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ORIGINAL PAPER

C. B. Faulds · A. I. Sancho · B. Bartolomé

Mono- and dimeric ferulic acid release from brewer's spent grain by fungal feruloyl esterases

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Abstract Ultraflo L, a β-glucanase preparation from *Humicola insolens* sold for reducing viscosity problems in the brewing industry, exhibited activity against the methyl esters of ferulic, caffeic, *p*-coumaric and sinapic acids, displaying mainly type-B feruloyl esterase activity. Ultraflo also contained the ability to release 65% of the available ferulic acid (FA) together with three forms of diferulate from brewer's spent grain (BSG). An "esterase-free" Ultraflo preparation greatly enhanced the ability of a feruloyl esterase from *Aspergillus niger*, AnFAEA, to release FA (from 23 to 47%) and its dimeric forms, especially the 8,5' benzofuran form, from BSG. While total release of these phenolic acids was not observed, this synergistic enhancement of ferulate release demonstrates that FA and its dimeric forms present in BSG require the addition of more than a xylanase. This suggests either that FA is not solely attached to arabinoxylan in the barley cell wall, or that the cell wall polysaccharides in BSG hinder the accessibility of enzymes to the ferulates, due to processing treatments.

Introduction

Brewer's spent grain (BSG), a waste residue from the brewing process, consists of 39% (w/w) hemicelluloses (mainly arabinoxylan), 24% protein, 17% cellulose, 6% lipids and 4% lignin (Valverde 1994). BSG contains a number of utilisable compounds, such as oligo- and polysaccharides and small phenolic compounds. One such compound, ferulic acid (FA) (Hernanz et al. 2001), exhibits a number of potential commercial applications, such as natural antioxidant, food preservative/antimicrobial agent, anti-inflammatory agent, photoprotectant, and as a food flavour precursor (Graf 1992). FA, in either the monomeric or dimeric form, has been shown to be ester linked to the C-5 position of arabinosyl residues in arabinoxylans. Diferulates cross-link cell wall polysaccharides and influence cell wall integrity (Bunzel et al. 2001). They also act as a nucleation site for lignification (Hatfield et al. 1999).

Inducible feruloyl esterases have been identified and characterised from mesophilic fungi (Williamson et al. 1998). These enzymes hydrolyse the ester bond linking FA to plant cell-wall-bound polysaccharides. Feruloyl esterases work in synergy with main-chain degrading enzymes, such as β-(1,4)-xylan endohydrolases (xylanases; Bartolomé et al. 1995) to increase the removal of FA and derivatives from the cell wall. Almost all the FA was released from wheat bran (Faulds and Williamson 1995) and sugar beet pulp (Thibault et al. 1998) using these feruloyl esterases in combination with other cell wall glycosyl hydrolases. Feruloyl esterases have been shown release FA from BSG (Bartolomé et al. 1997b) and maize bran (Faulds et al. 1995), although the amount of FA released by these enzymes was limited by other, as yet unknown, factors. Studies on enzymatic release of ferulate dehydrodimers are more limited. Feruloyl esterases from *Aspergillus niger* (AnFAEA) and *Pseudomonas fluorescens* subsp. *cellulosa* (XylD) were reported to release only the 5,5' dimer from BSG in the presence of a xylanase from *Trichoderma viride* (Bartolomé et al. 1997a). The release of the 8-*O*-4' diferulate by AnFAEA has been demonstrated only from presolubilised wheat bran (Kroon et al. 1999) or from diethyl diester model compounds (Garcia-Conesa et al. 1999). The release of 8,5' diferulate (benzofuran form) has been demonstrated only by a commercial *Humicola insolens* preparation (Ultraflo L) on BSG (Bartolomé and Gómez-Cordovés 1999) and by a tannase from *Aspergillus oryzae* on the diethyl diester compound (Garcia-Conesa et al. 2001).

In this paper, we demonstrate that a commercial xylanase preparation from *H. insolens*, Ultraflo L, con-

C.B. Faulds (✉) · A.I. Sancho
Food Materials Science Division, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK
e-mail: craig.faulds@bbsrc.ac.uk
Tel.: +44-1603-255152
Fax: +44-1603-255038

B. Bartolomé
Instituto de Fermentaciones Industriales (CSIC), Juan de Cierva 3, 28006 Madrid, Spain

tains high levels of feruloyl esterase activity. We also show that a feruloyl esterase from *A. niger*, AnFAEA, is able to release the three dimer forms (5,5', 8-O-4' and 8,5' benzofuran form) from BSG in the presence of an "esterase-free" preparation of Ultraflo. The specificity and temperature stability of the feruloyl esterases present in Ultraflo L is also discussed.

Materials and methods

Material

BSG (malt:maize, 80:20 w/w) was provided by Mahou SA (Madrid, Spain). BSG was oven dried at 60°C for 18 h and milled to a fine powder (particle size <50 μm). Recombinant *A. niger* AnFAEA was produced in the methylotrophic yeast *Pichia pastoris* (Juge et al. 2001). Ultraflo L was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark) and desalted through a PD-10 column into 100 mM 3-[*N*-Morpholino]-propane sulphuric acid (MOPS), pH 6.0, prior to use. *T. viride* xylanase M1, *Trichoderma* sp. xylanase M2, *Trichoderma longibrachiatum* xylanase M3, ruminal xylanase M6, and wheat arabinoxylan (medium viscosity) were purchased from Megazyme International Ireland (Bray, Ireland). Driselase was obtained from Sigma (Poole, Dorset, UK) and purified by the method of Borneman and co-workers (1990). Methyl ferulate, methyl caffeate and methyl sinapate were obtained from Apin Chemicals (Oxon, UK). Ferulic, sinapic and caffeic acids were obtained from Sigma. FA dehydrodimers were quantified according to the method of Waldron and colleagues (1996) using the following response factors (RF) against trans cinnamic acid at 280 nm: RF =0.21 for 5,5'diFA, RF =0.14 for 8-O-4'diFA, RF =0.18 for 8,5'diFA (open form), and RF =0.12 for 8,5'diFA (benzofuran form).

Enzyme assays

Feruloyl esterase activity was determined against methyl esters of hydroxycinnamic acids using the HPLC assay method described by Juge et al. (2001). One unit of esterase activity was defined as the amount of enzyme required to release 1 μmol hydroxycinnamic acid min^{-1} mg $protein^{-1}$ at 37°C, pH 6.0. Enzymatic release of FA and diferulates from plant cell wall material was determined in 100 mM MOPS buffer (pH 6.0) in a thermostatically controlled rotating incubator (5 rpm, 37°C). Plant cell wall material (10 mg) was incubated with different amounts and combinations of AnFAEA, Ultraflo L, Driselase, xylanase and α-L-arabinofuranosidase in a final volume of 500 μl for 3 h. Incubations were terminated by the addition of 200 μl of glacial acetic acid. After centrifugation (20,000 *g*, 5 min, 15°C), samples were filtered through a 0.45 μm filter and 100 μl aliquots were analysed by HPLC as described by Juge et al. (2001). All assays were prepared and analysed in duplicate. The amount of phenolic material released was quantified against standard curves. The total alkali-extractable hydroxycinnamate content was determined as described by Hernanz et al. (2001). Xylanase activity against 1% (w/v) wheat arabinoxylan in 10 mM McIlvaine's buffer, pH 5.5, was determined as described in Bailey et al. (1992).

Temperature optimum and thermal stability of the feruloyl esterase activity in Ultraflo L

To determine the temperature optimum of the Ultraflo L sample, 5 μl of a 10-fold dilution of an Ultraflo L sample in 100 mM MOPS, pH 6.0, after buffer exchange through a NAP-10 column (Amersham Pharmacia), was incubated with 1 mM methyl ferulate (MFA) for 10 min over a temperature range of 25 to 80°C, in 5°C increments. Reactions were terminated by the addition of glacial acetic acid and the amount of FA release determined by HPLC as described previously. The stability of the feruloyl esterase activity of Ultraflo L was determined at 60°C and 75°C. NAP-10 desalted fractions (diluted 10-fold) were incubated at the above temperatures for up to 90 min. Aliquots (5 μl) were removed every 10 min and assayed for residual activity against MFA (10 min, 37°C). A sample of enzyme was kept on ice for 90 min to measure general activity loss with time. Reactions were terminated with the addition of glacial acetic acid, centrifuged and 100 μl of the supernatant run on the HPLC as previously described.

Preparation of an "esterase-free" carbohydrate preparation

Ultraflo L (40 ml) was incubated with phenylmethyl sulphonyl fluoride (PMSF; 10 mM prepared in isopropanol) and aminoethyl-benzene sulphonyl fluoride (AEBSF; 10 mM prepared in 100 mM MOPS, pH 6.0) for 20 h at 4°C with constant agitation. Sample was then dialysed overnight against 50 volumes of MOPS buffer. Dialysed samples were assayed against wheat arabinoxylan and MFA for residual xylanase and feruloyl esterase activity, respectively.

Results

Feruloyl esterase activity in Ultraflo L

Ultraflo L exhibited activity against the methyl esters of ferulate (5.1 U ml^{-1}), sinapate (0.39 U ml^{-1}), *p*-coumarate (2.4 U ml^{-1}) and caffeate (6.7 U ml^{-1}). From these results, and according to the putative classification of feruloyl esterases proposed previously (Kroon et al. 1999), Ultraflo contains predominantly a type-B feruloyl esterase (active on MCA), with a small amount of type-A (active on MSA). When 500 mU (against MFA) was incubated with BSG at 37°C for 3 h, 65% of the FA was released, 41% of the 5,5'-dimer, 66% of the 8,5' dimer and 15% of the 8-*O*-4' dimer (Table 1). This release of all three major types of diferulates has not previously been demonstrated for any enzyme preparation. Addition of a recombinant feruloyl esterase from *A. niger*, AnFAEA (10 U), had no effect on the release of phenolic material from BSG (Table 2). In comparison, Driselase alone released only 3% of the available FA, but no dimers, while with the addition of AnFAEA, 42% of the FA and 18% of the 5,5' diferulate was released.

To test whether this release of monomeric and dimeric ferulate was due to the action of feruloyl esterases and xylanases alone, 200 U/g BSG of xylanase M2 (from *Trichoderma* sp, pI 5.5), xylanase M3 (from *T. longibrachiatum*) or xylanase M6 (from an undisclosed ruminal microorganism) were incubated with BSG together with AnFAEA (10 U g^{-1} BSG). Table 2 summarises the effect on the release of FA and 5,5' diferulic acid from BSG by these enzymes. The source of xylanase was seen to have little effect on the release of the phenolics, suggesting either that other plant cell wall-degrading enzymes are required to act with the xylanase, or that the xylanases used in this study have limitations in the solubilisation of feruloylated material present in BSG compared to the enzymes produced by *H. insolens*.

Table 1 Influence of the carbohydrate preparation in the release of phenolics from brewer's spent grain (BSG) by *Aspergillus niger* feruloyl esterase AnFAEA (28 U g^{-1} BSG). AnFAEA was incubated with BSG (10 mg) for 3 h in the presence of either *Trichoderma viride* xylanase (1,000 U g^{-1} BSG) or serine esterase-treated Ultraflo L preparation (700 U xylanase activity g^{-1} BSG). Comparison is also given with treated or untreated Ultraflo alone (700 U xylanase activity g^{-1} BSG). All assays were performed in duplicate. Values are expressed as a percentage of the total alkali-extractable levels of the phenolic acids in spent grain (Hernanz et al. 2001). Values in brackets represent the standard deviation

Enzyme combination	Amount of phenolic acid released			
	Ferulic acid	5,5'-diFA	8-O-4'-diFA	8,5'-benzofuran diFA
AnFAEA + *T. viride* xylanase (M1)	23 (±0.14)	10 (±0.24)	ND[a]	ND
Treated Ultraflo alone	5 (±0.62)	ND	ND	ND
AnFAEA + treated Ultraflo	47 (±0.33)	17(±0.61)	3 (±0.06)	23 (±1.46)
Untreated Ultraflo alone	65 (±1.61)	41 (±1.42)	15 (±0.61)	66 (±0.02)

[a] Phenolic compounds not detected in the assay supernatant

Table 2 Release of phenolics from BSG by AnFAEA (10 U g^{-1} BSG) in the presence of different xylanases (200 U g^{-1} BSG) or carbohydrase preparations (700 U xylanase g^{-1} BSG). Numbers after xylanases correspond to Megazyme codes. All assays were performed in duplicate. Values are expressed as a percentage of the total alkali-extractable levels of the phenolic acids in spent grain (Hernanz et al. 2001). Values in brackets represent the standard deviation

Source of xylanase	Ferulic acid (FA)	5,5' diFA
T. viride (M1)	23 (±0.14)	10 (±0.24)
Trichoderma species (M2)	24 (±0.66)	6 (±0.49)
Trichoderma longibrachiatum (M3)	30 (±7.43)	8 (±2.64)
Ruminal M6	20 (±0.69)	6 (±2.04)
Driselase (Basidiomycete)	42 (±2.14)	18 (±0.87)
Ultraflo (*Humicola insolens*)	65 (±1.61)	41 (±1.42)

Release of phenolic acids from BSG by AnFAEA in the presence of an "esterase-free" preparation of Ultraflo L

After treatment of Ultraflo L with AEBSF and PMSF for 24 h, only 0.1% of the activity against MFA remained in the preparation, but 82% of the xylanase activity was retained. On BSG, the treated sample released only 6.6% of the monomeric FA, but no diferulate. However, the ability of the preparation to release the three forms of diferulate from the barley cell wall was abolished (Table 1). This preparation was thus used as an "esterase-free" carbohydrase mixture to examine the release of ferulates by AnFAEA.

AnFAEA (28 U g^{-1} BSG) was incubated in the presence of treated Ultraflo (700 U xylanase g^{-1} BSG, i.e. the same level as used previously), and the amount of mono- and dimeric ferulate released was quantified (Table 1). The amount of FA released by AnFAEA increased from 33% to 47%, which indicates that the carbohydrases present in Ultraflo, apart from xylanase and arabinofuranosidase, further unravel the cell wall structure to allow the esterase greater access to the feruloyl groups. A significant improvement was also noticed in the release of diferulate from BSG by AnFAEA. In the presence of the xylanase from *T. viride*, only the 5,5'-dimer was released by the action of AnFAEA. With the other plant cell-wall-degrading enzymes present in Ultraflo, the 8-O-4' and the 8,5' benzofuran forms were also released, albeit in lower amounts than with untreated Ultraflo alone. AnFAEA had previously been shown to release only the 8-*O*-4' dimer from pre-solubilised wheat bran and never the 8,5'-dimer (Garcia-Conesa et al. 1999; Kroon et al. 1999). This is the first evidence of AnFAEA being able to release both the 8-O-4' and the 8,5 dimers from insoluble plant cell wall material without the need for presolubilisation (Garcia-Conesa et al. 1999).

Temperature optimum and stability of the feruloyl esterase activity in Ultraflo

Samples of Ultraflo were incubated with 1 mM MFA for 10 min over the temperature range 25–80°C and the amount of FA release was determined by HPLC. There was a clear temperature optimum between 60 and 65°C. To test the stability of the feruloyl esterase activity, samples were incubated at 60°C or 75°C and aliquots removed with time and assayed at 37°C for residual activity. The feruloyl esterase activity did not decrease with time while on ice. At 60°C, there is an initial loss of ~25% of the activity over the first 10 min, decreasing to 60% residual activity after 90 min.

Discussion

It is known that the composition of certain cereal cell wall fractions, such as maize bran (Saulnier et al. 2001), wheat bran (Faulds and Williamson 1995) and BSG (Sancho et al. 2001), sterically hinder the accessibility to, and activity on, cell wall polysaccharides of feruloyl esterases (and probably xylanases) (Faulds et al. 1995). Similarly, processing of cereal cell walls may restrict the capability of degradative enzymes, possibly through compression of the cell wall. AnFAEA and xylanase alone are insufficient to alleviate accessibility problems in barley-derived material. Only carbohydrase mixtures, such as Ultraflo, resulted in the increased release of mono- and dimeric ferulate (Table 2). This suggests either that other poly-

mers present in the barley cell wall interact with the arabinoxylan in such a way that they hinder the hydrolytic activity of xylanases, or even that the majority of the FA in barley is not associated with arabinoxylan as, according to Dervilly et al. (2002), arabinoxylan isolated from malted is easily degraded by xylanases.

Ultraflo is sold to brewers described as: "a heat-stable multi-active β-glucanase preparation produced by a selected strain of *H. insolens*. The most important side activities are cellulase, xylanase, pentosanase and arabinase". Feruloyl esterase activity is not described in the product documentation. These results indicate that Ultraflo contains feruloyl esterases as efficient as AnFAEA together with either a number of other feruloyl esterases, including some specific for the other dimers, or a more efficient esterase than AnFAEA. The combination of enzymes in the mix solubilises the feruloylated oligosaccharides sufficiently for the esterase to be able to act. However, neither FA nor dimeric forms were released in their entirety, which suggests that Ultraflo does not contain all the enzymes necessary to facilitate total hydrolysis. What constitutes the correct combination of enzymes from *Humicola* to act on cell-wall-derived material has yet to be elucidated.

Treatment of Ultraflo with AEBSF and PMSF removed almost all of the feruloyl esterase activity in the preparation against MFA. The small amount of FA released may have been due to the low level of feruloyl esterase activity remaining or by the presence of a non-serine esterase able to hydrolyse FA esters at low rates. As 82% of the xylanase activity was retained, this preparation could be used as an "esterase-deficient" carbohydrase preparation. A significant increase in the release of mono- and dimeric ferulate by AnFAEA was achieved using this preparation, without the need to presolubilise the cell wall material prior to the addition of feruloyl esterase (Table 1). With the inhibitor-treated Ultraflo, AnFAEA was able to release the 8,5' benzofuran dimer from spent grain. However, Garcia-Conesa et al. (1999) showed that AnFAEA could hydrolyse the diester bonds of the 5,5' and 8-O-4'diferulate only using model substrates, and that only a monoester intermediate was released with the 8,5'benzofuran diferulate diester. This suggests that there is an enzyme present in the Ultraflo preparation that is capable of hydrolysing the ester bond on the 8,5' benzofuran diferulate. AnFAEA can then act on this monoester, which will be still attached to either the insoluble cell wall or a solubilised feruloylated oligosaccharide. This enzyme is not similar to the tannase of *A. oryzae* (Garcia-Conesa et al. 2001), as this enzyme hydrolysed both ester bonds from the 8,5' benzofuran diferulate diester. Another explanation might be that AnFAEA exhibits different specificity towards model substrates and insoluble plant cell wall substrates such as BSG. The specificity profile of Ultraflo against the hydroxycinnamic acid methyl esters suggests that it contains predominantly a type-B feruloyl esterase, with lower levels of a type-A, based on the putative classification of Kroon et al. (1999). Dimer release has never been demonstrated for a type-B feruloyl esterase, and only the release of the 5,5' dimer has been previously reported from untreated material for a type-A feruloyl esterase. Esterases acting on all the major forms of the diferulates are also present in this preparation, and in the culture supernatant of *Humicola grisea* grown on wheat bran (unpublished data). While activity on 5,5' and 8-O-4' diferulates has been previously identified with type-A feruloyl esterases (Bartolomé et al. 1997a; Fillingham et al. 1999; Garcia-Conesa et al. 1999; Kroon et al. 1999, 2000), and activity on the 8,5' diferulate form by a tannase (Garcia-Conesa et al. 2001), there may be as yet undefined feruloyl esterases produced by *Humicola* species.

There was a clear temperature optimum between 60 and 65°C for feruloyl esterase activity in the Ultraflo preparation, which is slightly higher than that recorded for AnFAEA (55–60°C; Faulds and Williamson 1994). Activity quickly dropped above 65°C (55% at 70°C) and virtually disappeared after 10 min at 75°C. Since Ultraflo is sold to work at 75°C, there is either sufficient stability in the sample for the residual activity to be sufficient to act on the barley cell walls at 75°C, or the esterase works to release the phenolic compounds while the mashing mix heats up to 75°C in the brewery. The Ultraflo feruloyl esterase(s) also demonstrated a remarkable thermostability compared to FAEA from *A. niger*, which retained 33% of its activity after 60 min at 60°C (Juge et al. 2001).

In conclusion, we have shown that Ultraflo L, a β-glucanase preparation from *H. insolens*, exhibited activity against the methyl esters of ferulic, caffeic, *p*-coumaric and sinapic acids, displaying mainly type-B feruloyl esterase activity. Ultraflo also contained the ability to release 65% of the available FA together with three forms of diferulate from BSG. An "esterase-free" Ultraflo preparation greatly enhanced the ability of a feruloyl esterase from *Aspergillus niger*, AnFAEA, to release ferulic acid (from 33 to 47%) and its dimeric forms, especially the 8,5' benzofuran form, from BSG. While total release of these phenolic acids was not observed, this synergistic enhancement of ferulate release demonstrates that FA and its dimeric forms present in BSG require the addition of more than a xylanase.

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