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A PARTIAL PURIFICATION AND CHARACTERIZATION OF TWO AMINO PEPTIDASES FROM *CUCURBITA MAXIMA* COTYLEDONS

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Abstract—An enzyme fraction containing two similar aminopeptidases has been isolated from *Cucurbita maxima* Duch. var. Hubbard cotyledons in purified form using L-leucine amide as the principal substrate. The substrate specificity of one of the two enzymes was similar to leucine aminopeptidase and the other varied only in that it hydrolyzed L-leucylglycylglycine at about three times the rate of leucine aminopeptidase. Evidence is presented that showed that a third peptidase which hydrolyzed L-leucylglycine more rapidly than either of the two aforementioned enzymes was also a constituent of squash cotyledons. This third peptidase was removed during the purification procedure and not studied in detail. The hydrolysis of L-leucine amide by the purified fraction was activated by Mg^{++} and Mn^{++} and inhibited by EDTA, $(NH_4)_2SO_4$ and high concentrations of NaCl. The highest pH optimum of the purified fraction was between 8·0 and 8·5, however there was also a lower pH optimum at pH 7·0. The peptidase activity of the fraction increased at a relatively uniform rate from 25 to 45°, increasing about three-fold within this range. The K_m value was $2{\cdot}4 \times 10^{-2}$ M/L with L-leucine amide as the substrate at pH 7·6.

INTRODUCTION

PROTEIN is a frequent reserve material in seeds. This reserve protein is broken down during germination with a concomitant rise in amino acids and amides.[1, 2] This is followed by the translocation of the major portion of these degradation products to the growing parts of the embryo and the subsequent synthesis of protein.[3] A lesser portion of these degradation products are utilized in the storage organ for the synthesis of other essential proteins, e.g. α-amylase.[4]

Although it appears that proteases are active in seeds during germination, detailed information on the nature of these enzymes is woefully lacking. Numerous workers have demonstrated the presence of proteinase activity in extracts of germinating seeds using casein, gelatin, or edestin as substrates. Irving and Fontaine[5] extracted an enzyme from peanuts, *Arachis hypogaea*, which hydrolyzed benzoyl-L-arginine amide but was completely inactive toward carbobenzoxyglycylphenylalanine, carbobenzoxyglycyltyrosine, and carbobenzoxyglutamyltyrosine. As early as 1930, Mounfield[6] demonstrated that an extract from germinating wheat, *Triticum vulgare*, seeds was able to hydrolyze L-leucylglycine and glycylglycine. L-Leucylglycine was hydrolyzed more readily than glycylglycine. Guitton[7] found that an extract of germinating *Pinus pinea* L. seeds was able to hydrolyze several synthetic peptide substrates. Those containing N-terminal L-leucine were hydrolyzed to a greater degree than

[1] E. SCHULZE, and B. UMLOUFT, *Landwirtsch. Jahrb.* **5**, 819 (1876).
[2] E. SCHULZE, *Z. Physiol. Chem.* **20**, 327 (1895).
[3] Y. OOTA, R. FUJII and S. OSAWA, *J. Biochem.* (*Tokyo*) **40**, 649 (1953).
[4] J. E. VARNER and G. R. CHANDRA, *Proc. Natl Acad. Sci. U.S.* **52**, 100 (1964).
[5] G. W. IRVING and T. D. FONTAINE, *Arch. Biochem.* **6**, 351 (1945).
[6] J. B. MOUNFIELD, *Biochem. J.* **30**, 549 (1930).
[7] Y. GUITTON, *Bull. Soc. Franc. Physiol. Vegetale* **9**, 125 (1963).

those with N-terminal glycine. Tazawa and Hirokawa[8] reported that an extract from germinating soybean, *Glycine soja*, seeds was able to hydrolyze gelatin but not benzoylglycine, benzoylglycylglycine, or leucylglycylglycine.

There appears to be no reports in the literature on the purification of proteolytic enzymes from germinating seeds. However, there are two reports on the purification of proteolytic enzymes from dormant seeds.[8,9] The proteolytic enzyme which Soedigo and Gruber[9] purified 350-fold from pea, *Pisum sativum* L., seeds hydrolyzed casein, hemoglobin, and serum albumin but did not hydrolyze benzoylarginine amide, glycylphenylalanine amide, or glycylglycine. Akune and Takagi[10] purified a proteinase from azuki seeds 40-fold using casein as a substrate, but did not determine its substrate specificity.

Recently, Shain and Mayer[11] suggested that there are three distinct types of proteolytic activity in extracts from germinating lettuce, *Lactuca sativa* L., seeds.

The current research was undertaken to purify and characterize a peptidase from germinating squash, *Cucurbita maxima* Duch., seeds. Evidence is presented that there are at least three peptidase present. The partial purification and chracterization of two of these peptidases is given.

RESULTS AND DISCUSSION

The assay used to detect the activity with the various substrates was performed by an incubation and formal titration as described in detail in the experimental section. Various experiments were conducted to establish the validity of the assay. Using leucine amide as the substrate, the assay yielded linear relationships for substrate concentration, amount of enzyme used, and incubation time for both the crude enzyme (fraction I) and the purified enzyme (fraction V) within the ranges used in these experiments.

Purification

The flow sheet of the purification procedure is presented in Fig. 1. The data from a representative purification indicates that a 110-fold purification of these enzymes was obtained with a 43 per cent recovery, Table 1. This data also suggests that an inhibitor or inhibitors of these enzymes was removed by Sephadex G-50 and perhaps by protamine sulfate.

TABLE 1. PURIFICATION OF AMINO PEPTIDASES FROM *Curcubita maxima*

Fraction	Total volume	Protein (mg)		Activity		Specific activity (units/mg)	Recovery (%)	Purification (fold)
		Per ml	Total	Per ml (units*)	Total (units)			
I (crude)	247	47·6	11,757	0·119	29·4	0·0025	100	1
II (Sephadex)	270	39·6	10,692	0·229	61·8	0·0058	210†	2·3
III (protamine SO_4)	283	2·9	821	0·248	70·2	0·0855	239†	34·2
IV (DEAE-A)	22·3	0·31	69	0·650	14·5	0·2101	49	84·0
V (DEAE-B)	2·8	1·66	46	4·50	12·7	0·2761	43	110·0

* 1 unit = 1 ml of 0·1 M KOH/100 min.

† Greater than 100 per cent recovery suggests that an inhibitor was removed.

[8] Y. TAZAWA and T. HIROKAWA, *J. Biochem. (Tokyo)* **43**, 785 (1956).
[9] R. SOEDIGO and M. GRUBER, *Biochem. Biophys. Acta* **44**, 315 (1960).
[10] S. AKUNE and S. TAKAGI, *J. Agr. Chem. (Japan)* **36**, 63 (1962).
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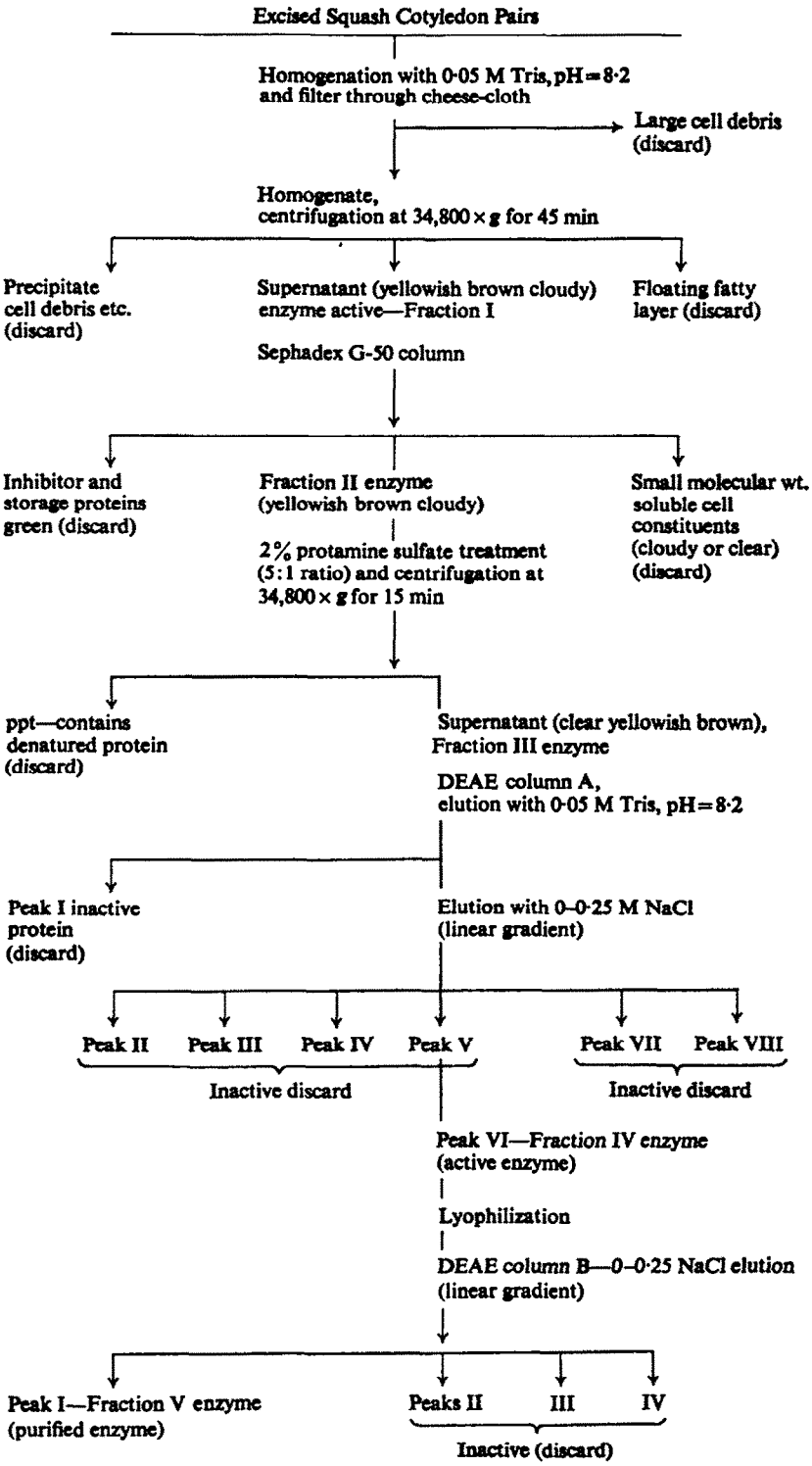

FIG. 1. ENZYME PURIFICATION—FLOW CHART FORM.

The presence of proteolytic activities in the crude enzyme extract had been detected in preliminary work using such substrates as L-leucine amide, L-leucylglycine, hippurylphenyllactic acid, L-leucylglycylglycine, L-phenylalanine amide, L-tyrosine amide, L-glycine amide and L-leucylleucine. To determine if certain of these activities were catalyzed by one or more than one enzyme, the changes in the ratios of the various activities during various purification procedures were measured. This was done using L-leucine amide and L-leucylglycine as substrates at all five levels of the purification procedure (Table 2). The ratios of L-leucylglycine activity to L-leucine amide activity varied from 19·6 in fraction I to 0·41 in fraction V. These data indicate that at least two different enzymes are present in the crude fraction according to the criteria mentioned by Irving *et al.*[12]

TABLE 2. CHANGE IN ACTIVITY RATIOS OF SUBSTRATE DURING PURIFICATION

Fraction	Substrate	Purification (fold)	Activity (units/ml enzyme)	Ratio of activities L-leucylglycine to L-leucine amide
I (crude)	L-Leucine amide	1	0·167	19·6
	L-Leucylglycine	1	3·260	
II (Sephadex)	L-Leucine amide	0·9	0·113	14·6
	L-Leucylglycine	0·67	1·646	
III (protamine SO_4)	L-Leucine amide	26·4	0·194	
	L-Leucylglycine	7·9	1·148	5·9
IV (DEAE-A)	L-Leucine amide	43·2	0·062	
	L-Leucylglycine	4·3	0·123	2·0
V (DEAE-B)	L-Leucine amide	70·8	0·322	
	L-Leucylglycine	1·5	0·133	0·41

The complete system for leucine amide activity contained 0·15 mmole of leucine amide, 2·0 ml enzyme fraction and 0·2 mmole of sodium phosphate buffer, pH 7·6, to make a total of 4·0 ml. The complete system for L-leucylglycine activity contained 0·450 mmole of L-leucylglycine, 1·0 ml enzyme fraction and 0·3 mmole of sodium phosphate buffer, pH 7·6, to make a total of 4·0 ml. In both systems the control system contained no substrate. The above procedure was repeated for all fractions, with 2·0 ml of the fraction for L-leucine amide and 1 ml for L-leucylglycine at each purification step. The reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formal titration method. One unit = 1 ml of 0·1 M KOH/100 min.

The protamine sulfate enzyme, fraction III, was placed on a DEAE cellulose column (A), eluted with a linear NaCl gradient, and the elutate monitored at 280 nm. The activity was found to be in a relatively broad band containing two peaks (Fig. 2). Subsequent studies, which will be described later, showed that these two proteins were similar enzymes which defied separation with the techniques available. Several inactive peaks were also observed.

Fraction IV was applied to a second DEAE cellulose column and eluted with a steeper NaCl gradient than used for column A. This resulted in all of the activity occurring in a single well-defined peak, Fig. 3. This is the final purified enzyme and is referred to as fraction V. Several inactive peaks were also observed.

Enzyme Properties

Fraction V preparation showed a substrate specificity similar to leucine aminopeptidase from hog kidney when compared to published data[13] or a commercial preparation (Worthing-

[12] G. W. IRVING, Jr., J. S. FRUTON and M. BERGMANN, *J. Biol. Chem.* **138**, 231 (1941).
[13] E. L. SMITH and D. H. SPACKMAN, *J. Biol. Chem.* **212**, 271 (1955).

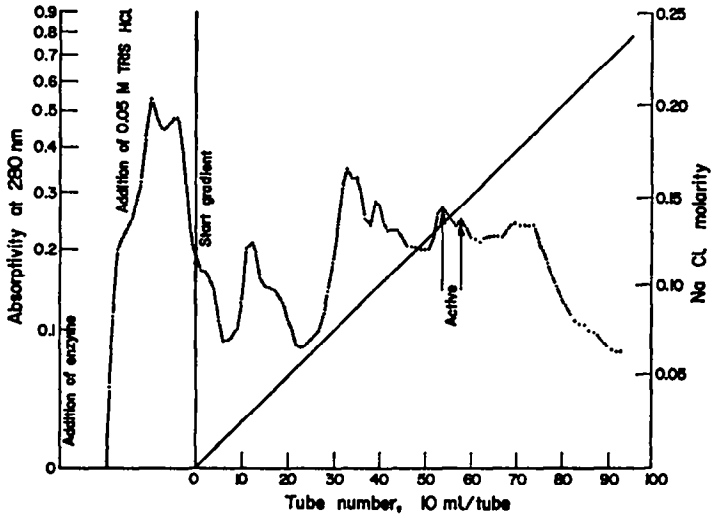

FIG. 2. COPY OF TYPICAL U.V. RECORDING OF DEAE COLUMN A. THE CONDITIONS FOR THIS COLUMN ARE GIVEN IN THE EXPERIMENTAL SECTION FOR DEAE COLUMN A. THE TOTAL TIME ELAPSED FOR THE RUN WAS APPROXIMATELY 20 HR.

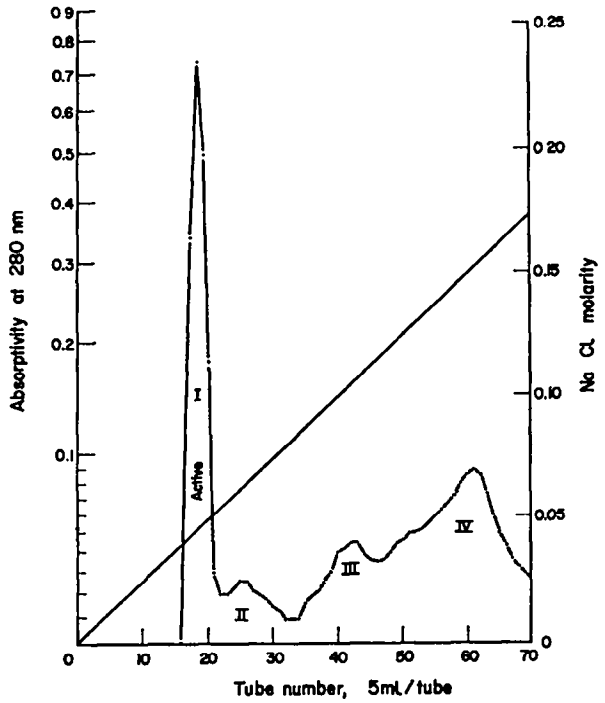

FIG. 3. COPY OF TYPICAL U.V. RECORDING OF DEAE COLUMN B. THE CONDITIONS FOR THIS COLUMN ARE GIVEN IN THE EXPERIMENTAL SECTION FOR DEAE COLUMN B. THE TOTAL TIME ELAPSED FOR THE RUN WAS APPROXIMATELY 12 HR.

ton enzymes) (Table 3). The substrates used in this experiment were L-leucine amide, L-leucylglycine, L-leucylglycylglycine, L-phenylalanine amide, L-tyrosine amide, L-glycine amide and L-leucyl-L-leucine.

The data on Mg^{++} and Mn^{++} activation, as well as EDTA inhibition of fraction V when L-leucine amide was used as the substrate, is shown (Table 4). The data indicated Mg^{++} and

TABLE 3. SUBSTRATE SPECIFICITY OF FRACTION V

Substrate	Relative hydrolysis (L-leucine amide = 100)		
	Commercial L.A.P.	Literature[13]	Fraction V
L-Leucine amide (0·0375 M)	100	100	100
L-Leucylglycine (0·1125 M)	40	86	36
L-Leucylglycylglycine (0·05 M)	83	120	129
L-Phenylalanine amide (0·05 M)	47	26	21
L-Tyrosine amide (0·05 M)	5	16	22
L-Glycine amide (0·05 M)	6	0·1	11
L-Leucyl-L-leucine (0·042 M)	82	100	127

The complete reaction mixture contained 2 ml of enzyme, 0·2 mmole of sodium phosphate buffer, pH 7·6, and substrate to give a final volume of 4·0 ml. The control system was the same as the complete system, but with no substrate. These reaction mixtures were incubated at the 34·5° temperature for 100 min and assayed using a modified formal titration method. Fraction V had a specific activity of 0·138 ml of 0·1 M KOH per mg protein per 100 min for L-leucine amide.

TABLE 4. ACTIVATION AND INHIBITION STUDIES WITH Mg^{++}, Mn^{++} AND EDTA

Type and concentration of additive	Activity (units/mg protein)	Per cent change
Undialyzed enzyme		
No addition	0·182	
0·005 M EDTA	0·135	−25·8
0·005 M EDTA + 0·005 M Mg^{++}	0·194	+6·7
0·005 M EDTA + 0·005 M Mn^{++}	0·176	−3·3
Dialyzed enzyme		
No addition	0·076	
0·005 M Mg^{++}	0·100	+31·6
0·005 M Mn^{++}	0·144	+89·3
0·005 M Mg^{++} + 0·005 M EDTA	0·076	0

The complete system contained 5·2 mg of fraction V (2·6 mg protein/ml), 0·15 mmole of L-leucine amide, 0·15 mmole Tris-HCl, pH 7·6, and H_2O + Mg^{++} or Mn^{++} EDTA or a combination to give a final volume of 4·0 ml. The control system was the same as the complete system, but without the L-leucine amide substrate. These reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formal titration method. One unit = 1 ml 0·1 M KOH/100 min.

Mn^{++} activation of dialyzed fraction V and the loss of the Mg^{++} activation using EDTA. It was also shown that the inhibition with EDTA on the undialyzed preparation was eliminated by addition of either Mg^{++} or Mn^{++}. Further studies on these metal-ion requirements were not pursued due to the conflicting results obtainable in more complex treatments as was shown by Smith and Spackman[13] of leucine aminopeptidase from hog kidney.

The pH optima experiment indicated two pH optima, a low one at pH 7·0 and a much higher and broader one at pH 8·0–8·5 (Fig. 4). Fraction V appeared to have the same relatively high temperature optima properties of many proteolytic enzymes which covers the range from 30 to 45°, Fig. 5. The heat stability of fraction V was quite high and declined

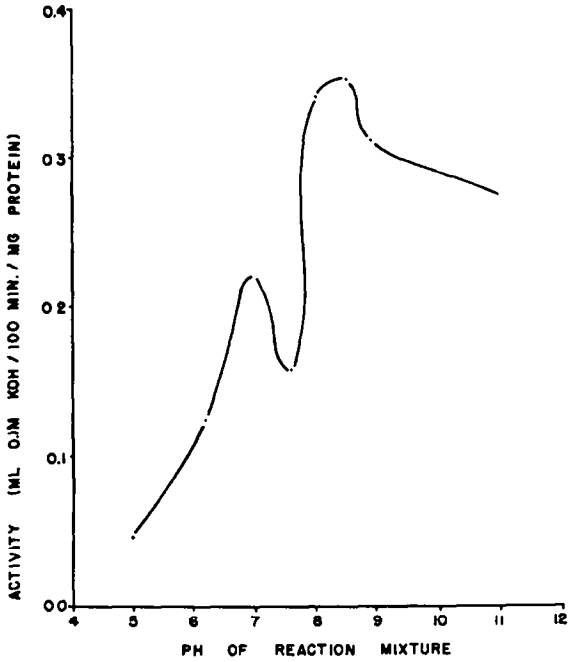

FIG. 4. pH OPTIMA OF PURIFIED ENZYME.

The complete system contained 3·2 mg of fraction V enzyme (1·6 mg/ml), 0·15 mmole L-leucine amide and 0·15 mmole of buffer at the given pH to give a final volume of 4 ml. The control system was the same as the complete system, but with deionized water in place of leucine amide substrate. These reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formol titration method.

rapidly at 55°, Fig. 6. Its activity was completely lost at 75° and above. The K_m determined for fraction V using L-leucine amide was $2{\cdot}4 \times 10^{-2}$ M/L. The stability of the purified enzymes over longer periods of time seemed to be preserved best by freezing at −10° in 0·05 M Tris-HCl, pH 8·2. The enzyme was stable to lyophilization as a means of concentration of the activity.

It was found that ammonium sulfate appeared to destroy the enzyme's activity with L-leucine amide as substrate in contrast to data on leucine aminopeptidase from hog kidney, which is stable to such treatment.[13] There was some inhibition of leucine amide activity at high NaCl concentrations (Table 5). At the concentration of NaCl present in the final preparation of fraction V enzyme (about 0·04 M) the inhibition was only about 10 per cent.

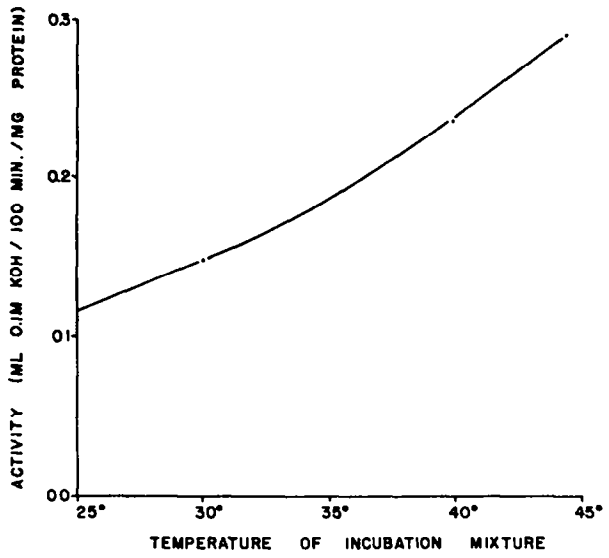

FIG. 5. TEMPERATURE OPTIMA.

The complete system contained 4·8 mg of fraction V enzyme (2·4 mg/ml), 0·15 mmole L-leucine amide and 0·2 mmole of sodium phosphate buffer, pH 7·6, to give a final volume of 4 ml. The control system was the same as the complete system, but with no leucine amide substrate. These reaction mixtures were incubated at the various temperatures for 100 min and assayed by the modified formal titration method.

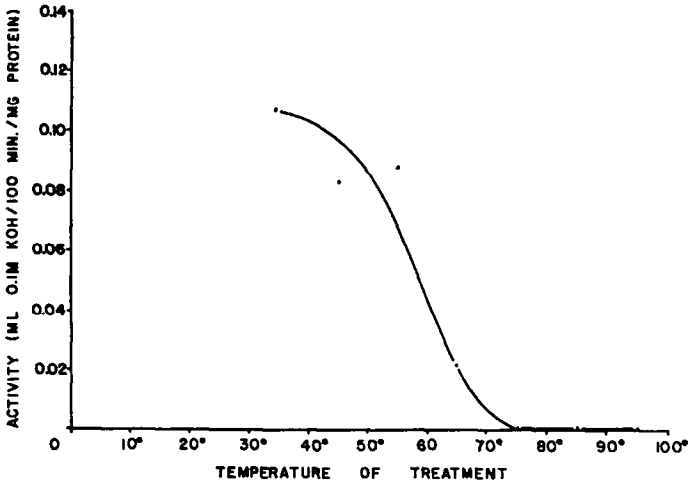

FIG. 6. TEMPERATURE OF INACTIVATION.

The complete system contained 2 ml of fraction V enzyme (2·3 mg/ml) which had been exposed to the given temperature for 5 min and centrifuges, 0·15 mmole L-leucine amide and 0·2 mmole sodium phosphate buffer, pH 7·6, to give a final volume of 4 ml. The control system was the same as the complete system, but with no leucine amide substrate. The reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formol titration method.

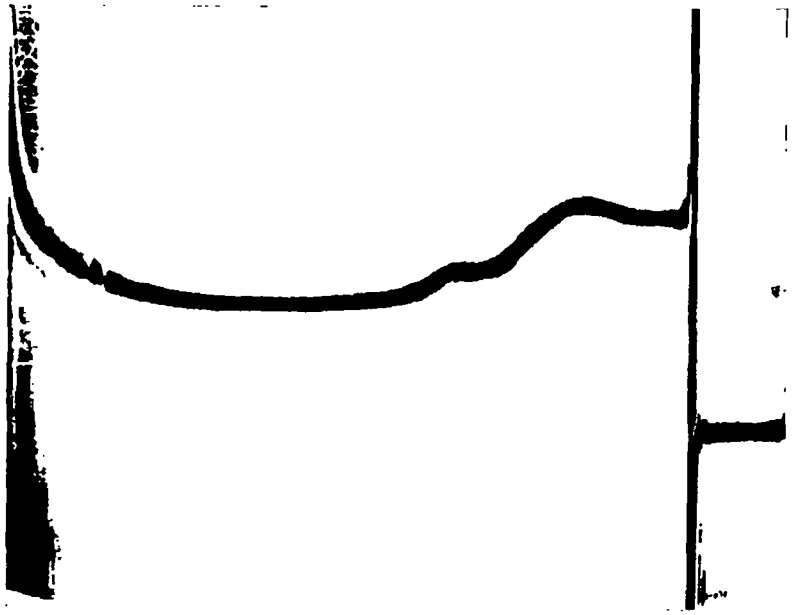

FIG. 7. ULTRACENTRIFUGE PATTERN OF FRACTION V ENZYME.

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TABLE 5. NaCl INHIBITION OF ACTIVITY

Molarity of NaCl present	Activity (units/0·75 ml)	Per cent inhibition of activity
0·000	0·148	0
0·0375	0·135	8·8
0·075	0·120	18·9
0·150	0·094	23·0

The complete system contained 0·15 mmole of L-leucine amide, 7·6 mg of fraction IV (3·8 mg protein/ml), 0·176 mmole of sodium phosphate buffer, pH 7·6, and NaCl plus deionized H_2O to make a total of 4·0 ml. The control systems were the same as the complete, but without substrate. The incubation was at 34·5° and after 100 min the activity was assayed using the formal titration method. One unit = 1 ml 0·1 M KOH/100 min.

The final enzyme preparation, fraction V, was 110-fold purified and was found to give two peaks in the ultracentrifuge treatment (Fig. 7), although only one peak appeared in the DEAE column B u.v. absorption recording (Fig. 3). The presence of two proteins in this final preparation, fraction V, was further supported by the presence of two protein bands in a vertical acrylamide gel electrophoretigram (Fig. 8). The possibility of separating these two proteins was extensively explored using various techniques. There was some indication of a small amount of separation in the DEAE column A (fraction IV) u.v. absorption recording (Fig. 2), which had shown the presence of two closely associated peaks. The application of various linear and nonlinear NaCl gradients for DEAE cellulose column elution of different milliequivalent values also failed to give a useful separation of these two proteins. Separation by molecular size was attempted by use of Sephadex G-100, Sephadex G-200, Biogel P-100 and Biogel P-200, however separation of these two proteins was not attained with any of these systems. Variations in the earlier steps of purification before the introduction of DEAE columns was not useful, e.g. ammonium sulfate, batch-wise calcium phosphate gel or hydroxyl

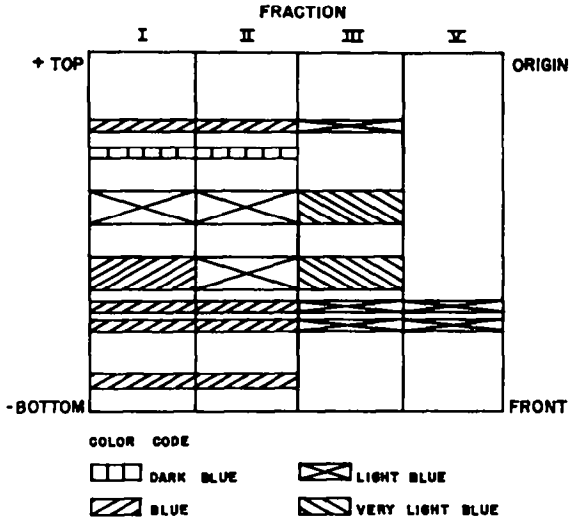

FIG. 8. VERTICAL GEL ELECTROPHORESIS OF FRACTIONS I, II, III, AND V.

apatite columns. Acetone powder preparations did give some purification. The introduction of acetone powder treatment after the protamine sulfate step purified the enzyme to 650-fold. However, the total activity recovered was so small that the properties of the enzyme could not be determined.

It was assumed that the two peaks which appeared on u.v. absorption recording of fraction IV (Fig. 2), as well as the ultracentrifuge analysis and the acryamide electrophoresis pattern of fraction V, were the same. In order to determine if both of these peaks were peptidases

TABLE 6. ACTIVITY RATIOS OF PEAK I AND II OF FRACTION IV

Substrate	Activity (units/mg protein)		Relative hydrolysis (L-leucine amide = 100)			
	Peak I	Peak II	Peak I	Peak II	Fraction V	Leucine amino peptidase[12]
L-Leucine amide (0·0375 M)	0·0649	0·0612	100	100	100	100
L-Leucylglycine (0·1125 M)	0·0304	0·0359	47	59	36	86
L-Phenylalanine amide (0·05 M)	0·0196	0·0189	31	31	21	26
L-Leucylglycylglycine (0·05 M)	0·0400	0·1690	62	276	129	120

The complete system contained 5·0 mg of protein from peak I or 5·3 mg of protein from peak II, 0·2 mmole of sodium phosphate buffer, pH 7·6, and substrate to give a final volume of 4·0 ml. The control system was the same as the complete system, but without substrate. The substrates used were L-leucine amide, L-leucylglycine, L-phenylalanine amide and L-leucylglycylglycine. These reaction mixtures were incubated at the 34·5° temperature for 100 min and assayed using the modified formal titration method. One unit = 1 ml 0·01 M KOH/100 min.

TABLE 7. pH RATIOS OF PEAKS I AND II OF FRACTION IV

Enzyme preparation	pH of reaction mixture	Activity (units/mg protein)	Activity ratio to activity at pH 7·6
Peak I	pH = 7·0 0·04 M KPO_4	0·094	1·38
Peak I	pH = 7·6 0·04 M Na PO_4	0·068	1·00
Peak I	pH = 8·0 0·04 Tris-HCl	0·147	2·16
Peak II	pH = 7·0 0·04 M KPO_4	0·048	1·0
Peak II	pH = 7·6 0·04 M Na PO_4	0·048	1·0
Peak II	pH = 8·0 0·04 M Tris-HCl	0·071	1·48

The complete system contained 3·2 mg of protein from peak I or 2·7 mg of protein from peak II, 0·15 mmole of L-leucine amide and 0·15 mmole of buffer at the given pH to give a final volume of 4·0 ml. The control system was the same as the complete system, but without the substrate. These reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formal titration method. One unit = 1 ml 0·1 M KOH/100 min.

and investigate some of their properties, fraction IV was prepared repeatedly, the two peaks separated, and each peak pooled and its corresponding peak from other runs until a sufficient amount of each peak was obtained for the critical assays. However, it should be noted that these two fractions are not pure but somewhat contaminated with the corresponding peak. These two peaks are referred to as peak I and peak II in the subsequent discussion. The relative specificity of these peaks were similar to each other as well as fraction V and published leucine aminopeptidase data for L-leucine amide, L-leucylglycine, and L-phenylalanine amide, Table 6. However, they varied markedly in their ability to hydrolyze L-leucylglycylglycine.

In view of the fact that two pH optima were obtained from fraction V when L-leucine amide was used as a substrate, the pH ratios were determined for peak I and II at pH 7·0, 7·6, and 8·0 for this substrate. The data is presented in Table 7. This data also suggests that we are indeed dealing with two different proteolytic enzymes in peaks I and II. Peak I had two pH optima, whereas peak II had only one.

EXPERIMENTAL

All experiments utilized seeds of *Cucurbita maxima* Duch., Chicago Warted Hubbard squash, which were soaked for 4 hr in distilled water and then grown in vermiculite for 3 days in the dark at 30°.

Assay Methods

Protein analysis was accomplished by use of the Lowry Method.[14] Determination of proteolytic activity in the form of increased acidity due to substrate hydrolysis was accomplished by the following procedure. Unless otherwise indicated the reaction mixture consisted of 0·15 mmole of leucine amide, 0·2 mmole sodium phosphate, pH 7·6, and enzyme to make a total volume of 4 ml. A control reaction mixture without substrate was run for each sample. The enzyme was added at 0 times and a sample was removed immediately and placed in ice until assayed. The reaction mixtures were incubated at 34·5° for 100 min in a shaking water bath. A sample was removed at 100 min and placed in ice until assayed. The analysis of samples was accomplished by a modification of the formal titration method of Sorensen.[15] A 0·75 ml volume of reaction mixture was added to 0·75 ml of 36% formaldehyde solution, pH 7·0, and titrated to pH 8·6 at 34° with 0·1 M KOH using an automatic titrator. The increased acidity was calculated as ml of 0·1 M KOH used to titrate the 100 min sample minus the amount of titrant used to titrate the 0 time sample and expressed in units (1 unit = 1 ml of 0·1 M KOH/100 min).

Purification Procedure

Crude enzyme (fraction I). Three-day-old cotyledons were excised and placed in a container in ice. The cotyledons were homogenized in a Virtis blender at high speed in a ratio of two cotyledon pairs for each one ml of 0·05 M Tris-HCl, pH 8·2. This homogenate was squeezed through cheese-cloth to remove large cellular debris. It was then centrifuged for 45 min at 34,800 *g* at 0° in a refrigerated centrifuge. The supernatant fluid, after removal of the fatty layer, is referred to as the crude enzyme. All subsequent manipulations were carried out at near 0° by use of ice buckets or working in a cold room.

Sephadex enzyme (fraction II). Approximately 120 ml of crude enzyme was added to a

[14] O. N. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol.Chem.* **193**, 265 (1951).
[15] S. P. L. Sorensen, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.* **7**, 1 (1907).

41 × 4·6 cm column of bead form Sephadex G-50. The Sephadex had been previously equilibrated with 0·05 M Tris buffer, pH 8·2. The column was eluted with 0·05 M Tris, pH 8·2, under a low pressure and the first clear fraction displaced by the enzyme solution was discarded. The second fraction which had a yellowish-brown color contained the active enzyme and is referred to as the Sephadex enzyme or fraction II.

Protamine sulfate enzyme (fraction III). A freshly prepared solution of 2% protamine sulfate (salmon sperm protamine sulfate from Cal-Biochem.) was cooled to 0° and slowly added to the cold fraction II enzyme which was constantly mixed by a magnetic stirrer; the volume ratio was 5 parts of enzyme to 1 part protamine sulfate. After a 10 min period of equilibration, this solution was centrifuged at 34,800 *g* for 15 min in a refrigerated centrifuge. The clear brownish supernatant fluid from this centrifugation is referred to as the protamine sulfate enzyme or fraction III.

DEAE-column A enzyme (fraction IV). A column of 0·75 meg/g DEAE cellulose, 30 × 2 cm, was prepared using 0·05 M Tris-HCl buffer, pH 8·2, according to the methods of Peterson and Sober.[16] Approximately 140 ml of the protamine sulfate enzyme (300–350 mg of protein) was added to the column at 1 ml/min using a constant flow pump. The column was then eluted with about 100 ml of 0·05 M Tris-HCl buffer, pH 8·2, and the displaced fraction discarded. The column was then eluted with a linear gradient of 0–0·25 M NaCl in 0·05 M Tris-HCl buffer, pH 8·2, at a rate of 1 ml/min using a constant flow pump. The fraction collection was 10 ml per tube and a recording u.v. absorption analyzer was used to detect peaks. The active fraction was present in tubes 50–60 or peak VI on the u.v. absorption recording at 280 nm. This fraction is referred to as DEAE column A enzyme or fraction IV. A total of from 80 to 100 ml of this fraction was dialyzed twice for 2 hr in the cold room against 2-l. volumes of 0·0005 M Mg^{++} in 0·005 M Tris-HCl buffer, pH 8·2, to remove the NaCl. It was then lyophilized to dryness in about 7 hr. This same procedure was repeated in order to obtain sufficient enzyme for the subsequent procedures. The two preparations were combined and diluted to about 10–16 ml with 0·05 M Tris-HCl, pH 8·2, and is referred to as lyophilized fraction IV.

DEAE column B enzyme (fraction V). A column of 0·75 meg/g DEAE cellulose, 30 × 2 cm, was prepared in the 0·05 M Tris-HCl, pH 8·2, using the same procedure as used for preparing DEAE Column A. Ten to sixteen millilitres of lyophilized fraction IV enzyme (50–60 mg of protein) was added to the column at 0·5 ml/min flow rate. The column was eluted with a linear 0–0·25 M NaCl gradient in 0·05 M Tris-HCl buffer, pH 8·2, at 0·5 ml/min flow rate using the constant volume pump. This gradient was twice as steep as the gradient used in DEAE column A. Volumes of about 5 ml per tube were collected during this run. A u.v. absorption recorded set at 280 nm was used to detect the peaks. A total of four peaks were detected and only the first symmetrical peak I at tubes 15–20 (about 0·04 M NaCl) had activity. This fraction volume was about 25 ml and it is referred to as DEAE column B enzyme or fraction IV.

Splitting of DEAE-column A enzyme into two fractions. The u.v. absorption recording of the fraction referred to as DEAE column A enzyme indicated the partial separation of two fractions of protein which overlapped. Small peak portions from these two fractions were pooled separately, dialyzed and concentrated by lyophilization from a number of runs, using the same procedure as for the concentration of fraction IV. These separated fractions after suitable dilution in 0·05 M Tris-HCl, pH 8·2, are referred to as peak I enzyme and peak II

[16] E. A. PETERSON and H. A. SOBER, In *Methods in Enzymology* (Edited by S. B. COLOWICK and N. O. KAPLAN), Vol. 5. Academic Press, New York (1962).

enzyme. These fractions were used to try to characterize further the two proteins in our final purified fraction V enzyme.

Ultracentrifuge pattern. A sample of fraction V was subjected to ultracentrifugation, Fig. 7. The sample contained 7·2 mg of protein per ml in 0·03 M Tris-HCl buffer, 0·1 M sodium phosphate, and 0·003 M Mg^{++}, pH 7·5 The sample had been concentrated by lyophilization for about 3 hr. The data suggests that the mol. wt. of peak I is about 40,000 and peak II about 70,000.

Vertical gel electrophoresis. Enzyme fractions I, II, III, and V were submitted to vertical gel electrophoresis under the following conditions, Fig. 8. The electrophoresis medium contained 5% acrylamide gel in 0·1 M Tris buffer, pH 9·2 (0·09 M Tris, 0·0025 M EDTA and 0·0075 M boric acid). The recirculating buffer was 0·1 M Tris, pH = 9·2. The gel was stained with amidoblack dye. The conditions of the run were at a constant voltage of 280–290 V and amperage varied from 250 A at the beginning of the run to 190 A at the end. The gel was prerun for 45 min before adding samples and continued for an additional 3 hr after adding the samples. The positive electrode was on top and the negative on the bottom. The dialyzed samples which were added to the top contained 2·7, 3·6, 5·9, and 3·5 mg of protein for fractions I, II, III, and V, respectively. The electrophoresis apparatus was the EC 470 Vertical Gel Electrophoresis system from the E-C Apparatus Corporation.

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