilisation only occurred over 180°C.

The release of individual sugars and ferulic acid as a function of pretreatment temperature are shown in Figs. 1 and 2. Arabinose, xylose and galactose, which are components of heteroxylans, are solubilised in a similar way, and up to 80% of these sugars were solubilised under the most severe conditions (210°C). Glucose coming from cellulose was little solubilised by flash-explosion treatment. Among the sugars solubilised, about 50% of arabinose, 14% of xylose and 30% of galactose were found to be monomers. This result reflected the very high sensitivity of arabinose to acid hydrolysis whereas most of xylose and all the cellulosic glucose solubilised were found as oligomers or small polymers. Ferulic acid was solubilised in a similar way to arabinose, xylose and galactose. The ester linkage survived the

Table 4
Conditions of flash-explosion and recovery of insoluble residue and supernatant

Temperature (°C)	160	170	180	190	200	210
Pressure (bars)	4	6	8	12	15	19
pH	4	4	4	4	3.5	3.5
Yield						
Residue	92.3	87.4	75.0	65.2	42.4	32.2
Soluble	5.0	6.8	19.4	30.0	48.6	56.7
$\sum$	97.3	94.2	94.4	95.2	91.0	88.9

conditions of pressure and temperature used, and ferulic acid remained esterified to neutral sugars.

Although the solubilisation extent from the bran of the different components of heteroxylans (Ara + Xyl + Gal) and ferulic acid increased with autoclaving temperature, the total recovery of these components, i.e. the sum of these different constituents in the insoluble residue and the supernatant, decreased over 190°C indicating degradation reactions. This loss of material was particularly distinct for arabinose and ferulic acid for which total recovery was less than 80% under the most severe conditions.

Pretreatment had a solubilizing effect on the bran, especially at high temperature, but might also have improved the solubilizing efficiency of the enzyme preparation on the pretreated insoluble residue. Insoluble residues isolated after pretreatment at 160, 170, 180 and 190°C were therefore treated with two N342 preparation dosages: 1% w/w protein/bran for 16 h at 40°C (N342 — 1%) or 2% w/w protein/bran for 16 h at 40°C with the subsequent addition of a fresh preparation (1% w/w protein/bran) and an extra incubation time of 8 h (N342 — 2 + 1%). The solubilisation of individual sugars and ferulic acid from the different pretreated insoluble residues are shown in Figs. 3 and 4. In all cases, arabinose, xylose and galactose exhibited a similar behaviour. Pretreatments at 160 and 170°C solubilised small amounts of neutral sugars or ferulic acid from the bran, and the action of N342 on these pretreated insoluble residues was only slightly improved as compared with the action of

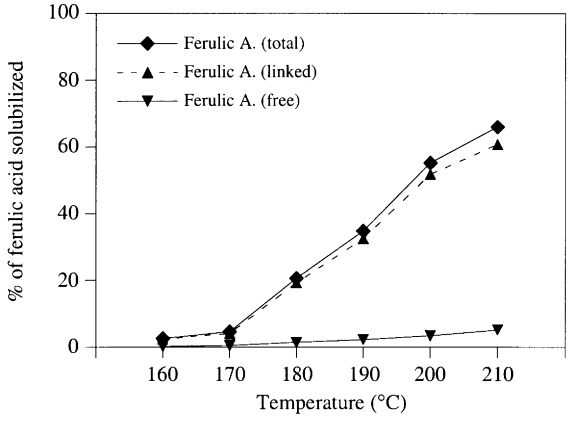

Fig. 2. Effect of the temperature of flash-explosion on the solubilisation of ferulic acid from the bran.

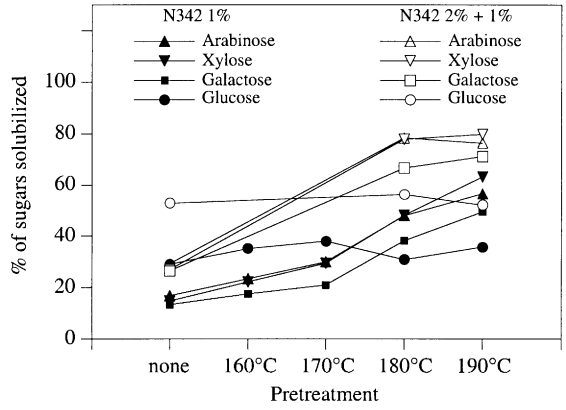

Fig. 3. Effect of Novozym 342 on the solubilisation of neutral sugars from insoluble residues obtained after flash-explosion of maize bran.

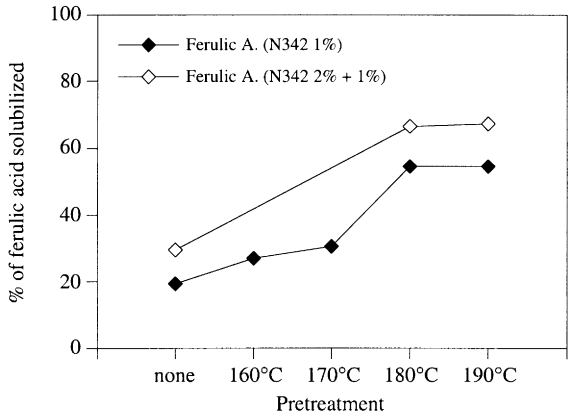

Fig. 4. Solubilisation of ferulic acid by Novozym 342 from insoluble residues obtained after flash-explosion of maize bran.

N342 on the native bran. Pretreatments at 180 and 190°C solubilised a significant amount of material from the bran, and also improved the solubilisation by N342 of the arabinose, xylose and galactose contained in the insoluble residues. Up to 70–80% of these sugars were solubilised from the 180 and 190°C insoluble residues with the highest enzyme preparation dosage (N342 — 2 + 1%). Therefore, pretreatment at 180 and 190°C enhanced enzyme accessibility to residual heteroxylans present in insoluble residues.

The amount of heteroxylans (calculated as the sum of arabinose + xylose + galactose) released from the bran by the 190°C pretreatment itself was 40.8% (w/w). The action of enzyme preparation at the dosage N342 — 1% or N342 — 2 + 1% on the insoluble residue left after pretreatment, solubilised 31.3 or 40.4% of heteroxylans (g/100 g of heteroxylan in the bran), respectively.

Similar behaviour was observed for ferulic acid (Fig. 4). As reported for the treatment of the bran by N342, about 70% of ferulic acid was found in a free form, but 30% was still esterified to sugars.

The fate of glucose was different as it was little solubilised during pretreatment, but the solubilisation of glucose

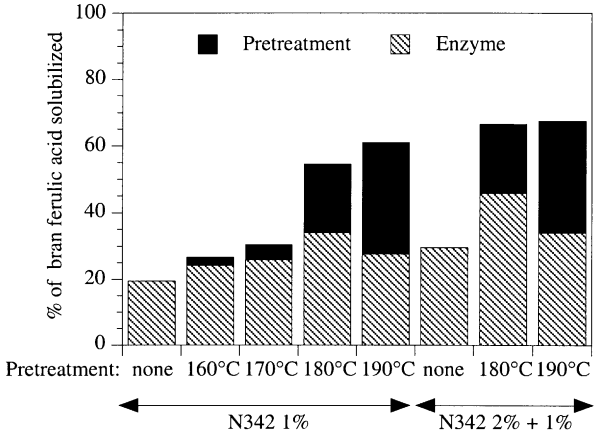

Fig. 5. Total solubilisation of ferulic acid from maize bran by a combined action of flash-explosion and Novozym 342.

by N342, which was rather high on the starting bran, was little or not increased on pretreated insoluble residues (Fig. 3).

Therefore flash-explosion is an efficient method to solubilise feruloylated material and to improve enzyme accessibility to insoluble residues. However, under the conditions tested (short heating time and fast cooling) the effects were important only over 180°C. As shown in Fig. 5, there is a balance between direct solubilisation by the pretreatment alone (with possible degradation of ferulic acid under severe conditions) and the enzymic solubilisation from the insoluble residues left after pretreatment. Overall, pretreatment at 180 or 190°C and subsequent treatment with N342 — 2 + 1%, gave the best results. Nearly 70% of the ferulic acid (in esterified and acid form) contained in the bran was solubilised; about 1/3 and 1/2 of total ferulic acid solubilisation was due to the 180 and 190°C flash-explosion pretreatment, respectively.

Ferulic acid solubilised during flash-explosion was mainly esterified to neutral sugars, so that feruloyl esterases action was necessary to convert it into free acid. Supernatant obtained after flash-explosion of the bran at 190°C was therefore incubated with N342 or FAEA. FAEA was able to convert almost all feruloyl esters into free ferulic acid, but total conversion into free ferulic acid was not obtained with N342 (Table 5). Based on specific activity for feruloyl esterases present in N342, it would have been necessary to use a protein/bran ratio of 40% (w/w) to reach the same activity level with N342 as with FAEA. The feruloyl esterase activity of Novozym 342 is therefore too low to convert in reasonable conditions (time, amount of enzyme preparation) all feruloyl esters into free ferulic acid.

3.3. Autoclaving treatment of maize bran

Although flash-explosion pretreatment followed by N342 treatment was efficient to free the major part of ferulic acid from the bran, it required a specific equipment due to the high pressure developed. Autoclaving treatment of the brans at 160°C was therefore investigated.

As shown in Figs. 6 and 7, the solubilisation of arabinose and xylose reached a plateau value after 1 h of treatment and up to 85% of heteroxylans were solubilised, which corresponds to the best result obtained using flash-explosion. After 2 h of treatment, degradation of the sample occurred, especially for arabinose. For longer times (4 h) sugars were degraded probably in furfuric derivatives and solutions turned dark brown.

Ferulic acid followed a similar pattern of solubilisation as that of the neutral sugars and maximum release was reached after 1–2 h of treatment. The amount solubilised was similar to that observed with flash-explosion treatment. For longer times (2 h) degradation of ferulic acid occurred, possibly due to its instability at high temperature. Ferulic acid was still mainly esterified (~95% of total ferulic acid

Table 5
Effect of Novozym 342 and FAEA on feruloyl esters contained in supernatant obtained by flash-explosion of maize bran at 190°C

Ferulic acid	Free	Linked
Starting supernatant	6.8	93.2
Enzyme treatment[a]		
N342 1%	30.3	69.7
N342 2% + 1%	49.3	50.7
FAEA	95.3	4.7

[a] % w/w N342/freeze-dried supernatant at 40°C in water for 16 h for FAE III 12.5 nKat for 100 mg of freeze-dried supernatant at 40°C for 16 h.

released) to sugars, and feruloyl esterases treatment was necessary to convert it into its free form.

The supernatant obtained after autoclaving 0.5 and 1 h at 160°C was then incubated with Novozym 342 (1% w/w) in water at 40°C for 16 h. Novozym had little effect on neutral sugars which were already present as small oligomers or monomers. As an example, about 45% of released arabinose was present as monomers after autoclaving; this proportion increased to about 60% after enzyme incubation. About 30% of the released ferulic acid was converted into the free form after N342 treatment, which corresponded to the results reported in Table 5 with a similar amount of enzymes.

4. Conclusions

Maize bran cell-walls are extremely resistant to enzyme attack, probably due to the high degree of cross-linking of heteroxylans through diferulic bridges, as well as the high substitution degree of the xylan core by side-chains, both having negative impacts on the degradability of bran tissue, probably by restricting the accessibility of enzymes within the wall. None of the enzyme mixture tested exhibited the appropriate combination of cell-wall degrading enzymes able to solubilise extensively the xylan core of heteroxylans and feruloylated oligosaccharides, which are substrates for

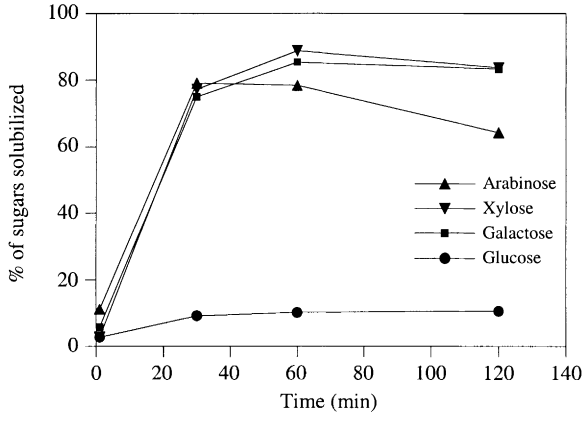

Fig. 6. Effect of autoclaving time on the solubilisation of neutral sugars from maize bran. Autoclaving temperature 160°C.

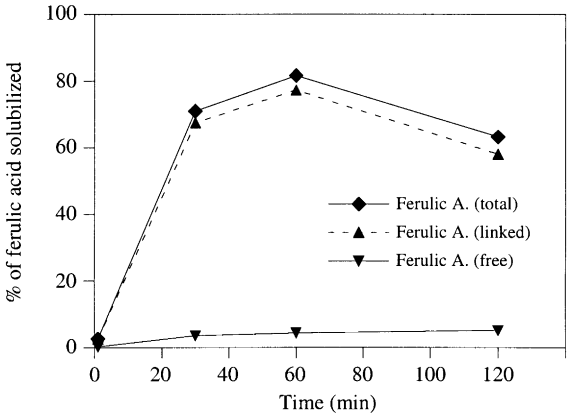

Fig. 7. Effect of autoclaving time on the solubilisation of ferulic acid from maize bran. Autoclaving temperature 160°C.

feruloyl esterases. The best enzyme complex tested (Novozym 342) was only able to solubilise ~30% of ferulic acid from the wall, but due to its low feruloyl esterase activity, feruloyl esters were only partly converted into free ferulic acid.

Thermal pretreatment of the wall at a high temperature was necessary to facilitate enzyme action. Flash-explosion treatment solubilised feruloylated material from maize bran, by autohydrolysis, and improved the subsequent action of Novozym 342 on the remaining insoluble material. The flash-explosion treatment (180–190°C) solubilised ferulic acid (25–35% of ferulic acid initially contained in the bran) as feruloylated esters and Novozym 342 solubilised ferulic acid from the insoluble residue and partly converted feruloyl esters into free ferulic acid. Overall, about 70% of ferulic acid was solubilised from the bran by flash-explosion treatment at 180 or 190°C and subsequent action of Novozym 342, but ferulic acid was incompletely converted into free acid.

Autoclaving the bran at 160°C for 1 h solubilised ~70–80% of the ferulic acid as feruloylated oligosaccharides. A pure feruloyl esterase, like FAEA, was then able to convert feruloyl esters into free acid. Autoclaving treatment of the bran with the subsequent action of a feruloyl esterase is therefore the most efficient treatment to produce free ferulic acid from maize bran. New sources of enzyme preparation with higher feruloyl esterase activity than Novozym 342 are now under investigation.

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