

Partial Purification of Ferulic Acid Esterase from Malted Barley

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ABSTRACT

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A partial purification of ferulic acid esterase, which degrades feruloyl glycerol, has been achieved from barley malt in small yields. Coloured and viscous materials were removed from the malt extract using batch-elution anion exchange chromatography. Further steps included gradient elution anion exchange chromatography and gel filtration chromatography. Estimations of the molecular weight varied greatly from 22KDa to 158 KDa, possibly because the protein interacted with the matrix of the gel exclusion chromatography column and because multiple forms of the enzyme were present. The partially purified feruloyl esterase had an apparent K_m of 0.46% feruloyl glycerol. However more than one enzyme may be present and the substrate contains two isomers and so Michaelis-Menten kinetics may not be appropriate.

Key words: Barley malt, ferulic acid esterase, partial purification.

INTRODUCTION

The purification of feruloyl esterase from malted barley was undertaken to open the way for investigating enzyme degradation of arabinoxylans in malting barley and in mashing. Arabinoxylans are a major constituent of the cell walls of the aleurone layer (about 85%) and starchy endosperm (about 23%)⁸. These pentosans are substituted with ferulic and acetic acids joined to the polysaccharides by ester links. During malting, channels are digested through the cell walls of the aleurone layer to release enzymes into the starchy endosperm^{14,15,31} and the cell walls of the starchy endosperm are progressively degraded²⁴.

It is believed that the feruloyl and acetyl substituents impede the breakdown of the polysaccharide by carbohydrases. It is likely therefore that the removal of the aryl substituents is necessary for the breakdown of the cell wall pentosans. The cell walls of the aleurone layer, the crushed cell layer, the sheaf cells and some parts of the embryo fluoresce strongly in ultraviolet light, while the

cell walls of the starchy endosperm fluoresce less strongly^{2-4,8,11}. The fluorescence is due to the ferulic acid (4-hydroxy-3-methoxy cinnamic acid), which is attached to the C-5 position of some arabinose residues^{1,25}.

Evidence for the action of ferulic acid esterase activity in the germination stage of malting is lacking, but ferulic acid release has been detected during kilning²¹ and mashing^{22,27} when the optimum temperatures were 44–45°C^{23,27}. Ferulic acid occurs in beer²², it may give rise, via decarboxylation, to 4-vinyl guaiacol, a strongly flavoured substance^{9,13,20,26,28,30}. When isolated barley aleurone layers were incubated with gibberelic acid some cell wall degradation occurred, but the ferulic acid was still attached to arabinoxylan oligomers^{15,16}, and so in this case ferulic acid esterase was not active. Both soluble and insoluble esterases have been detected in barley¹⁸. The work reported here has been carried out on the soluble material.

Feruloyl esterase has been detected in barley malt^{17,29} and an extract of this malt released ferulic acid from 'spent barley'⁶. Several ferulic acid esterases have been detected. A purified enzyme from *Aspergillus niger* releases ferulic acid from ground barley malts⁵. The enzyme in the malt extract released only a small proportion of the total extractable ferulic acid but when a xylanase from *Trichoderma viride* was added the release increased substantially. This synergism has also been noticed with mixtures of microbial enzymes¹² and may indicate that feruloylated oligomers are preferable substrates to the 'parent' polysaccharides.

MATERIALS AND METHODS

Barley samples

Pipkin barley (1996 harvest) was supplied by Carlsberg-Tetley, Burton-on-Trent.

Reagents

DEAE cellulose was obtained from Sigma and Sephacryl S-200HR was from Pharmacia Biotech. All other reagents were of high quality grades.

Extraction of ferulic acid esterase from barley malt

Extraction was achieved using the method previously described by Humberstone and Briggs¹⁸. Barley malt (3g) was ground and then finely dispersed with a Polytron homogeniser with extraction buffer (20mL; tris(hydroxymethyl)aminomethane hydrochloride [Tris·HCl], 50mM, pH 8.0; reduced glutathione, 25mM; Triton-X-100, 1%

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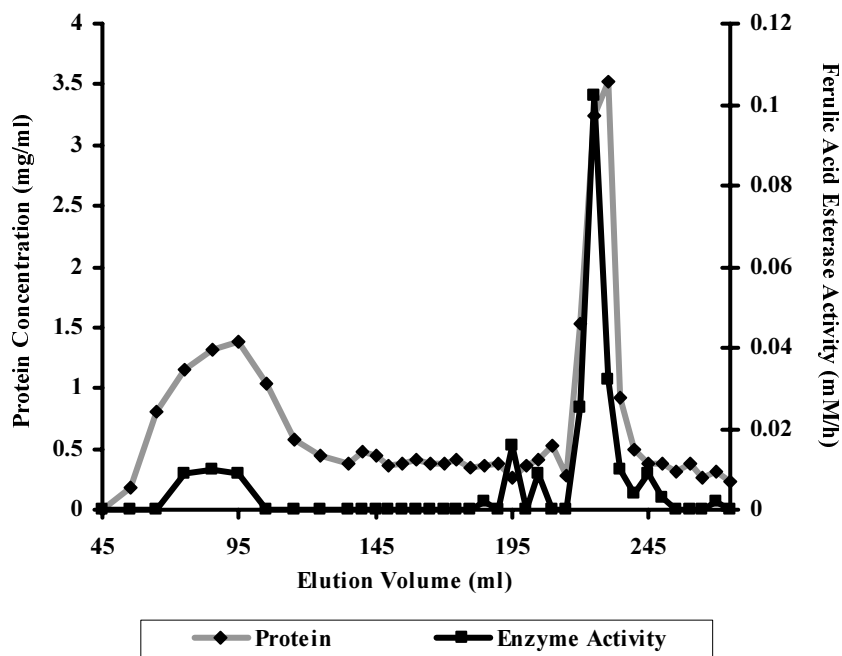


FIG. 1. Purification of feruloyl esterase from barley malt using batch-elution anion exchange chromatography with DEAE cellulose as an initial step. Crude enzyme was bound to the column at pH 6.0 and eluted with buffer containing 0.5M NaCl. The elution profile shows release of enzyme activity and protein during the wash step and then the protein elution.

(w/v); polyvinylpolypyrrolidone [PVPP], 0.2g). The extract was centrifuged to remove residual solids.

The estimation of soluble ferulic acid esterase

Ferulic acid esterase was measured using the method previously described¹⁸. Feruloyl glycerol was used as the substrate and the ferulic acid released during incubation was estimated using high performance liquid chromatography (HPLC). Enzyme activity was defined as the increase in ferulic acid concentration per hour (mM/h) in the standard reaction mixture. Specific activity is enzyme activity per mg of protein (mM/h/mg).

Protein estimation, micromalting and gel electrophoresis

These were carried out as previously described¹⁹.

Ion exchange chromatography

DEAE cellulose was used as a batch treatment for the initial stage of purification. DEAE cellulose (fibrous form) was swollen in piperazine buffer (50mM, pH 6.0) and packed into a Quickfit chromatography column (2cm × 40cm) to a volume of 90mL. The column was rinsed with the same piperazine buffer and then with lower strength piperazine buffer (10mM, pH 6.0). Crude enzyme extract was dialysed against piperazine buffer (10mM, pH 6.0) and 50mL was applied to the column, which was then washed thoroughly with piperazine buffer (150mL; 10mM, pH 6.0). Enzyme was eluted with buffer containing salt (125mL; piperazine, 10mM, pH 6.0; NaCl, 0.5M). Fractions (5mL) were collected and assayed for feruloyl esterase activity and protein concentration. Active fractions were pooled.

DEAE cellulose gradient elution chromatography was used for the further purification of ferulic acid esterase. Enzyme preparation from the batch preparative column was dialysed against buffer (2l; piperazine, 10mM, pH 6.0) to remove salt. A DEAE cellulose column (1.6cm × 40cm) of volume 100mL was prepared in buffer (piperazine, 10mM, pH 6.0). Dialysed enzyme (40mL) was applied to the column at a flow rate of 1mL/min. The column was washed with buffer (83mL). Then a salt gradient (63mL; 0–0.5M NaCl in buffer) was applied to the column and 3mL fractions were collected. The gradient was followed by elutions with 0.5M NaCl (39mL) and finally 2M NaCl (55mL) to ensure that all protein was removed. Fractions were assayed for feruloyl esterase activity. Active fractions were pooled.

Gel filtration chromatography

This was carried out as previously described¹⁹.

RESULTS

Partial purification of ferulic acid esterase

Batch-elution anion exchange chromatography was successful in removing the coloured material and reducing the viscosity of the barley malt extract. The resulting extract was clear, colourless and not noticeably viscous. Fig. 1 shows a typical elution profile. Some minor peaks of activity were also detected, suggesting multiple enzymes, but only the major peak was pooled and progressed for further purification.

DEAE cellulose gradient elution chromatography was used as the second step of the purification. A distinct main

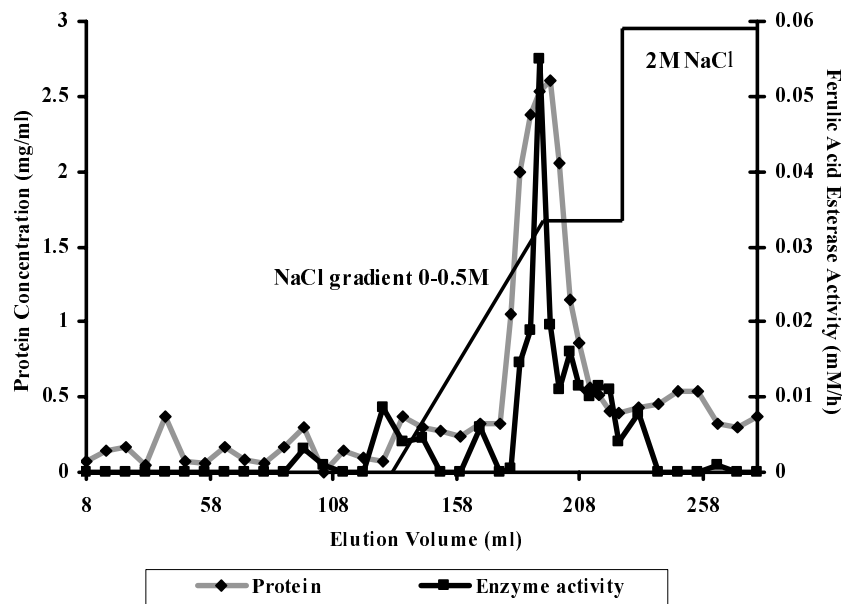


FIG. 2. Purification of ferulic acid esterase from barley malt, using anion exchange chromatography as a second step. Enzyme, partially purified on a DEAE cellulose column using batch-elution was bound to the column at pH 6.0 and eluted at pH 6.0 with a gradient of 0–0.5M NaCl. The elution profile shows release of enzyme activity and protein during the wash and elution steps.

peak of enzyme activity was eluted, well separated from much of the remaining protein (Fig. 2). Some minor peaks were also detected.

Gel filtration was used as the third step in the purification. Previous gel filtration experiments used to purify acetyl esterase from barley malt¹⁹ had shown that the best running buffer for the column contained both glucose (0.5M) and NaCl (2.5%). These additions had reduced interactions of the extract proteins with the column matrix. A elution profile is shown in Fig. 3, total activity measured across all fractions was used to calculate enzyme recovery.

Two purification procedures were completed and estimates were made of the molecular weights of the active fractions for the gel filtration runs (Table I). A partial purification of feruloyl esterase from barley malt is summarised in Table II.

TABLE I. Estimated molecular weights of feruloyl esterases, determined from gel filtration results.

Purification run	Estimated molecular weights ^a (kDa)		
	1	138	119
2	158	138	25 22

^aFigures in bold refer to the MW calculated from the most active fraction for each gel filtration run.

TABLE II. Purification of ferulic acid esterase from barley malt.

Purification step	Total protein (mg)	Total activity (mM/h)	Specific activity (mM/h/mg)	Purification (%)	Recovery
Extraction	681	47.2	0.069	1	100
DEAE cellulose 1	82.5	14.1	0.171	3.96	50
DEAE cellulose 2	13.8	2.74	0.199	2.88	5.8
Gel filtration	0.308	1.96	6.36	148	4.2

Determination of apparent Km

The apparent Km values for the partially purified feruloyl esterase was estimated from Hanes-Woolf plots (Fig. 4) to be 0.46% feruloyl glycerol.

DISCUSSION

The initial barley malt extract was highly coloured and viscous. The initial batch-elution anion exchange chromatography was efficient at removing both the coloured and viscous materials. Enzyme recovery was low (50%) which was probably because only the major peak was recovered. The gradient-elution anion exchange column was expected to produce a greater purification than that achieved during the first step. However this did not appear to be the case (Table II). The average purification after the second step was 2.9 (n = 2) compared to 5.2 (n = 2) for the first step. In two trials there was less purification achieved than after the initial DEAE cellulose column separation. The recoveries also decreased significantly after this chromatography step (which gave an approximate 89% loss), indicating that the enzyme had either been lost, had been inactivated or that a synergistically acting enzyme had been removed. It is unlikely that any enzyme remained bound to the column material as this was washed with a

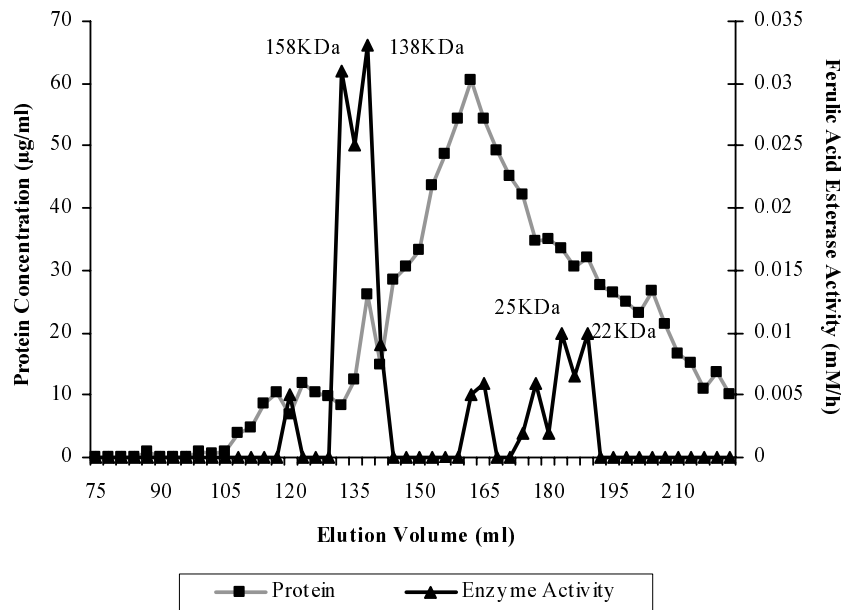


FIG. 3. Purification of feruloyl esterase by gel filtration using Sephacryl HR-200. Enzyme partially purified using anion exchange chromatography with DEAE cellulose was applied to the column with buffer (Tris\HCl, 10mM, pH 7.5) containing glucose (0.5M) and NaCl (2.5%). The elution profile shows the release of enzyme activity and protein. The estimated molecular weights for the elution volume of feruloyl esterase are indicated.

strong salt solution and no enzyme was detected in the eluate.

Gel filtration chromatography of the feruloyl esterase enzyme gave several peaks of activity (Fig. 3) indicating the presence of enzymes having different molecular weights. The estimated molecular weights ranged from 158kDa to 22kDa although the most active peak in both

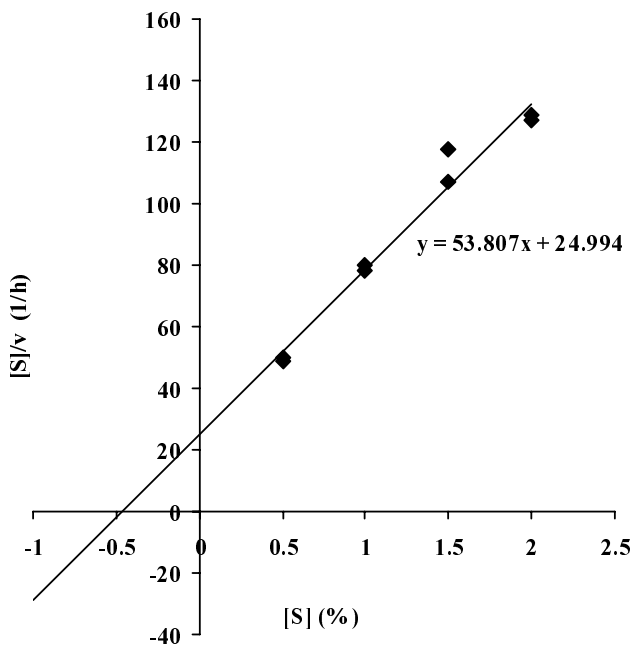


FIG. 4. Hanes-Woolf plot for partially purified ferulic acid esterase from barley malt. Calculation of the intercept on the negative substrate axis allows the estimation of the K_m .

cases corresponded to a molecular weight of 138kDa. Microbial feruloyl esterases that have been purified varied in molecular weight from 24kDa (FAE-II from *Neocallimastix* strain MC-27) to 132kDa (FAE-I from *Aspergillus niger*¹⁰). In the case of *Aspergillus niger* three feruloyl esterases have been characterised and their estimated molecular weights are 132kDa (FAE-I), 29kDa (FAE-II) and 36kDa (FAE-III)^{10,11}. This suggests that such a variation in molecular weights is not unusual.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) gels of active feruloyl esterase fractions showed that a progressive purification has been achieved. However after gel filtration the protein concentration was very low and even with silver staining, detecting bands on a gel was difficult. In order to progress this work it would be advantageous to develop a suitable method for detecting the native esterase enzyme on the gel. The K_m determined is reduced in value by the use of a mixed substrate (1-feruloyl glycerol and 2-feruloyl glycerol) and the fact that multiple forms of the enzyme appear to be present.

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