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PURIFICATION AND PROPERTIES OF AN ENDOPEPTIDASE OF THE DORMANT LUPIN SEED

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Key Word Index—*Lupinus albus*, Leguminosae, dormant seed, endopeptidase, BAPAase; legumin-like proteins; storage proteins

Abstract—An endopeptidase present in the albumin fraction of the dormant lupin seed has been purified over 3000-fold. The preparation was virtually homogeneous on gel filtration, ion exchange chromatography, non-denaturing PAGE and SDS-PAGE

The enzyme consists of one polypeptide chain of M_r *ca* 70 000. It acts on legumin-like proteins of lupin and pea seeds and causes limited proteolysis of their acidic polypeptide(s). The activity becomes apparent after a 24–36 hr incubation at 37°. The enzyme cleaves the α III carboxymethylated subunit of pea legumin stepwise to fragments part of which are below the detection limit of SDS-PAGE. Activity on this substrate and on benzoyl-D,L-arginine-*p*-nitroanilide is manifest straightaway

The enzyme has no effect on other substituted aminoacids, including Leu-*p*-nitroanilide and Ala-*p*-nitroanilide, on synthetic peptides or on aminoacid esters. Its optimal pH is 7.4 with the protein substrate and 8 4 with the synthetic substrate. The enzyme is inhibited by the sulphydryl reagents, leupeptin and salmin.

INTRODUCTION

In legume seeds a number of proteolytic events are involved in the storage of proteins in the protein bodies and in making them available for digestion by proteases at germination [1–3] Enzymes responsible for co- and post-translational proteolysis have not been identified and only a limited number of proteinases present in the resting seed have been described. few of them have been purified [4]. In several instances proteolytic activity was followed using either heterologous or synthetic substrates (for a review see ref [5]) and the purified enzymes failed to cleave storage proteins [6, 7]. The role of most of these enzymes in the proteolytic processing of storage proteins is not clearly understood

Two proteinases have been isolated from germinating lupin seeds. One, which specifically hydrolyses conglutin β (a vicilin-like storage globulin), has an M_r of 27 500 and an optimal pH of 4.0 [8]. The other with M_r *ca* 60 000 and optimal pH 4.5 has been partially purified. it cleaves only chemically modified storage globulins and does not act on the native proteins [9] The limited proteolysis that modifies legumin precursors in developing lupin seeds has been detailed [10], though the enzyme(s) involved have not been identified

In a previous paper, we described the proteolytic degradation *in vitro* of pea legumin, pea vicilin precursor and jack bean vicilin brought about by a partially purified endopeptidase from mature lupin seeds [11] In the present work we have purified the enzyme involved and determined some of its properties.

Abbreviations BAPA benzoyl-D,L-arginine-*p*-nitroanilide; ATEE. *N*-acetyl-L-tyrosine ethyl ester; TAME. tosyl-L-arginine methyl ester, LeuPA. L-leucine-*p*-nitroanilide, AlaPA L-alanine-*p*-nitroanilide; NEM *N*-ethylmaleimide; pHMB *p*-hydroxy mercury benzoate; PMSF phenylmethylsulphonyl fluoride, 2ME 2-mercaptoethanol

RESULTS

Quantitative data on the purification are given in Table 1 The final purification was over 3000-fold based on activity measured on the synthetic substrate Proteolytic activity on storage proteins was not quantified, but was checked at all purification steps. Adsorption on salmine-Sepharose concurs with the finding that salmin inhibits both BAPAase and proteinase activity (see below).

Homogeneity and apparent M_r

Only one protein band was detected in the final preparation by non-denaturing PAGE and SDS-PAGE both under reducing and non-reducing conditions. Trace contamination appeared on ion exchange chromatography and gel filtration (Fig 1). In non-denaturing procedures the protein peak coincided with the site of activity on BAPA.

The polypeptide had on SDS-PAGE an apparent M_r of 77 000 Somewhat smaller figures (63 000 and 68 000) were obtained by gel filtration on Ultrogel, HPLC on Protein Pack 300 SW or FPLC on TSK G3000 SW Technical details are given under Experimental.

Substrate specificity

The proteolysis of lupin legumin as followed by SDS-PAGE is shown in Fig. 2. A 30–40 hr incubation at 37°

Table 1 Purification of lupin proteinase

Purification step	Protein (mg)	Sp activity (BAPA hydrolysed μmol/min mg)	Purification factor	Activity yield
Water extract pH 5 0	3178	0 009	1	100
Gel filtration Ultrogel AcA44	591	0 041	5	86
Ion exchange chrom , DEAE cellulose pH 7 5	98	0 239	27	83
Ion exchange chrom , DEAE cellulose, pH 8 5	9 8	1 510	170	52
Affinity chrom , Salmine-Sepharose 4B	1 5	8 290	930	44
Ion exchange chrom , TSK DEAE 5 PW, FPLC	0 3	29 100	3,270	36

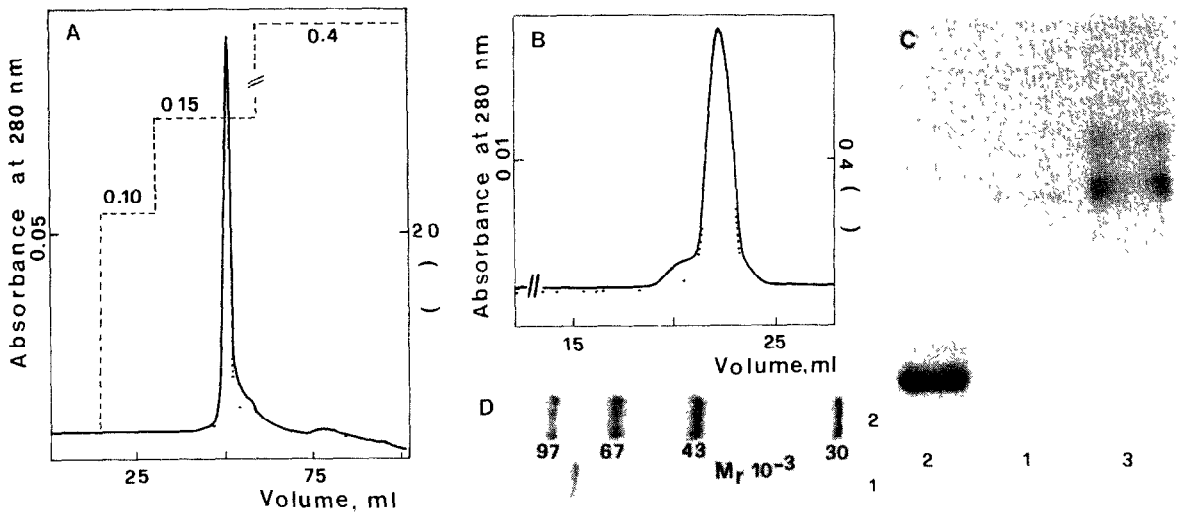

Fig 1 Analysis of the final enzyme preparation (A) FPLC on DEAE 5 PW (7 5 × 0 8 cm column) protein, 1 5 mg, was eluted first with 50 mM Tris HCl buffer, pH 8 5, then NaCl was added at the molar concentrations indicated (– – –) –, A_{280}, activity on BAPA (μmol hydrolysed/ml/min) (B) FPLC on TSK G3000 (30 × 0 8 cm column) protein, 0 25 mg, was eluted with 50 mM Na phosphate buffer, pH 7 5 containing 0 1 M NaCl and 0 02% sodium azide Absorbance and activity as in (A) (C) Non-denaturing PAGE The enzyme 3 μg, was in lane 1 BAPAase activity coincided with the protein band Lane 2 β-lactoglobulin (7 μg), lane 3 haemoglobin (7 μg) (D) SDS-PAGE in the presence of 2% 2 mercaptoethanol lane 1, enzyme (5 μg), lane 2, protein markers (15 μg) as detailed in the text Assay methods are described in the Experimental section

was necessary before the activity of the enzyme became evident on lupin legumin. Its action was then completed within a few hours Only the protomer of M_r 81 000 (unreduced) was visibly attacked, and was transformed into the protomer of M_r 54 000 (unreduced) which is present also in the original legumin. Reducing conditions showed that the acidic polypeptide of M_r 54 000 was the site of action of the protease Indeed the basic chain (M_r 21 000) remained unaffected Some new material (M_r 35 000) was seen under non-reducing conditions, but none appeared when 2-mercaptoethanol was present The legumin was slightly modified when the enzyme was omitted but this did not conceal the effects of the enzyme

Bacterial contamination as a cause of proteolysis was excluded by experiments carried out under sterile conditions and by the differences observed between samples with or without the enzyme

Degradation appeared straightaway and was more rapid and extensive with the carboxymethylated α III subunit of pea legumin, which has no bound basic polypeptide, bound sugar or protein folding. Cleavage occurred stepwise at specific sites Chains of M_r 39 000–36 000 and 15 000 were early major products The chain of M_r 15 000 became one of M_r 14 000 which, in turn, was degraded further However, below this M_r only a faint band at 10 000 was visible as the products were present in too small an amount to be detected or they were cleaved as they formed Polypeptides of M_r 25 000–23 000 were also produced Their rate of appearance/disappearance does not fit well in the pattern out-

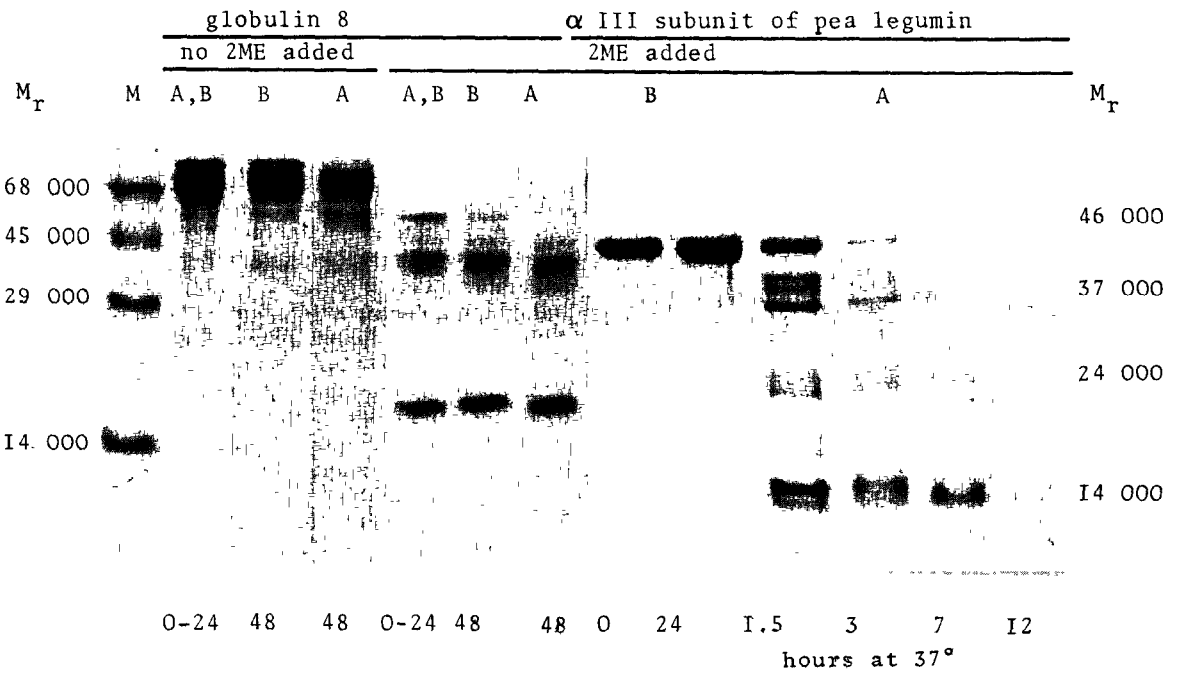

Fig 2 Effect of the purified proteinase on globulin 8 and on the α III subunit of pea legumin The protein substrate, 1 mg, in 0 98 ml 50 mM sodium phosphate buffer, pH 7 5, containing 0 22 M NaCl and 0.05% sodium azide was incubated with (A) and without (B) 20 μl purified lupin proteinase (activity on BAPA 75 nmol hydrolysed/min) Aliquots of the incubation mixture were analysed by SDS-PAGE in the presence or absence of 2% 2 mercaptoethanol (2ME) as indicated Amounts loaded were 15 μg of unreduced or 10 μg of reduced lupin legumin and 15 μg of the α-III carboxymethylated subunit of pea legumin M protein markers

lined above, therefore they may well have been formed by a separate pathway Degradation of substrate in the absence of enzyme was extremely small

The purified enzyme acted also on BAPA and was virtually saturated at 0 33 mM substrate With this substrate activation was unnecessary. The enzyme did not hydrolyse AlaPA, LeuPA, benzoyl-glycine-L-phenylalanine, ATEE, TAME and benzoyl-glycine-L-lysine. Enzymes hydrolysing the first two substrates were present in the unfractionated albumin extract and were separated during purification (Duranti M , unpublished results).

The turnover number calculated with BAPA on the basis of apparent M_r and the measured specific activity was 37 mol of substrate hydrolysed per mol of enzyme per second

Optimal pH

The effect of pH on enzyme activity is shown in Fig. 3 The purified enzyme was active on globulin 8 only between pH 6 0 and 8.5 with the maximum at pH 7.4 Activity on BAPA had a wider pH range with the maximum at pH 8 0 When total albumins acted on BAPA, a flatter curve with a maximum at pH 9 0 was obtained and some activity was present also at pH 4.0. This suggests that in the total extract more than one enzyme acted on BAPA Base catalysed hydrolysis of BAPA above pH 10.0 made it difficult to evaluate the activity of the enzyme In the pH range assayed the molar absorbance at 405 nm of BAPA remained constant.

Inhibition studies

The inhibition pattern of the BAPA-hydrolysing activity of the purified enzyme is given in Table 2 Thiol reagents as well leupeptin and salmin were effective Typical serine proteinase inhibitors such as PMSF and soybean trypsin inhibitor, and EDTA had no effect. Salmin also inhibited the cleavage of lupin globulin 8 (not shown).

DISCUSSION

The presence of only one polypeptide under either reducing or non-reducing conditions and overlap of activity and protein in non-denaturing separation procedures indicate that the enzyme consists of a single polypeptide. The higher apparent M_r measured on SDS-PAGE may be due to reduced electrophoretic mobility of the long unfolded SDS-bound polypeptide. Inhibition by NEM and mercuric chloride indicate the presence of an active cysteine

The information provided by SDS-PAGE indicates that the enzyme is an endopeptidase acting on acidic polypeptides of legumin-like proteins in lupin and in the pea. The extent of cleavage depends on protein folding. Thus cleavage is limited to high M_r acidic polypeptides in native globulin 8 and native pea legumin [11] and is much more extensive and rapid with the isolated carboxymethylated III subunit of the latter protein. The limited number and sharp definition of intermediates and

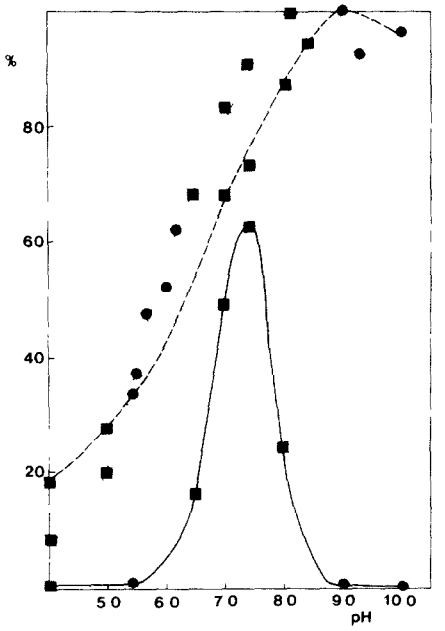

Fig 3 pH activity curve of lupin proteinase – – –, Total albumin extract, — —, a 170-fold purified enzyme preparation, , purified enzyme Activity on (a) lupin globulin 8 (· —) is given as per cent of M_r 81 000 protomer disappearing in 36 hr and (b) BAPA (– – –, ·) as per cent of the highest activity measured Blanks have been subtracted Buffers used were 50 mM Na phosphate (■) and Na pyrophosphate (●) The ionic strength was adjusted at 0 33 M by adding NaCl The pH did not change during the assay Activity on 33 mM BAPA at pH 8.1 was the same in phosphate and pyrophosphate buffer Other conditions as in Fig 2

Table 2 Inhibition of lupin proteinase

Inhibitor	Concentration (mM)	Residual activity on BAPA (%)
N-Ethyl maleimide	0 10	26
	1 00*	10
Mercuric chloride	0 26	0
Tosyllysinemethyl chloroketone	0 05	13
	0 10	0
Leupeptin	0 025	0
PMSF	0 08	100
Salimine	0 005	11
	0 010	8
	0 050	2
Soybean trypsin inhibitor	0 02	100
	0 09	100
EDTA	1	100
	10	95
	10*	97

Activity was measured as described under Experimental

*Activity measured after 5 min preincubation of the enzyme with the inhibitor at 37°

products formed from this substrate indicate that cleavage occurs stepwise at specific sites Formation of small peptides or free amino acids cannot be followed by SDS-PAGE nevertheless, if it occurred, it did not visibly modify the larger fragments originating at each splitting

The incubation period required before activity becomes evident on native proteins is probably due to modifications occurring in the enzyme Indeed when fresh substrate was added to a working enzyme, it was cleaved immediately (Duranti M, unpublished results) This probably reflects the access of substrate to the active site. Indeed the unfolded polypeptide and the small BAPA molecule are cleaved immediately

Homologies with other proteases described in dormant seeds or at early germination are not easily found A BAPAase isolated from *Vicia sativa* has an apparent M_r of 70 000. However, the preparation displayed other bands of comparable intensities on SDS-PAGE and the authors were led to conclude that the enzyme consisted of four M_r 17 000 subunits, that may variably associate [12]

An endopeptidase acting on BAPA, of similar M_r, pH optimum and high affinity for arginine residues as the lupin enzyme has been isolated from buckwheat (*Fagopyrum esculentum* L) seeds. However the enzyme is a serine protease, and does not cleave albumins, globulins or the 13S reserve protein of the seed [13]

Endopeptidase activities causing limited proteolysis of the acidic polypeptides of 11S seed globulins at neutral/alkaline pH and which are inactive on the β chains have been characterized in the total extract of mature soybean cotyledons [14] Others have been shown to be active at pH 6 8 in the low ionic strength extract of ungerminated pumpkin seeds [15]

The enzyme described in the present work has an apparent M_r approaching the one of neutral aminopeptidases of resting seeds It also has a similar optimal pH to these enzymes and like them acts on BAPA This activity, however, may be a consequence of the affinity of the enzyme for arginine residues, as has been established in previous work on the pea vicilin precursor [11], for which the amino acid sequence is known This affinity is confirmed by the binding of the enzyme to and inhibition by salmin, a protein that contains more than 70% arginine residues, and by inhibition by leupeptin The enzyme does not act on LeuPA or on dipeptides of hydrophobic amino acids as aminopeptidases do and its mode of action is apparently that of an endopeptidase

Though the substrates split are similar, the enzyme also differs from proteinases A and B described by Vaintraub and Shutov [3], because of its optimal pH in the neutral range, the higher apparent M_r and its presence in the dormant seed

A definite role of the enzyme within the pattern of proteolysis in the dormant seed or when germination is starting, is still uncertain In view of the activation period required, it is possible that the proteinase is present in an inactive form in the dormant dry seed until access of water at imbibition modifies and activates it

EXPERIMENTAL

Chemicals and standard proteins were the purest available from Merck and Sigma DEAE cellulose was purchased from Whatman, Ultrogel AcA 44, TSK DEAE 5PW and TSK G3000 SW from LKB, Sepharose CNBr 4B from Pharmacia and

Protein Pack 300 SW from Waters Carboxymethylated α III-subunit of pea legumin was a gift of Dr R R D Croy Mature dry seeds of *L. albus* were of the sweet Multolupa variety (alkaloid content 0 05%)

Enzyme purification Lupin flour which had been defatted in a Soxhlet apparatus with pentane for 3 hr at 36° was extracted overnight at 4° in dist H_2O (100 g/200 ml) adjusted to pH 5 0 The supernatant obtained after centrifugation at 10000 *g* for 30 min was used in this study Material precipitating between 33 and 50% $(NH_4)_2SO_4$ was resuspended in 50 mM Na-Pi buffer, pH 7 5, and fractionated on a column of Ultrogel AcA 44 (40 × 2 5 cm) at a flow rate of 34 ml/hr The active fraction, which was eluted between 114 and 146 ml, was adsorbed on a column of DEAE cellulose (40 cm × 3 cm) equilibrated in the same buffer as above; the buffer containing 0 1 M NaCl at a flow rate of 140 ml/hr eluted the enzyme between 254 and 330 ml

This fraction, diluted with a same vol of buffer without NaCl, was applied to a column (20 × 2 cm) of the same ion exchanger equilibrated in 50 mM Tris–HCl buffer, pH 8 5 the column was washed at a flow rate of 125 ml/hr with the buffer containing 0 1 M NaCl and the enzyme eluted between 56 and 91 ml with a buffer containing 0 13 M NaCl This fraction was loaded on a salmine–Sepharose column (5 × 1 cm) equilibrated with 50 mM Na-Pi, pH 7 5 An active peak was eluted between 45 and 55 ml with the buffer containing 0 5 M NaCl This pool was dialysed against 50 mM Tris–HCl, pH 8 5 at 4° overnight and then loaded on a TSK DEAE 5 PW column (7 5 × 0 8 cm) equilibrated with the same buffer in a FPLC apparatus Elution of the active peak was obtained by adding 0 15 M NaCl to the buffer This fraction was either frozen at −30° or used immediately FPLC and CC on salmine–Sepharose were performed at room temp No significant loss of activity was observed in control samples kept at the same temp. for an equivalent time. All other procedures were carried out at 4°

Salmine-Sepharose was prepared by covalent immobilization of salmine (23 mg/g dry resin) according to ref. [16] Protein concentration was determined by micro biuret [17] or micro Bradford [18] depending on the amount of protein

Enzyme activity Activities on synthetic substrates were followed for 60 sec at 37° in 50 mM Na-Pi buffer, pH 8.0, containing 0 15 M NaCl in a recording spectrophotometer at 405 nm for BAPA, LeuPA and AlaPA [19], 254 nm for synthetic peptides [20], 237 nm for ATEE [21] and 247 for TAME [22]

Qualitative detection of enzyme activity on gel slabs was carried out by overlaying the separation gel with a 0 3% agarose gel slab in 0 3 M Na-Pi buffer, pH 8 0, containing 0 33 mM BAPA The gels were kept 10–15 min at 40° when the active band developed a yellow colour

Proteolytic activity was assayed at 37° in 50 mM Na-Pi buffer, pH 7 5, containing 0 15 M NaCl and 0 05% NaN_3 with the enzyme and the proteins at the concentration indicated in the figures The incubation was stopped by adding the same vol as the incubation mixture of a soln containing 0.25 M Tris–HCl, pH 6 8, 30% glycerol and 2% SDS, plus 2% 2-mercaptoethanol when necessary, and heating at 100° for 5 min The mixture was then analysed by SDS-PAGE.

Electrophoretic analysis SDS-PAGE was run according to ref [23] in the absence or presence of 2% 2-mercaptoethanol on a 15% (w v) acrylamide gel for assays of protomer composition and on a 11–19% acrylamide gradient for apparent M_r determinations Densitometer scannings of the Coomassie Blue-stained gels were performed with an LKB Ultroscan Laser densitometer interfaced with an Apple/E computer Protein markers were phosphorylase b, bovine serum albumin, egg albumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin

PAGE under non-denaturing conditions was on a 7 5% acrylamide gel in 0 06 M Tris–glycine buffer, pH 9 0, protein markers were haemoglobin and β-lactoglobulin

Gel filtration The apparent M_r of the enzyme was determined by chromatography on Ultrogel AcA 44 (50 × 1 cm) which was equilibrated with 50 mM Na-Pi buffer, pH 7 5, containing 0 1 M NaCl at a flow rate of 5 ml/hr The eluate was monitored at 280 nm with a Uvicord 2138 S, LKB, and activity was measured as described before Alternatively a column (30 × 0 8 cm) of TSK G3000 SW on a FPLC apparatus or of Protein Pack 300 SW on a Waters HPLC apparatus were used Protein markers were bovine serum albumin, transferrin, β-lactoglobulin, trypsinogen, myoglobin, cytochrome *c*

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