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A CYSTEINE ENDOPEPTIDASE FROM BARLEY MALT WHICH DEGRADES HORDEIN

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Key Word Index—*Hordeum vulgare*; Gramineae; barley malt; cysteine endopeptidase; purification; hordein degradation; substrate specificity.

Abstract—A cysteine endopeptidase of M_r 29 000 which we have named malt endopeptidase-1 (MEP-1) was purified to homogeneity from a four-day green malt of barley (*Hordeum vulgare* cv Schooner). It consists of two main species of p*I* 4.2 and 4.3, has a pH optimum of 4.5 for the hydrolysis of hordein and accounts for over a half of the hordein degrading activity in the malt. It is inhibited by *p*-chloromercuriphenolsulphonic acid and leupeptin. MEP-1 also hydrolyses haemoglobin and azocasein and while the activity on the latter is stimulated two-fold by 5 mM 2-mercaptoethanol (2-ME) the hydrolysis of hordein is increased 11-fold at this concentration of the thiol. MEP-1 hydrolysed a range of *N*-*t*-butoxycarbonyl-L-amino acid-*p*-nitrophenyl esters; the highest activity was obtained with the derivatives of glutamine, alanine and leucine. A polyclonal antibody to MEP-1 cross reacted with a 37 000 M_r endopeptidase present at low activity.

INTRODUCTION

Hydrolysis of starch and protein in the endosperm of the barley grain during germination is the basis of malting and brewing processes. Compared with our knowledge of the amylases which degrade starch [1, 2] and the involvement of *endo*-β-glucanases in the dissolution of β-glucan in the endosperm cell wall [3] we have a poor understanding of the degradation of the protein reserves [4]. Alcohol soluble hordeins comprise the main protein component in the barley grain [5].

Enari and Mikola [6] established that cysteine dependent enzymes accounted for more than 90% of the endopeptidase activity in green malt. In subsequent studies a range of species were identified by ion exchange chromatography [7, 8] and isoelectric focusing [9] while Baxter [10] found that the main endopeptidase was acidic and did not bind to a cation exchange column at pH 3.8. Estimates of the M_r values of the endopeptidases by gel filtration varied considerably [9, 10]. None of these endopeptidases were purified to homogeneity, and with the exception of the work of Baxter, hordein was not tested as substrate. The malt carboxypeptidases have been more thoroughly characterized [11, 12].

Recently there has been a renewed interest in the elucidation of the properties of the endopeptidases in the germinating barley grain [13–15] and Koehler and Ho [15] have identified two main endopeptidase fractions, which degrade haemoglobin and azocasein, with M_r values of 37 000 and 30 000. Both appeared to be cysteine dependent enzymes and the larger species, which was well characterized, was considered to be the main endopeptidase secreted by embryo-less half seeds supplied with gibberellic acid. We have studied the endopeptidase activity in green malt where the predominant endopeptidase appears to be similar to the smaller species identified by Koehler and Ho [15]. The isolation and characterization of this endopeptidase including its degradation of hordein, is described in this paper.

RESULTS

Purification of malt endopeptidase-1

The maximum recovery of endopeptidase activity measured with hordein, azocasein or haemoglobin as substrate (Table 1) was obtained with an extraction medium of pH 4.0–4.5. When crude extract prepared at pH 4.5 was dialysed overnight at pH 6.0 there was a loss of about a half of the hordein degrading activity.

Passage of the crude extract through a Sephadex G-25 column resulted in a small increase in endopeptidase activity and the separation of all the peptidase activity from two large fractions of UV absorbing material. Most of the activity recovered after ammonium sulphate precipitation (*ca* 70%) was in the fraction obtained with 35–70% saturation (Table 2).

Table 1. Influence of pH on the extraction of endopeptidase activity from green malt

Measurement	pH of extraction medium* 3.0	4.0	4.5	6.0
Protein (mg/ml)	0.50	1.53	1.45	1.84
Hordein degradation†	5.67	11.86	10.32	5.94
Azocasein degradation†	0.73	2.47	4.04	3.06
Haemoglobin degradation†	8.35	16.20	18.24	11.86

*Crude extracts prepared at the pH indicated were dialysed for 16 hr against 20 mM sodium acetate pH 4.5 containing 50 mM NaCl and 1 mM 2-ME.

†Units/ml as defined in Experimental.

Table 2. Purification of malt endopeptidase-1 from four-day barley malt

Purification step		Protein (mg)	Activity (units)*	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
	Crude extract	343	510	1.48		100
(1)	Sephadex G-25	302	536	1.77	1.2	105
(2)	$(NH_4)_2SO_4$ pellet	190	394	2.08	1.4	77
(3)	Sephacryl S-200	150	390	2.6	1.8	76
(4)	CM-Sephadex	18	184	10.2	6.9	36
(5)	Mono-Q	1.1	66	59.4	40	13

*Hordein degrading activity was measured as described in the Experimental

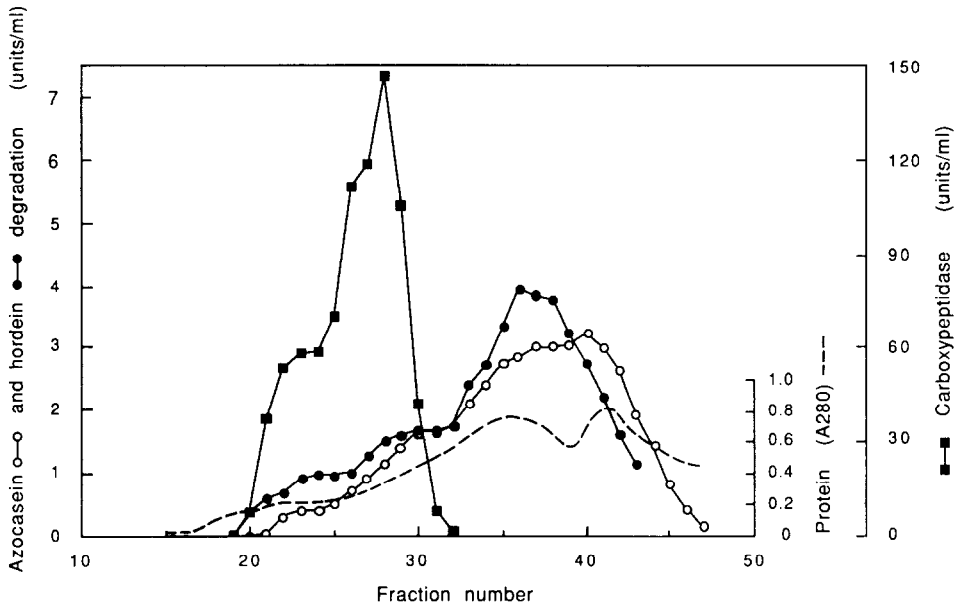

Fig. 1. Separation of carboxypeptidase and endopeptidase activity on a Sephacryl S-200 column. The units of enzyme activity are described in the Experimental. Fractions 32–43 were pooled for further study.

When this fraction was separated on a Sephacryl S-200 column (Fig. 1) the hordein and azocasein degrading activities showed similar elution patterns with 85% of the activity eluting after the carboxypeptidase activity. Haemoglobin degradation differed in that a higher level (30% of the total recovered) eluted with the carboxypeptidase (data not shown). The endopeptidase fraction selected for further study eluted after ovalbumin (M_r 45 000).

A major proportion of the endopeptidase activity (65% of that recovered) did not bind to CM-Sephadex equilibrated with 0.1 M acetate pH 4.5 containing 50 mM sodium chloride and 1 mM 2-ME (Table 2, step 4). The bound fraction was eluted when the sodium chloride concentration of the buffer was increased to 200 mM and the pH adjusted to 5.2. Preliminary fractionation and inhibitor studies indicate that it consisted of at least three endopeptidase species, one of which is sensitive to pepstatin A (data not shown).

When the fraction that did not bind to the CM-Sephadex column was chromatographed on a Mono Q (anion exchange) column at pH 5.3 (Fig. 2) all the endopeptidase activity was retained and the main fraction (97% of that recovered) was consistently eluted in a double peak with 45–60 mM sodium chloride. Attempts to separate the peaks further were unsuccessful. Each peak was homogeneous as judged by SDS-PAGE (Fig. 3) and migrated as a single species with an apparent M_r of 29 000 ± 500. We have designated this fraction malt endopeptidase-1 (MEP-1). A minor peak of endopeptidase activity (3% of that recovered) eluted from the Mono Q column with 250 mM sodium chloride (Fig. 2) and was estimated to have a M_r of 37 000 by SDS-PAGE (Fig. 3).

Isoelectric focusing showed that MEP-1 consisted of two main components of p*I* 4.2 and 4.3 and a minor component of p*I* 3.8 (data not shown). A sample of homogeneous MEP-1 which had been kept at −15° for two months showed only faint bands at p*I* 4.2 and 4.3 and a more pronounced band at p*I* 3.8. This sample still migrated as a single band of M_r 29 000 on SDS-PAGE and retained its original activity.

The overall purification of MEP-1 achieved (Table 2) was 40-fold and the yield 13%. Initial extraction at pH

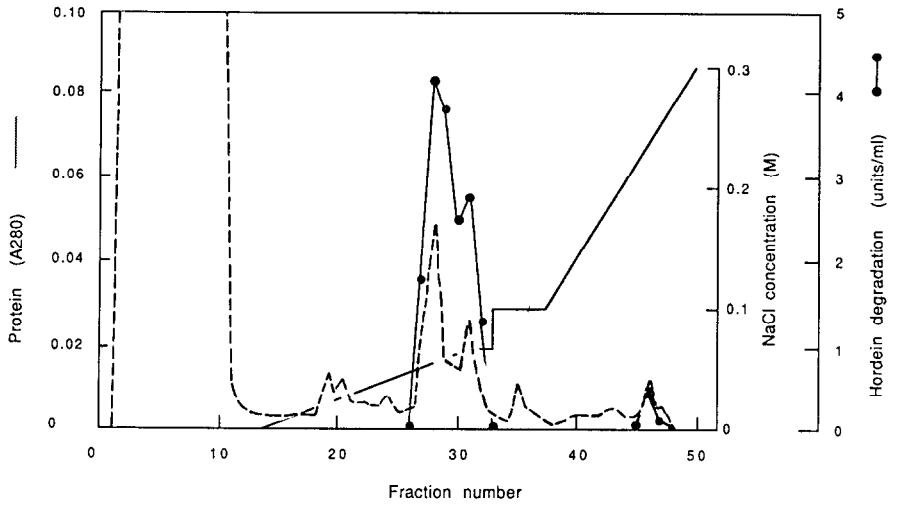

Fig. 2. Final purification of the main endopeptidase fraction on a FPLC-Mono Q column. The elution procedure is described in the Experimental.

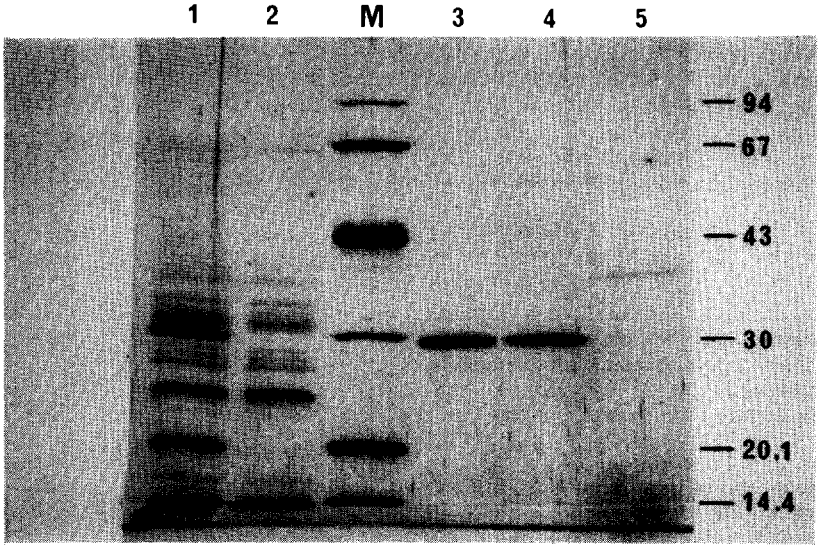

Fig. 3. SDS-PAGE of the main endopeptidase fractions isolated on the Mono Q column. Lane 1, sample loaded; lane 2, unbound fraction; lane M, M_r markers ($M_r \times 10^{-3}$ indicated in margin); lanes 3 and 4, peak fractions of MEP-1 (28, 31); lane 5, fraction 46.

4.5 resulted in a four-fold lower protein yield than in an extract at pH 7.5. Thus the overall purification obtained is underestimated by this factor. Other endopeptidases also contributed to the activity measured in the preliminary steps of the purification procedure.

Properties of malt endopeptidase-1

On an SDS-PAGE gel stained with periodic acid Shiff's reagent, MEP-1 was detected as a pink band, indicating that it is a glycoprotein. The pH optimum of MEP-1 activity on hordein was 4.5, on azocasein, 5.5 and on haemoglobin 4.0. MEP-1 can be stored at $-15°$ in 10% (v/v) glycerol and 1 mM 2-ME for at least three months without loss of activity, and at $-70°$ for at least six months under the same conditions. At 4° there is a gradual loss of activity accompanied by some autolysis. It is very labile above pH 6.5.

The activity of MEP-1 was tested on a range of *N-t*-butoxycarbonyl-L-amino acid-*p*-nitrophenyl esters (Boc-amino acid-ON_p). Highest activity was obtained with the glutamine, alanine and leucine esters (Table 3). No activity was obtained with *N*-carbobenzoxy(Cbz)-proline-ON_p and Cbz-benzyl cysteine-ON_p or with benzoyl arginine ethyl ester, acetyl tyrosine ethyl ester and benzoyl arginine *p*-nitroanilide (data not shown).

The most effective active site inhibitors of MEP-1 (Table 4) were leupeptin and *p*-chloromercuriphenolsulphonic acid (*p*CMPS), the latter being a specific inhibitor of cysteine dependent endopeptidases. Inhibition by

Table 3. Hydrolysis of amino acid esters by malt endopeptidase-1

Substrate*	Activity (units/mg protein)
Boc-glutamine-ON_p†	12.7
Boc-alanine-ON_p	9.4
Boc-leucine-ON_p	7.4
Boc-tyrosine-ON_p	3.6
Boc-tryptophan-ON_p	3.3
Boc-asparagine-ON_p	1.0
Boc-phenylalanine-ON_p	0.5

*Each substrate was tested at a final concentration of 10 μM in 0.2 M sodium acetate (pH 4.5) as described in Experimental.

†Boc = *N*-*t*-butoxycarbonyl; ON_p = *p*-nitrophenyl ester.

Table 4. Effect of inhibitors on malt endopeptidase-1

Inhibitor	Concentration*	Activity†%
Leupeptin	20 μM	4
p-Chloromercuriphenol sulphonic acid	250 μM	0
N-Ethylmaleimide	5 mM	10
$HgCl_2$	1 mM	0
Lodoacetate	1 mM	10
Phenylmethylsulphonyl fluoride	1 mM	100
EDTA	10 mM	100
o-Phenanthroline	1 mM	114
Pepstatin A	5 μM	100

*Purified MEP-1 (free of 2-ME) was preincubated at 37° for 15 min with the inhibitor at the concentration indicated, as described in the Experimental.

†Hordein-degrading activity.

*p*CMPS but not leupeptin was reversed by 2-ME. *N*-Ethylmaleimide, mercurous chloride and iodoacetate were also inhibitory. Inhibitors of the other three types of endopeptidases, phenylmethylsulphonyl fluoride (serine), EDTA and *o*-phenanthroline (metal) and pepstatin A (aspartic acid), did not inhibit MEP-1.

The activity of MEP-1 on hordein was stimulated 11-fold by 5mM 2-ME (Fig. 4). The degradation of azocasein and Boc-glutamine-ONp was only enhanced about two-fold and increase in 2-ME concentration from 1 to 5 mM had a relatively small effect.

Preliminary studies with a polyclonal antibody to malt endopeptidase-1

The antibody to MEP-1, which gave a strong reaction to the MEP-1 fraction from the Mono-Q column used as antigen, also recognized the M_r 37000 endopeptidase species recovered from this column (Fig. 5). Several cross-reacting species were detected in the adsorbed fraction from the CM-Sephadex column. No additional cross-reacting species were detected in the large M_r fraction of the Sephacryl column (Fig. 1).

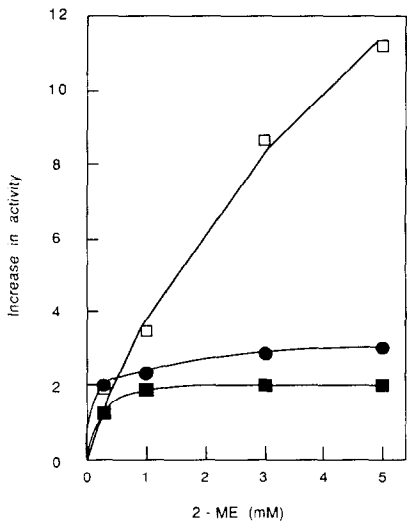

Fig. 4. Influence of 2-ME on the activity of malt endopeptidase-1. Hordein degrading activity (□—□), azocasein degrading activity (●—●) and hydrolysis of BOC-glutamine-ON_p (■—■) were assayed in the presence of the levels of 2-ME shown.

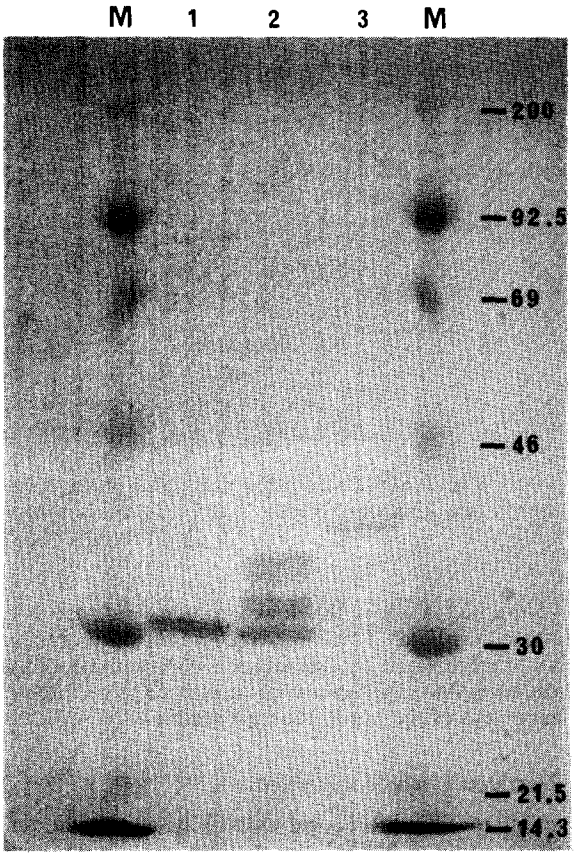

Fig. 5. Immunoblot of proteins detected by antibody to malt endopeptidase-1. Lane M, M_r markers ($M_r \times 10^{-3}$); lane 1, MEP-1; lane 2, adsorbed fraction from CM-Sephadex column; lane 3, 37000 M_r endopeptidase from Mono Q column.

DISCUSSION

Cysteine endopeptidases are considered to be the most important enzymes in the degradation of the protein reserves in the barley endosperm [6]. These consist mainly of the alcohol soluble prolamins referred to as hordeins [5]. We have purified a cysteine dependent endopeptidase (MEP-1) from a four-day green malt of barley which accounts for over 50% of the hordein degrading activity. Its pH optimum on hordein is 4.5, close to that of the starchy endosperm (4.9–5.1) [16].

MEP-1 has a M_r of 29 000 and appears to be the same enzyme studied by Baxter [10] and Jones and Poulle [14] who estimated M_r values of 28 000 and 30 000 respectively. Koehler and Ho [15], who recently purified an endopeptidase from germinating barley seeds with a M_r of 37 000, also reported the presence of a 30 000 M_r endopeptidase. Cysteine endopeptidases characterized in wheat [17], rice [18] and maize [19] appear to have lower M_r values, between 21 and 23 000, but the wheat and maize estimates were based on gel filtration which we found to give lower values for MEP-1 than that determined by SDS-PAGE.

MEP-1 is a glycoprotein as are carboxypeptidases I–III [12, 20], the two 1,3; 1,4-β-glucan endohydrolases [3] and α-amylase II, [21] of germinating barley. It is likely that all these hydrolases are synthesized in the aleurone or scutellum before secretion into the endosperm.

We have shown that MEP-1 is the main endopeptidase in four-day green malt while Koehler and Ho [14] found that the 37 000 M_r endopeptidase was the most abundant species secreted by embryo-less half seeds of barley. The activity gel they used, containing haemoglobin as a substrate, would have underestimated the amount of MEP-1 as this enzyme is very labile at the pH of the gel (8.3). We found that the 37 000 M_r endopeptidase accounted for only 3% of the total hordein degrading activity in four-day intact seedlings. The difference in the amount of these two endopeptidases could be due to choice of cultivar and that Koehler and Ho studied the endopeptidases secreted into the medium from embryo-less half seeds. The proportion of α-amylase isozymes 1 and 11 produced by embryo-less half seeds of barley also differed from that of germinated intact seeds [22].

When purified to homogeneity, MEP-1 consisted of two main species that were only partly separated by ion exchange chromatography. We estimated their p*I*s by isoelectric focusing to be 4.2 and 4.3, which is in agreement with Baxter [10] who found the main endopeptidase fraction to have an asymmetric profile with an average p*I* of 4.3. A minor component which we observed with a p*I* of 3.8 appears to be formed during storage. Koehler and Ho [15] found that the 37 000 M_r species comprised three isozymes differing in a small number of amino acids at the amino terminal end. Differences in amino acid composition could also account for the charge difference between the two forms of MEP-1. Alternatively there could be differences in their carbohydrate content, as has been suggested for carboxypeptidase 1 from malted barley which consists of two species of p*I* 5.65 and 5.73 [23].

Cysteine endopeptidase activity is stimulated *ca* two-fold by exogenous thiols [13, 18]. This has been demonstrated for MEP-1 using azocasein or Boc-glutamine-ON_p as substrate. However, the hydrolysis of hordein by MEP-1 was stimulated more than 10-fold by 2-ME. The B-hordeins, which are the main component (80–90%) of the prolamin reserve in barley, have a high cysteine content contributing to intermolecular and intramolecular disulphide bonds [5]. It appears that the degree of reduction of the sulphydryl groups in the hordein, and perhaps its conformation, influence its susceptibility to MEP-1.

MEP-1 exhibited a fairly broad specificity for amino acyl groups at its active site. We have shown that it hydrolyses a range of amino acid esters while Jones and Poulle [14], who have isolated what appears to be the same endopeptidase species, found that it cleaved a range of small proteins at a number of sites. We found that MEP-1 had some specificity for glutamine at its active site, and Jones and Poulle [14] demonstrated that two peptide bonds on the carboxy terminal side of cysteinyl residues of α1-purothionin were most susceptible to hydrolysis by this endopeptidase. A characteristic of the hordeins is their high content of glutamine, and the main hordein fraction is also rich in cysteine residues [5]. Hordeins also have a high content of proline but we found that MEP-1 had no activity on the proline ester.

A polyclonal antibody to MEP-1 also recognises the 37 000 M_r endopeptidase and five proteins in the fraction which adsorbed to the CM-Sephadex column. These species must have a high degree of homology of tertiary structure with MEP-1 and since we have observed that 30% of the hordein degrading activity is in this fraction and is due to several endopeptidases, then some or all of the cross-reacting species are likely to be endopeptidases similar to MEP-1. The two 1,3;1,4-β-glucanases in germinating barley share common antigenic determinants [3].

This study shows that MEP-1 accounts for most of the endopeptidase activity in a four-day green malt ($\sim$60%), with other cysteine dependent endopeptidases responsible for some of the remainder. Isolation and characterization of all these endopeptidases, especially their substrate specificities is required before an assessment can be made of their relative importance in the degradation of protein during malting.

EXPERIMENTAL

Plant material and malting procedure. Hordeum vulgare cvv. Schooner and Clipper were supplied by the Agronomy Department of the Waite Agricultural Research Institute. Seeds of Schooner were surface-sterilized by treatment for 5 min in NaClO (6% available chlorine) followed by several rinses in sterile H_2O and then germinated on sterilized moist paper in petri dishes in the dark at 15° and 85% relative humidity. The green malt was harvested at 4 days, when the rootlets were 1–2 cm and the coleoptile *ca* 0.5 cm.

Preparation of crude extract. The extraction medium was 0.1 M NaOAc pH 4.5 containing 2 mM 2-ME and 2.5% (w/v) insoluble PVP. Freshly harvested malt was macerated for three 0.5 min periods in a Sorvall Omni-mixer with 2 ml chilled extraction medium per g tissue fresh weight and the mixing container held on ice. The extract was mixed for a further 1 hr, at 4° and then centrifuged at 20 000 *g* for 10 min. (All centrifugation steps were at 2°.)

Purification. Preliminary stages of purification were undertaken at 4°. The supernatant from above (200 ml) was loaded on a Sephadex G-25 column (3.3 × 105 cm) equilibrated and eluted with 0.1 M acetate pH 4.5 containing 1 mM 2-ME. The eluent

collected (300 ml) was equivalent to the first main *A* peak (280 nm).

$(NH_4)_2SO_4$ (194 g/l) was added to the pooled fractions from the Sephadex G-25 column to give 35% saturation. The sample was then stirred for 30 min at 4° and centrifuged at 20 000 *g* for 10 min. Additional salt (218 g/l) was added to the supernatant to give 70% saturation and after stirring and centrifugation as above the ppt. was resuspended in 0.1 M NaOAc pH 4.5 containing 50 mM NaCl and 1 mM 2-ME. The final vol. was *ca* 20 ml.

14 ml of redissolved $(NH_4)_2SO_4$ ppt. was loaded onto a Sephacryl S-200 column (2.2 × 85 cm), equilibrated and eluted with 0.1 M NaOAc pH 4.5 containing 50 mM NaCl and 1 mM 2-ME. Endopeptidase fractions pooled were concd to 0.3 vol. with an Amicon ultrafiltration cell using a YM 10 membrane at 350 kPa. A 2.1 × 3 cm column of CM-Sephadex C-50 was equilibrated with the Sephacryl S-200 buffer and 25 ml of the concd sample from the Sephacryl column loaded. The column was washed with the above buffer and finally eluted with 0.1 M NaOAc pH 5.2 containing 1 mM 2-ME and 0.2 M NaCl.

The final stages of the purification were undertaken on a Pharmacia FPLC system at room temp. The endopeptidase fraction which was not adsorbed to the CM-Sephadex column was dialysed against 20 mM piperazine–HCl pH 5.3 containing 1 mM 2-ME and 10% (v/v) glycerol and centrifuged at 10 000 *g* for 10 min. It was loaded on a Mono Q (HR 5/5) column which had been equilibrated with the above buffer. The NaCl concn was increased from 0 to 60 mM over a period of 30 min, then held at this concn until the A_{280} decreased to the baseline. A step of 0.1 M NaCl was followed by an increase in salt to 0.3 M over 25 min.

Enzyme assays. The ninhydrin procedure for determination of α-amino N, the assay of azocasein and haemoglobin degradation and carboxypeptidase assay with *N*-benzoyloxycarbonyl-L-phenylalanyl-L-alanine have been described previously [24]. Azocasein degradation was measured at pH 5.5 in 0.1 M citrate phosphate buffer with 5 mM 2-ME. Haemoglobin (1 mg) was incubated in 0.3 ml 0.1 M NaOAc pH 4.0 and the reaction terminated with an equal vol. of 10% (w/v) TCA. All assays were performed at 37°, usually 2 hr with azocasein and haemoglobin and 0.5 hr for carboxypeptidase. The units used are as follows: carboxypeptidase, μmol alanine released/hr; haemoglobin degradation, μmol α-amino N released/hr and azocasein degradation, ΔA_{440}/hr.

Assay of hordein degradation. A hordein sample was extracted from pearled barley of the cultivar Clipper with 50% (v/v) *iso*-PrOH containing 0.6% (v/v) 2-ME. This was precipitated by exhaustive dialysis against H_2O and the sample finally lyophilized. A 0.1 ml aliquot of a suspension of hordein (50 mg/ml) in H_2O was incubated at 37° with 0.1 ml, 0.2 M NaOAc pH 4.5 and 0.1 ml enzyme. 2-ME was included in the assay to give a final concn of 5 mM. The reaction was stopped after 1 hr by the addition of 0.3 ml 10% (v/v) TCA, and after 10 min on ice the mixture was centrifuged and 0.2 ml of the supernatant tested by the ninhydrin procedure [24]. One unit is equivalent to 1 μmol α-amino N released/hr.

Hydrolysis of ester derivatives of amino acids. The reaction mixture (1 ml) contained 0.2 M NaOAc pH 4.5, 5 mM 2-ME and 0.03 ml 0.33 mm substrate dissolved in MeOH. The A_{322} was measured for 2 min and corrected for any change in the absence of the enzyme. The unit of activity used is ΔA_{322}/min.

Active site inhibitors. iso-PrOH (5% v/v final in assay) was used to dissolve phenylmethylsulphonyl fluoride and *o*-phenanthroline. Pepstatin A was dissolved in MeOH (7% v/v final). Appropriate controls with these solvents indicated no inhibition of enzyme activity.

Electrophoresis. SDS-PAGE was carried out at 25° according to the procedure of ref. [25] using a 10% (w/v) acrylamide slab resolving gel run at 35 mA constant current. Proteins were visualized using the BioRad silver stain kit except that the reducing agent dithiothreitol (5μg/ml) was used in place of the oxidizer.

Western blots [26] were incubated with a specific antibody raised in rabbit to MEP-1 and then incubated with alkaline phosphatase labelled goat anti rabbit IgG $F(ab')_2$ fragment (Sigma). Immunoreactivity was visualized using naphthol AS-BI phosphate/Fast Red TR salt substrate (Sigma). Isoelectric focusing was performed at 4° using 0.4 mm ultrathin gels over a pH range of 3.5–5.0. The anode soln was 0.1 M NaOH, the cathode soln was 0.1 M H_2SO_4 and the gel was focused for 4 000 volt hr.

Protein estimation. Protein concn was measured by the dye binding method [27] using bovine serum albumin as a standard.

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