

Barley Contains Two Cationic Acetylxylan Esterases and One Anionic Feruloyl Esterase

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ABSTRACT

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Various carbohydrate-active esterases are detected in extracts of malted barley when analyzed by polyacrylamide gel electrophoresis. The slowest migrating and most heat-resistant of these are relatively cationic acetylxylan esterases. Two such activities, one with a high affinity for esterase substrates including acetylated xylan, and one with a low affinity, are indicated. These enzymes did not hydrolyze methyl ferulate. A relatively

heat-labile anionic feruloyl esterase has also been purified. It has some, albeit low, ability to act on acetylated xylan. The feruloyl esterase effects extensive release of ferulate from endosperm cell walls isolated from barley, whereas the acetylxylan esterases are only capable of very limited release of acetate.

Various enzymes are involved in the degradation of components of the starchy endosperm of barley during malting. Many of these have been extensively investigated, notably the endo- β -glucanases (Stuart et al 1986), endo-xylanases (Slade et al 1989), endo-peptidases (Zhang and Jones 1995), carboxypeptidases (Dal Degan et al 1994), α -amylases (MacGregor and MacGregor 1987), β -amylase (Buttner and Briggs 2000), and limit dextrinases (MacGregor et al 1994). The action of the amylases and limit dextrinases is less extensive than is that of the other hydrolases during germination due to the limited substrate susceptibility of large starch granules, although there is extensive digestion of small starch granules (Palmer 1972).

One series of enzymes that has attracted relatively limited attention has been the esterases. The barley kernel contains a range of water-extractable esterases detectable by gel electrophoresis using a generalized stain (Kahler and Allard 1970; Prentice 1970; Huston et al 1988; Jones and Heisel 1991). In an initial characterization of these various activities, the most cationic components had the greatest thermotolerance, are located in the starchy endosperm, and are most likely acetylxylan esterases (Ward and Bamforth 2002). It was hypothesized that during the germination process these enzymes are involved in the digestion of the starchy endosperm cell walls, notably through the splitting of acetic acid residues from the arabinoxylan that coats these entities (Kanauchi and Bamforth 2002). As such, they are likely a component of the so-called "solubilase" complex of enzymes that functions in the trimming of components from the cell walls, rendering access for the β -glucanases to the principle wall component (Bamforth et al 1979, 1997). The most intensely staining band, however, was extremely mobile in the gels (highly anionic) and was located in the aleurone but not the starchy endosperm (Ward and Bamforth 2002). In the present work we have explored these two fractions further with the aim of identifying their likely primary function.

MATERIALS AND METHODS

Materials

Except where noted, all chemicals, were purchased from Fisher Scientific (Fair Lawn, NJ). Barley (var. Stander) was a gift from Great Western Malting Company (Vancouver, WA).

Determination of Protein

Soluble protein was routinely determined by the spectrophotometric method of Kalb and Bernlohr (1977), in which protein is determined on the basis of relative light absorbance at 230 and 260 nm. In purified preparations, protein was determined by the Coomassie blue-binding method of Bradford (1976) using bovine serum albumin as standard.

Enzyme Assays

p-Nitrophenyl acetate-hydrolyzing esterase activity was assayed according to the procedure of Johnson et al (1988). Enzyme solution was mixed with a freshly prepared solution (0.4 mL) of 10 mM *p*-nitrophenyl acetate (*p*NPA, dissolved in dimethyl sulphoxide) in 50 mM sodium phosphate buffer, pH 6.5, in a total assay volume of 4.0 mL. The reaction was started by the addition of enzyme. The formation of *p*-nitrophenoxide was followed spectrophotometrically at 410 nm and 25°C (the molar extinction coefficient employed was 0.0171/M/cm).

α -Naphthyl acetate-hydrolyzing esterase activity was assayed according to Poutanen and Sundberg (1988). The reaction mixture included enzyme solution (added to start the reaction), 50 mM phosphate buffer, pH 7.0, 0.1 mL of Fast Blue RR salt (24 mM), and 0.4 mL of α -naphthyl acetate (10 mM) in a total reaction volume of 3.1 mL. Both α -naphthyl acetate and RR Fast Blue were predissolved in ethanol. The RR Fast Blue solution was filtered using a 0.45- μ m filter membrane (Millipore, Billerica, MA) before use. The progress of α -naphthol release was measured at 500 nm and 25°C (molar extinction coefficient employed was 0.0645/M/cm).

Acetylxylan esterase (AXE) activity was measured using as substrate a chemically acetylated xylan prepared according to Johnson et al (1988). Dry xylan (10 g) from oat spelts (Sigma Chemical Co., St. Louis, MO) was slowly added to 250 mL of dimethyl sulfoxide with gentle stirring at room temperature. Once xylan was suspended, the preparation was heated for 20 min at 55°C until solubilization was complete. Solid potassium borate (0.2 g) was added to a final concentration of 0.8% (w/v). The solution was stirred for an additional 10 min at 55°C. Acetic anhydride (200 mL), preheated to 60°C, was added over a 20-min period with continuous stirring. The reaction mixture was dialyzed (Fisher Scientific, molecular weight cut off 3,500) against running tap water at 4°C for five days. Following a further 24 hr of dialysis against deionized water, the sample was freeze-dried overnight. AXE activity was measured with a reaction mixture including 100 μ L of 0.5M sodium phosphate buffer, pH 6.0, 200 μ L of enzyme solution, and 200 μ L of 10% (w/v) acetylated xylan. Enzyme was used to start the reaction. Incubation was at 25°C for 2 hr before the reaction was stopped by boiling for 10 min. Samples were clarified in a bench-top centrifuge, and supernatants

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were analyzed for acetate release using a commercial kit (R-Biopharm, Marshall, MI). To determine the degree of acetylation of the substrate, 1 mg of sample and 1 mL of deionized water were heated for 20 min at 50°C until the solutions were clear. The sample was then diluted with 2 mL of 2M NaOH and left for 16 hr at 50°C. Samples were then assayed for acetic acid content using the procedure described above. Acetylation of xylosyl residues was 8.5% (w/w) based on a 65% yield (w/w) of xylan.

Feruloyl esterase (FAE) activity was measured using the methyl ester of ferulic acid as substrate. The latter was synthesized by the method of Borneman et al (1990). The substrate was solubilized in methanol and then added to a buffer solution (100 mM 3-(*N*-Morpholino)-propanesulfonic acid at pH 6.0) to a final concentration of 5 mM. The assay mixture included 1 mL of methyl ferulate and 0.5 mL of enzyme solution (used to start the reaction) and was incubated at 30°C for 3 hr. Release of free ferulic acid was measured by the fluorescence method of Fincher (1976) with excitation at 343.5 nm and emission at 382.5 nm. In all cases, one unit of activity is defined as the amount of enzyme that liberates 1 μ mol of product/min under the assay conditions.

Gel Electrophoresis

Crude extracts and purified enzyme solutions were fractionated by native polyacrylamide gel electrophoresis (PAGE) (Protean II, electrophoresis apparatus, Bio-Rad, Hercules, CA). All gels were run and stained for esterase activity according to the method of Jones and Heisel (1991). A stacking gel of 3% polyacrylamide gel at pH 6.8 and a separating gel of 7.5% polyacrylamide at pH 8.3 were employed. Esterase activity was detected by steeping gels in a solution containing 0.04% Fast Blue RR salt and α -naphthyl acetate in 0.05M sodium phosphate buffer at pH 6.0. After staining, gels were fixed in 12% (w/v) trichloroacetic acid.

Heat Stability Studies

Aliquots of partially purified enzyme (0.32 mg/mL of protein) were incubated at 25, 45, 65, or 85°C. Portions were removed at 5, 10, 15, 30, and 60 min and rapidly cooled in ice. Samples were centrifuged at 11,950 \times g for 5 min at 4°C before assay.

Enzyme Kinetics

Aliquots (0.2 mL) of partially purified esterase were incubated either with *p*NPA (0.025–1.75 mM) in a final volume of 4 mL, with α -naphthyl acetate (0.2–2.3 mM) in a final volume of 3.1 mL, acetylated xylan (5–56%, w/v) in a final volume of 0.5 mL, or methyl ferulate (0.6–3.75 mM) in a final volume of 0.8 mL.

Enzyme Purification

Barley was extracted as described by Ward and Bamforth (2002). Barley (300 g) was ground at gap setting 10 in a pin mill (Miag, Dresden, Germany), and mixed 1:2 (w/v) with 50 mM sodium phosphate buffer, pH 6.5, containing 6 mM dithiothreitol. The mixture was maintained at 25°C for 5 hr and centrifuged at 11,950 \times g for 20 min at 4°C. The crude extract (100 mL, 4°C) was taken to various successive degrees of saturation by the slow addition of solid ammonium sulfate. Precipitates were removed by centrifugation at 26,890 \times g for 20 min at 4°C. Pellets were resolubilized in 8 mL of 50 mM phosphate buffer, pH 6.5. The 30–50% and 50–70% pellet fractions were combined and heated at 65°C for 20 min before removing the precipitate by centrifugation at 11,950 \times g for 20 min at 4°C. The supernatant was taken to 100% saturation with ammonium sulfate and recentrifuged at 11,950 \times g for 20 min at 4°C. The pellet was taken up in 5 mL of 50 mM phosphate buffer, pH 6.5, and dialyzed for 24 hr against 3L of the same buffer at 4°C. Ion exchange chromatography was performed on DE23 cellulose (Whatman Co., Maidstone, England), prepared according to the manufacturer's specifications. A column 2.0 cm in diameter was filled to a height of 18 cm, and the resin was

equilibrated with 20 mM Tris-HCl buffer, pH 8.3, for 48 hr at 4°C. The enzyme was loaded and the column was washed with 100 mL of the same buffer. Elution was with a linear gradient of 0–0.5M NaCl at a flow rate of 1mL/min (Fig. 1A). Active fractions were pooled and concentrated by precipitation with ammonium sulfate (100% saturation) before dialyzing for 24 hr against 3L of 20 mM Tris-HCl at 4°C. Gel permeation chromatography was performed in a column (1 cm \times 62 cm) of Sephadex G-150 equilibrated in 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl (4°C). Dialyzed enzyme (3 mL) from the ion exchange stage was loaded into the gel permeation column, which was subsequently eluted with the equilibration buffer at a flow rate of 5 mL/hr. Active fractions were pooled and concentrated by ammonium sulfate precipitation (100%) and then dialyzed for 24 hr against 3L of 20 mM Tris-HCl at 4°C.

In a refinement of this procedure, slight changes were made to the ion-exchange protocol, namely a flow rate of 0.4 mL/min through a column of 1 cm \times 20 cm (Fig. 1B).

Ferulic acid esterase was purified using the same procedure as above except that no heating stage was employed.

Release of Acetic Acid and Ferulic Acid from Isolated Cell Walls

Barley cell walls were isolated as described by Kanauchi and Bamforth (2002). Partially purified AXE (4 units) or FAE (12 units) was added to 1.0 g of isolated barley cell wall in 5 mL of 100 mM sodium phosphate pH 6.5 and incubated at 35°C. Controls containing distilled water in place of the specific enzyme were used. After various time periods, 0.5 mL of trichloroacetic acid was added to stop the reaction and the supernatant recovered by centrifuging before analysis for acetic acid and ferulic acid.

RESULTS AND DISCUSSION

We have previously shown (Ward and Bamforth 2002) that a range of esterase enzymes from barley extracts are detectable by PAGE using the substrate α -naphthyl acetate. Furthermore, we showed that the slowest moving bands (most cationic) are the most heat stable and the fastest moving band (most anionic) is the most intensely stained but also the most thermolabile. It was further proposed that the diffuse pattern observed in the slow migrating bands did not reflect a multitude of isozymes but rather one or, at most, two enzymes that differ slightly in their surface charge characteristics, and hence their mobility. The mean molecular weight of this fraction (which was monodisperse when passed through a GPC column) was \approx 47,000. By contrast, the fastest moving anionic band was extremely sharp and clearly included a solitary enzyme with a molecular weight of 62,000.

In confirming a pattern of bands very similar to that previously reported (Ward and Bamforth 2002), we have ventured to purify the two main enzyme fractions (cationic and anionic portions, respectively). In view of the relative thermotolerance of the slower moving bands, a heating stage formed part of the initial purification route. This strategy removed all of the intermediate and fast moving esterase bands, but the slowest moving material remained. Routinely, we find that these slow moving fractions on gels do not stain crisply with the esterase assay applied to the gel and it is difficult to ascertain the precise number of bands located in this region. We suspect there are two.

It is customary whenever esterases have been studied in cereal and other systems to employ general substrates that are particularly amenable to spectrophotometric assays or that lead to useful staining on gels. While valuable tools for the enzymologist, these substrates have no physiological significance, so it is important to further investigate the activity that the enzymes display when tested with more relevant substrates.

The preparation obtained displayed esterase activity with *p*NPA and α -naphthyl acetate but also with acetylated xylan, confirming

the proposal of Ward and Bamforth (2002) that the slow migrating esterases are AXE. Humberstone and Briggs (2002a) previously reported an acetic acid esterase from malt. The rate of thermal inactivation of esterase activity in this preparation was similar (Fig. 2), irrespective of which substrate was used to assay the enzyme, further suggesting that all activities are due to the enzymes detected on the gel and not to trace quantities of some highly active enzyme in the preparation that is not detected using α -naphthyl acetate. It should be noted that the enzyme in crude preparations is rather more resistant to heating than this (Ward and Bamforth 2002), hence the use of a thermal event during the purification protocol. Apart from the fact that the enzyme is likely to be more resistant to heat when in crude preparations, the prior heating stage probably also potentiates more heat susceptibility in the enzyme when it is heated a second time in a more refined state.

Kinetic analysis revealed that nonlinear Lineweaver-Burk plots were obtained for each substrate. This is illustrated in Fig. 3 for the three substrates studied. Such biphasic plots are most readily interpreted by there being two enzymes in the preparation, one enzyme being a relatively high affinity esterase and the other dis-

playing a lower affinity for the substrates but a higher maximum velocity. The affinity constants calculated from these plots are given in Table I. The two putative enzymes may be nominally referred to as Esterase Y (the high affinity enzyme) and Esterase Z (the low affinity enzyme).

In an attempt to obtain a preparation with only a solitary slow moving esterase, a slightly revised purification protocol was used (Table II). Refinements to the ion exchange protocol led to the successful fractionation of two peaks of esterase activity, one eluting at 0.24M NaCl and one at 0.28M (Fig. 1B).

Linear Lineweaver-Burk plots were obtained when the enzyme fraction eluting at the higher salt concentration was examined kinetically for the three separate substrates (Fig. 4). Comparison of the Michaelis constants now obtained (Table I) with those derived with the first enzyme preparation indicates that the faster moving esterase is the one with the higher affinity for the substrate (Esterase Y). Neither enzyme preparation was capable of hydrolyzing methyl ferulate.

A very similar but separate purification protocol, one which omitted the heating stage, was employed to isolate the fastest moving intensely staining esterase found in barley and malt. When the product of this regime was tested by PAGE, only a single band of relative mobility at 0.95 was observed. We believe this to be the fastest moving band report by Ward and Bamforth (2002). By contrast with the cationic fractions described earlier, this enzyme

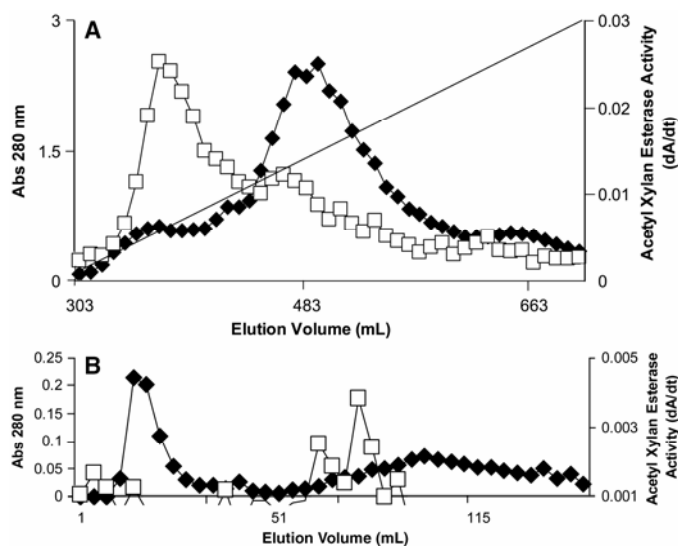


Fig. 1. Purification of cationic esterases using anion exchange chromatography. \blacklozenge , A280; \square , esterase activity. Straight line indicates NaCl gradient of 0–0.5M. A similar gradient was employed in the revised procedure (not shown). **A**, original procedure. **B**, revised procedure.

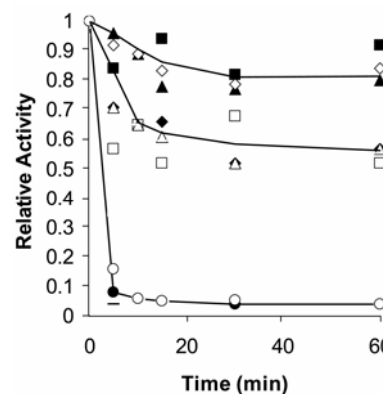


Fig. 2. Relative heat stability of esterase activity. Temperatures and substrates: 25°C acetyxylan (\blacksquare), α -naphthylacetate (\blacktriangle), *p*-nitrophenylacetate (\blacklozenge); 45°C acetyxylan (\square), α -naphthylacetate (\blacklozenge), *p*-nitrophenylacetate (\blacktriangle); 65°C acetyxylan (\bullet), α -naphthylacetate (\circ), *p*-nitrophenylacetate (\ominus).

TABLE I
Kinetic Parameters for Cationic Esterases Isolated from Barley

Substrate	Original Purification Protocol				Revised Purification Protocol	
	K_m Component Y	K_m Component Z	V_{max} Component Y	V_{max} Component Z	K_m	V_{max}
Acetylxylan (AX)	22.7% AX	143% AX	0.7 mM/min	2.5 mM/min	15.4% AX	0.3 mM/min
<i>p</i> -Nitrophenylacetate	0.37 mM	40.9 mM	11.2 mM/min	5.6 mM/min	0.96 mM	43.5 mM/min
α -Naphthylacetate	0.36 mM	17.4 mM	0.9 mM/min	16.9 mM/min	0.84 mM	5.1 mM/min

TABLE II
Purification Table for Acetyl Xylan Esterase from Barley Malt^a

Assay	Activity ($\mu\text{mol/mL/min}$)	Total Activity ($\mu\text{mol/min}$)	Protein Concentration (mg/mL)	Total Protein (mg)	Specific Activity ($\mu\text{mol/min/mg}$)	Fold Purification	Yield (%)
Crude extract	3.8	377.8	5.3	530.0	0.71	1.00	100
Combined fraction	39.2	313.3	76.3	610.7	0.51	0.72	82.9
Heat treatment (65°C, 20 min)	33.8	169.0	44.6	223.2	0.75	1.06	44.7
DEAE ion exchange	47.2	141.6	2.6	7.8	18.1	25.5	37.5
Size exclusion	21.0	63.1	0.3	1.0	65.7	92.1	16.7

^a Assay using acetylated xylan as substrate.

always displays bold, sharp staining in the esterase assay on gels. It displayed linear Lineweaver-Burk plots and had activity with methyl ferulate ($K_m = 1.27 \text{ mM}$), *p*-nitrophenylacetate (2.31 mM), α -naphthyl acetate (4.36 mM), and acetyl xylan (92.8%, w/v). Most microbial feruloyl esterases isolated to date display some acetyl xylan esterase activity (Crepin et al 2004), while some, but not all, AXE have associated feruloyl esterase activity (Ferreira et al 1993; Egaña et al 1996). In acetyl esterases from *Penicillium purpogenum*, activity against methyl ferulate was on average 1,500-fold lower than their activity against α -naphthyl acetate, and 100-fold lower than their activity on acetylated oat spelt xylan (Egaña et al 1996). Therefore, we believe this anionic species to be a feruloyl esterase. Such a heat-sensitive activity has previously been inferred by the release of ferulic acid during the mashing of malt (McMurrugh et al 1996). Feruloyl esterase activity has also been described in barley malt by Bartolomé et al (1996), Bamforth et al (1997), and Humberstone and Briggs (2002b). The substantially lower levels of this activity in malt as compared with barley are possibly due to its thermolability (Bamforth et al 1997; Ward and Bamforth 2002) as well as to a progressive decline in its level during germination (Sancho et al (1999). This observa-

tion would suggest that this enzyme has a very early role in the modification of the cell walls of the starchy endosperm in barley by facilitating the increased hydrolysis of both arabinoxylan and β -glucan from endospermic cell walls by main chain-acting carbohydrases such as endo- β -glucanase. However, feruloylated pentosan-derived oligosaccharides have been located in malt (Gubler et al 1985), suggesting that an initial removal of ester-linked ferulic acid from arabinoxylan is not a requirement for pentosan digestion during modification. This further suggests that the primary aim of endosperm-acting esterases is to increase the solubilization of feruloylated polysaccharides (Kanauchi and Bamforth 2002).

A quantity of AXE theoretically capable of releasing acetic acid at a rate of $240 \mu\text{g}/\text{min}$ actually released only $\approx 1 \mu\text{g}$ of acetate in a 2-hr period when incubated with 1 g of cell wall substrate. FAE was only able to release 1/20th of this amount. Levels of acetate measured in whole grain are of the order of $2.9 \mu\text{g}/\text{mg}$ of whole

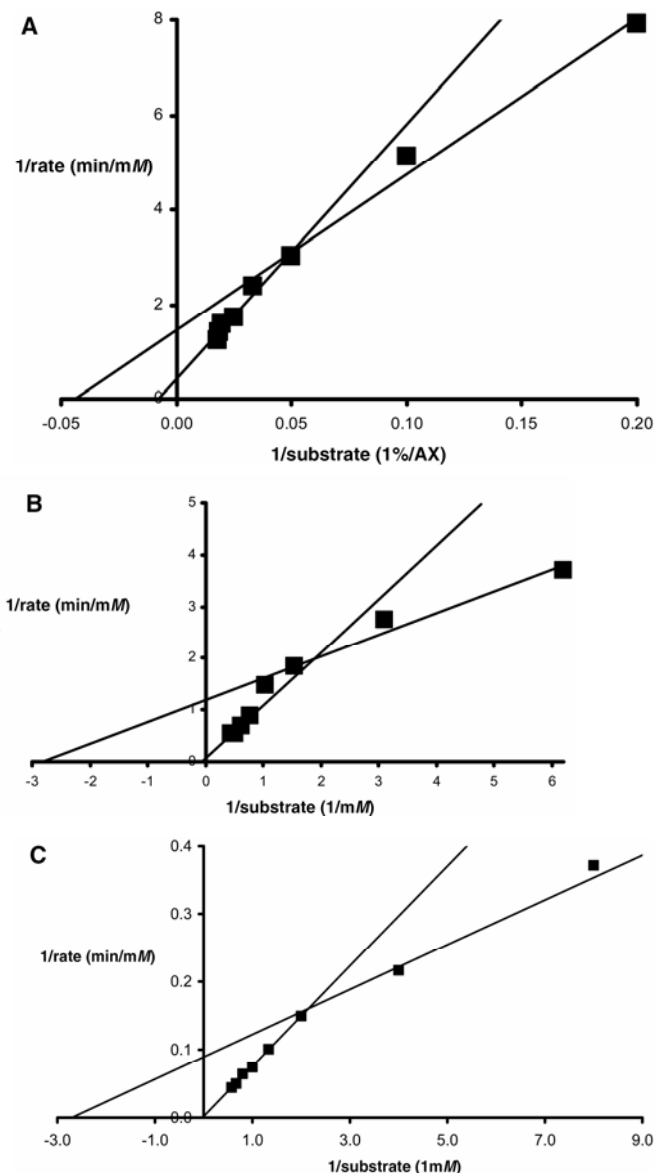


Fig. 3. Lineweaver-Burk plot for partially purified esterases from barley malt using as substrate (A) acetylxylan, (B) α -naphthyl acetate, (C) *p*-nitrophenylacetate.

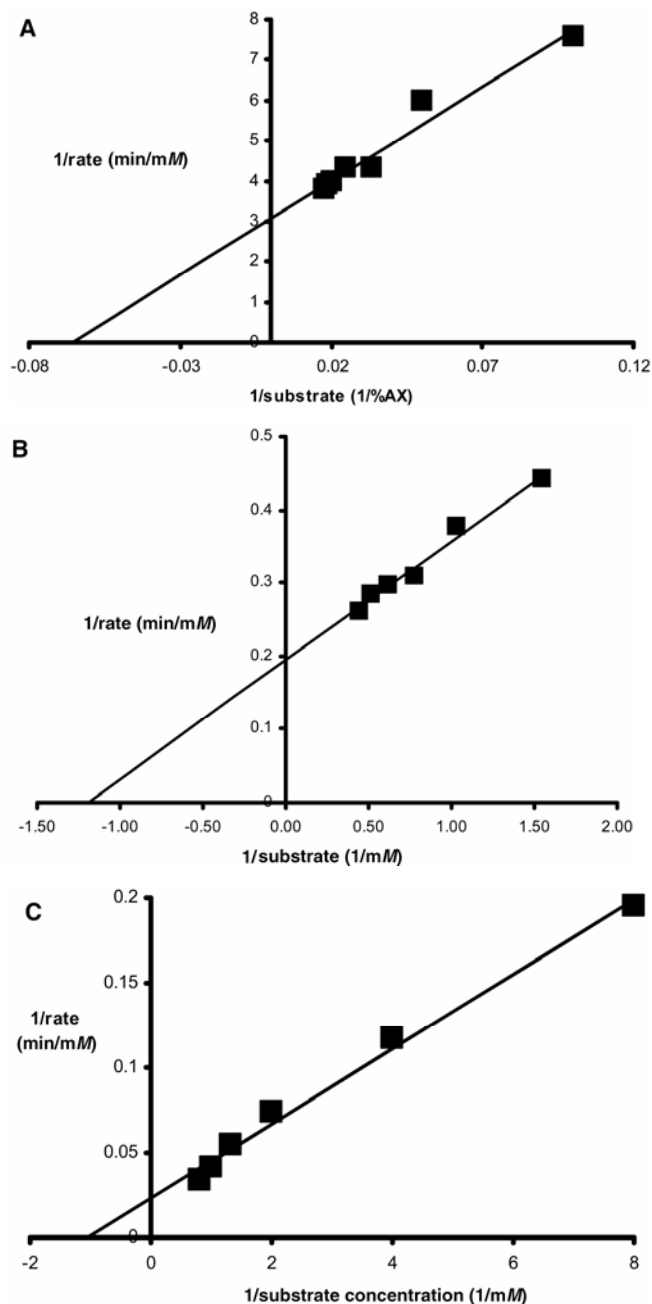


Fig. 4. Lineweaver-Burk plot for more extensively purified esterase from barley malt using as substrate (A) acetylxylan; (B) α -naphthyl acetate; (C) *p*-nitrophenylacetate.

grain (unpublished data). Assuming that the cell walls comprise 5% of the dry matter of grain and estimating that 50% of the acetate is located in the walls of the starchy endosperm, it may be inferred that ≈ 70 μg of acetate is available in the walls. This would suggest that, in the wall preparation, the enzyme has limited accessibility to acetyl groups. This may reflect, in part, the location of the acetyl groups in the arabinoxylan, with esterification being on the arabinosyl residues.

Cell walls of barley have been variously estimated to contain as little as 50 μg of ferulic acid/100 mg (Fincher 1976) and 60 μg of ferulic acid/100 mg (Ahluwalia and Fry 1986), or as much as 690 μg of ferulic acid/100 mg (Kanauchi and Bamforth 2002). In the present study, purified FAE was able to release 4 mg of ferulic acid from 1 g of isolated cell wall within 1 hr, whereas AXE was incapable of releasing any. This would suggest that the higher estimate for ferulate level in cell walls is the more realistic; furthermore, most of the feruloyl residues in cell wall arabinoxylan are available to attack by the FAE. Even so, it has been demonstrated that substantial quantities of esterified feruloyl remain in brewers spent grains after mashing (Bartolome and Gomez-Cordoves 1999), suggesting that there is incomplete action of FAE during malting and consistent with the relative thermal sensitivity of this enzyme that precludes its action during conversion temperatures in mashing.

CONCLUSIONS

Various carbohydrate-active esterases are present in malted barley. The most heat-resistant of these are relatively cationic and are acetylxylosterases. There are probably two such enzymes, one with a substantially higher affinity for acetylated xylan. Malt also possesses a relatively heat-labile feruloyl esterase that is very anionic. It has some ability, albeit low, to act on acetylated xylan. Release of ferulate from cell walls by the latter enzyme is more efficient than is that of acetate by the acetylxylosterases, however it is clear that the degradation of cell wall material during malting and mashing is not dependent on the initial removal of acetyl or feruloyl residues from cell wall polysaccharides.

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