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Purification and Characterization of two Proteolytic Enzymes in the Cotyledons of Germinating Lentils

H. Guerra and G. Nicolás

Departamento de Fisiología Vegetal
Facultad de Biología
Universidad de Salamanca
(España)

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Two proteases, one peptidehydrolase and one aminopeptidase, have been purified to homogeneity from cotyledons of germinating seeds of *Lens culinaris* Med. Peptidehydrolase has an apparent molecular weight of 89,000 and an isoelectric point of 4.7. Peptidehydrolase activity was not affected by metal chelators but it was affected by N-bromosuccinimide, phenylmethylsulfonyl fluoride and N-ethylmaleimide, suggesting the presence of tryptophan and serine residues together with free —SH groups in its active site. Peptidehydrolase activity was maximally active from pH 6.0 to 9.0 being practically zero below pH 5.0. It was stable at temperatures up to 40° C, and complete inactivation was obtained at or over 70° C. Aminopeptidase has an apparent molecular weight of 83,000 and an isoelectric point of 4.5. Its activity was affected by N-bromosuccinimide, suggesting the presence of tryptophan residues in its active site. The aminopeptidase presents its maximal activity at pH 5.5. It was stable at temperatures up to 40° C, and complete inactivation was detected at over 70° C.

Studies on proteolytic activity during seed germination have shown a considerable degree of complexity, and besides the intensive studies in this field during the last years the mechanism of protein degradation is still not well understood. One of the most fruitful lines of investigation has been the purification and characterization of plant proteases (2, 5, 7, 9, 11, 12, 16). In a previous paper we reported the presence during germination of lentil seeds of different kinds of proteolytic activities involved in the depletion of reserve proteins. The present paper describes the purification and characterization of one aminopeptidase and one peptidehydrolase isolated from the cotyledons of germinating lentil seeds.

Materials and Methods

Lentil seeds (*Lens culinaris* Med = *Lens esculenta* Moench) were germinated as previously described (8) and were harvested after 120 h of germination. After removing the testa and the embryo, the cotyledons were transformed to a fine

powder in a coffee-mill and used immediately.

Enzyme purification. The powdered cotyledons (200 g) were extracted with 500 ml of water containing 25 mM 2-mercaptoethanol and centrifuged at 12,000 x *g* for 20 min at 5° C. The resulting pellet was extracted twice with the same solution and centrifuged as before. The resulting three supernatants were pooled, adjusted to pH value of 6.3 with 1 N NaOH and kept overnight at 4° C. The pH was then adjusted to pH 10.0 with 1 N NaOH, and after heating to 30° C for 1 h was again adjusted to 6.3 with 2 N acetic acid. The solution was precipitated with 55% saturated $(NH_4)_2SO_4$, adjusted to pH 6.0, and after standing for 1 h it was centrifuged at 12,000 x *g* for 20 min at 5° C. The supernatant was precipitated with 65% saturated $(NH_4)_2SO_4$. The precipitate was resuspended in 20 mM Tris-HCl buffer pH 7.5 containing 5 mM 2-mercaptoethanol and dialysed against the same buffer for two days. The dialysate was passed through a DEAE-cellulose column (2 x 23.5 cm) equilibrated with 20 mM tris-HCl buffer pH 7.5 containing 5 mM 2-mercaptoethanol. The column was eluted with a 20-500 mM tris-HCl buffer pH 7.5 gradient, and the rest of the proteins were extracted with 500 mM tris-HCl buffer pH 7.5 containing 2 M NaCl. The fractions eluted from the DEAE-cellulose column containing the enzyme activity (aminopeptidase and peptidehydrolase) were precipitated with 80% saturated $(NH_4)_2SO_4$ and resuspended in 20 ml of 50 mM tris-HCl buffer pH 7.5 containing 5 mM 2-mercaptoethanol and then passed through a Sephadex G-200 column (2.5 x 34 cm) equilibrated and eluted with the same buffer. The fractions containing the enzyme activities were precipitated again with 80% saturated $(NH_4)_2SO_4$ and resuspended in 4 ml of the elution buffer. Then, 4 ml of -20° C acetone was added and after 30 min had elapsed the precipitate containing the enzyme activities was recovered by centrifugation at 30,000 x *g* for 20 min.

The following parameters were determined in all steps of the enzyme purification: proteins (10); enzyme activity using LNA and BAPA as substrates as previously described (8); and electrophoretic behaviour according to the method of BLOEMENDAL (3). The gels were stained with amido black except for the identification of aminopeptidase in wihch the substrate (LNA) fixed with Fast Garnet GBC was used.

The molecular weight of the enzymes was determined by dodecylsulfate/polyacrylamide gel electrophoresis (17). The isoelectric point of the enzymes was determined by electrofocusing in a LKB column with a 0-50% sucrose gradient in a 1% ampholine solution in the pH 3-5 range. The column was loaded with 92 mg of protein and run for 24-48 h at 400 V.

Effect of enzyme inhibitors. The following inhibitors of protease activity were used: phenylmethylsufonyl fluoride, N-ethilmaleimide, N-bromosuccinimide, O-phenantroline and ethylenediamine tetraacetic acid. Aliquots of the purified enzymatic solution were preincubated with the different inhibitors for 30 min at the concentration shown in table II. The enzymatic activity was determined as previously described (8).

pH optima for activity. 0.1 M citrate-phosphate buffer at pH 2.5-8.0 and 0.1 M tris/maleate/NaOH buffer at pH 5.5-10.0 were used to determine the pH optima of the purified enzymes.

Abbreviations: BAPA, M-Benzoyl-DL-arginin-4-nitroanilide HCl; LNA, L-leucyl-β-naphthylamide HCl; Fast Garnet GBC, o-Aminoazotoluene diazonium salt; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; NBS, N-bromosuccinimide; EDTA, ethylenediamine tetraacetic acid.

Results

Enzyme purification. The extraction with water containing 2-mercaptoethanol decreases the solubility of part of the proteins, mainly globulins, and the -SH groups are maintained in the reduced form. Proteins without protease activity are precipitated by alcaline pHs, whereas the proteases are precipitated between 55-65% saturated $(NH_4)_2SO_4$. Dialysis allows the elimination of a brown inactive precipitate. When the resulting soluble fraction was passed through a DEAE-cellulose column all the protease activity was adsorbed on the matrix, whereas most of the protein was eluted with the loading buffer. After addition of 2 M NaCl the peptidehydrolase and amino peptidase activities were eluted as two single peaks (figure 1). The pooled fractions containing the activity were concentrated by 80% saturated $(NH_4)_2SO_4$ and passed through a Sephadex G-200 column (figure 2) to eliminate low molecular-weight contaminants. Both enzymes were eluted between fractions 28-83

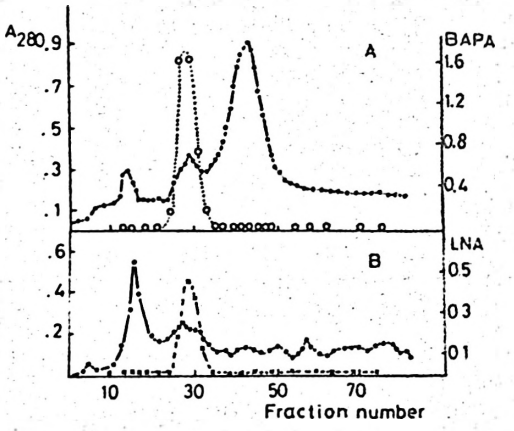

Fig. 2. *Cromatography on Sephadex G-200 of lentil peptidehydrolase (BAPA, O.....O) (A) and aminopeptidase (LNA, x-----x) (B).*
Absorbance at 280 nm (● —— ●).

which could suggest similar molecular weights. The last step, precipitation with 50% acetone at -20° C favoured the purification of peptidehydrolase while it completely inactivated aminopeptidase. Table I shows the purification accom-

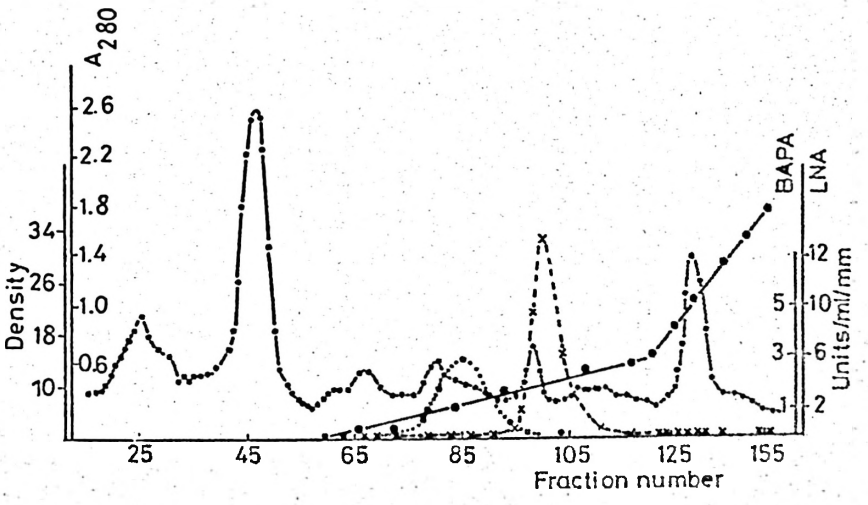

Fig. 1. *Fractionation of lentil peptidehydrolase (BAPA, O.....O) and aminopeptidase (LNA, x.....x) on DEAE-cellulose column.*
Absorbance at 280 nm (· —— ·) 20-50 mM Tris-HCl gradient containing up to 2 M NaCl (● —— ●).

Table I. *Purification of lentil peptidehydrolase (BAPA) and aminopeptidase (LNA).*

Purification step	Volume (ml)		Protein (mg/ml)		Activity (units/ml/min)		Specific activity (units/mg protein)		Purification factor	
	BAPA	LNA	BAPA	LNA	BAPA	LNA	BAPA	LNA	BAPA	LNA
Crude extract	355	355	19.75	19.75	97.5	346.25	4.93	17.52	1	1
$(NH_4)_2SO_4$ 55-65 %	23	23	8.0	8.0	106.5	417.5	13.31	52.18	2.69	2.97
DEAE-cellulose	5.3	5	1.625	1.05	163.5	453.33	100.61	431.74	20.40	24.62
Sephadex G-200	4	5	0.65	0.19	160.5	240.0	246.15	1263.15	49.92	72.05
Acetone	4	—	0.091	—	146.0	—	1604.39	—	325.43	

plished after each of the purification steps described above. Both enzymes were electrophoretically homogeneous and it was no possible to detect more than one protein by electrophoresis (figure 3).

Molecular weight and isoelectric point. The molecular weight of both enzymes, estimated by dodecylsulfate/polyacrilamide gels electrophoresis gave a value of 83,000 for aminopeptidase and 89,000 for peptidehydrolase (figure 4). The isoelectric point determined by electrofocusing (figure 5) gave a value of 4.5 for aminopeptidase and 4.7 for peptidehydrolase. Part of the activity was lost by electrofocusing.

Temperature stability. No loss in enzymic activity could be measured when the two enzymes were incubated for 30 min at 40° C (data not showed). A rapid decrease in activity was observed at temperatures above 40° C and complete inactivation was obtained at temperatures over 70° C. These results seem to indicate

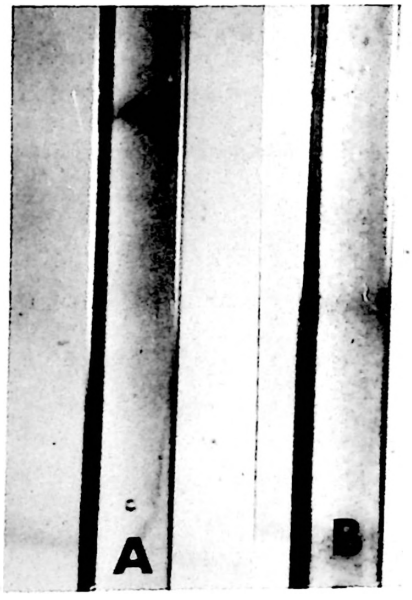

Fig. 3. *Polyacrylamide gel electrophoresis of the purifies peptidehydrolase (A) and aminopeptidase (B).*
In A, the gels were stained with amido Black, and in B, the substrate (LNA) was fixed with Fast Garnet GBC.

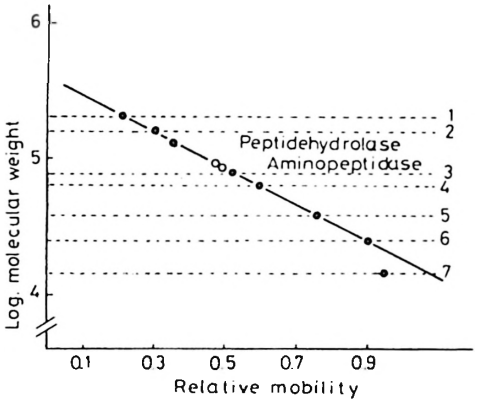

Fig. 4. *Determination of molecular weight of lentil peptidehydrolase and aminopeptidase by dodecylsulfate/polyacrilamide gel electrophoresis.*
Proteins of known molecular weight were used as references. 1, Ferritin; 2, γ-globulin; 3, transferrin; 4, bovine serum albumin; 5, alcohol dehydrogenase; 6, tripsin; 7, lysozyme.

Table II. *Effects of protease inhibitors on the activity of lentil peptidehydrolase and aminopeptidase.*
Enzymatic activity expressed as units/ml/min after 30 min incubation.

Protease inhibitor	Peptidehydrolase			Aminopeptidase		
	Inhibitor [M]	Enzymatic Activity	Inhibition %	Inhibitor [M]	Enzymatic Activity	Inhibition %
None	—	104	—	—	203	—
Phenylmethyl sulphonylfluoride	5×10^{-5}	102	—	1×10^{-4}	204	—
	5×10^{-4}	102	—	5×10^{-4}	207	—
	5×10^{-3}	46	56	5×10^{-3}	205	—
N-ethylmaleimide	2×10^{-4}	80	23	1×10^{-4}	203	—
	5×10^{-4}	60	42	5×10^{-4}	207	—
	5×10^{-3}	37	65	5×10^{-3}	218	—
N-bromosuccinimide	5×10^{-4}	100	—	1×10^{-4}	191	6
	2×10^{-3}	34	67	5×10^{-4}	152	25
	5×10^{-3}	20	81	5×10^{-3}	8	96
	1×10^{-2}	0	100			
EDTA	2×10^{-2}	103	—	2×10^{-2}	210	—
o-Phenantroline	5×10^{-3}	105	—	5×10^{-3}	204	—

a remarkable thermostability of both enzymes.

Effect of enzyme inhibitors (table II). Peptidehydrolase activity was not affected by metal chelators such a EDTA or o-phenantroline. However, it was considerably affected by NBS at a concentration of 2 x 10^{-3} M, by PMSF at 5 x 10^{-3} M and by NEM at 5 x 10^{-3} M. Aminopeptidase activity was only affected by 5 x 10^{-3} M of NBS.

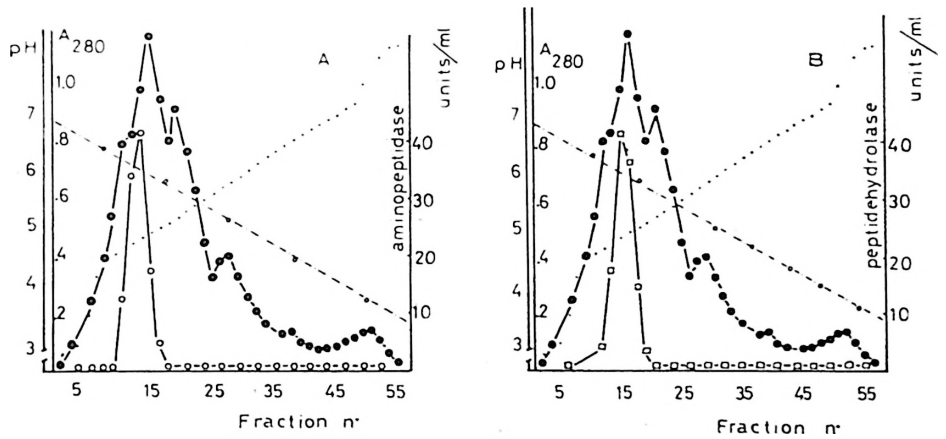

Fig. 5. *Isoelectric point determination of aminopeptidase (O———O) (A) and peptidehydrolase (□———□) (B) by electrofocusing in a LKB column with a 0-50% sucrose gradient (—·—·—) in a 1% ampholine solution in the range pH 3-5.*
Absorbence at 280 nm (●———●).

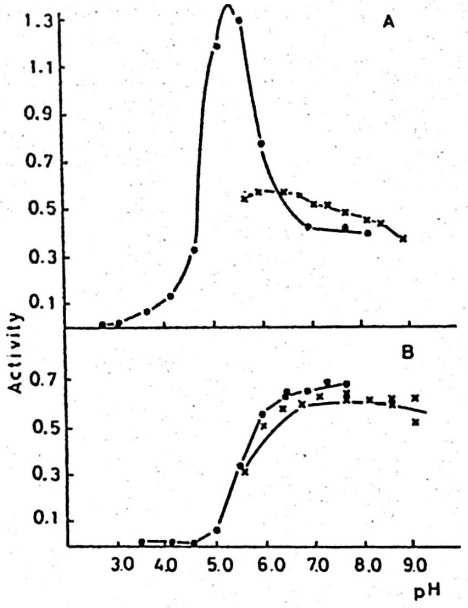

Fig. 6. *pH optimum of lentil aminopeptidase (A) and peptidehydrolase (B).*
0.1 M citrate-phosphate buffer, pH 2.5-8.0 (●—●); 0.1 M Tris/maleate/NaOH buffer pH 5.5-10.0 (x———x).

Effect of pH (figure 6). Lentil peptidehydrolase was maximally active from pH 6.0-9.0, being practically null below pH 5.0. Lentil aminopeptidase presented one peak of maximal activity at pH 5.5. The effect of two buffers is similar to that described for *Neurospora* (15).

Discussion

The purification procedure used in this work leads to two enzyme preparations purified to homogeneity as determined by polyacrylamide gel electrophoresis. Proteases are classified in four different groups based on the relationships of their enzymatic mechanisms: serine proteases, sulfydryl proteases, acid proteases and metallo proteases, distinguished by their susceptibilities to specific inhibitors. Studies with various types of inhibitors have shown that both lentil peptidehydrolase and aminopeptidase are considerably inhibited by NBS, indicating that tryptophan residues in both enzymes play an essential role probably in maintaining the active conformation of the enzyme (7). Peptidehydrolase activity was also inhibited by PMSF and by NEM, suggesting also the presence in its active site of serine residues and free -SH groups (11, 14), but as yet a tryptophan protease has only been found in sorghum (7). Neither of the two enzymes was inactivated by metal chelators, thus ruling out the participation of metal ions.

Peptidehydrolase activity from lentil shows a pH response similar to that obtainned in soybean (7) and in wheat (13). Aminopeptidase activity shows a pH response unusual in higher plants since the pH optima described in the literature for this enzyme are in the alkaline zone, as shown for *Phaseolus aureus* (4) and pea (6). Thermostability, molecular weight and isoelectric point determinations are in agreement with those determined in proteases purified from other seeds (1, 2).

Resumen

Se han purificado dos proteasas, una peptidohidrolasa y una aminopeptidasa, de los cotiledones de semillas de *Lens culinaris* Med. germinadas. La peptidohidrolasa tiene un peso molecular aproximado de 89.000 y un punto isoeléctrico de 4,78. Esta actividad no es afectada por queladores metálicos pero sí por N-bromosuccinimida, fenilmetil-sulfonilfluoruro y N-etilenmaleimida, lo que indica la presencia de restos de triptófano y serina junto con grupos -SH libres en el centro activo. La actividad peptido hidrolásica presenta su máximo a pH 6,0-9,0 siendo prácticamente nula por debajo de un pH de 5.0. El enzima es estable a 40° C y es totalmente inactivado por encima de los 70° C. La aminopeptidasa tiene un peso molecular aparente de 83.000, punto isoeléctrico de 4,52 y la actividad es afectada por N-bromosuccinimida, lo que indica la presencia

de triptófano en el centro activo. La aminopeptidasa presenta la máxima actividad a pH de 5,5 y es estable a temperaturas de 40° C, inactivándose por encima de los 70° C.

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