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PURIFICATION AND CHARACTERIZATION OF AN AMINOPEPTIDASE FROM *CUCURBITA MAXIMA* COTYLEDONS

F. M. ASHTON and W. J. DAHMEN

Department of Botany, University of California, Davis, California, 95616, U.S.A.

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Abstract—An aminopeptidase has been isolated from *Cucurbita maxima* Duch. "Chicago Warted Hubbard" cotyledons and purified 500 fold. Glycylglycylglycine was used as the principal substrate. This enzyme required a free amino group next to the bond cleaved. The terminal amino acid with the free amino group was split off leaving a dipeptide. This enzyme preparation exhibited no activity towards dipeptides, but did exhibit a low rate of hydrolysis on L-leucine amide, L-phenylalanine amide and L-tyrosine amide. Chloroacetylglycylglycine, hippuryl-L-leucine amide, and the carboxypeptidase substrate hippuryl-DL-phenyllactic acid were not hydrolyzed by this aminotripeptidase preparation. Tetraglycine was readily hydrolyzed, but pentaglycine was hydrolyzed much less and polyglycine hydrolysis was not detectable. There were no indication of activation of glycylglycylglycine hydrolysis by Mg^{2+} and inhibition was indicated by Mn^{2+}, Co^{2+}, Zn^{2+}, EDTA and cysteine. The loss in activity by dialysis or EDTA was not recoverable by the above metal ions. The pH optima was 8·0 for glycylglycylglycine with as a substrate. The peptidase activity was stable to a 5 min heat treatment up to 45° but decreased rapidly above this temperature. The K_m for glycylglycylglycine was $7{\cdot}3 \times 10^{-3}$ M at pH 7·6. This aminopeptidase appeared to be the same enzyme as the aminopeptidase contaminant in the previously reported leucine aminopeptidase,[1] and was distinctly different from the purified dipeptidase,[2] as well as the two proteinases.[3] All five of these proteolytic enzymes are present in 3-day-old squash cotyledons.

INTRODUCTION

THE significance of the protein reserves and the state of knowledge of their degradation and the enzymes involved in germinating seeds has recently been reported.[1–3]

The current research was undertaken to purify and characterize an aminopeptidase from germinating squash, *Cucurbita maxima* Duch. "Chicago Warted Hubbard", seeds. Additional evidence is presented which supports the fact that at least three peptidases are present in the early stages of germination. In addition to these peptidases there are at least two proteinases present.[3]

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. The purified enzyme activity (fraction IV) with time of incubation and with amount of enzyme was found to be linear over the ranges used. The amount of glycylglycylglycine required to saturate the purified enzyme (fraction IV) was 0·05 M.

[1] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* **6**, 641 (1967).
[2] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* **6**, 1215 (1967).
[3] D. PENNER and F. M. ASHTON, *Plant Physiol.* **42**, 791 (1967).

Purification

The flow sheet of the purification procedure is presented in Fig. 1. The quantitative data for the preparation of the enzyme is given in Table 1. The data from a representative purifica-

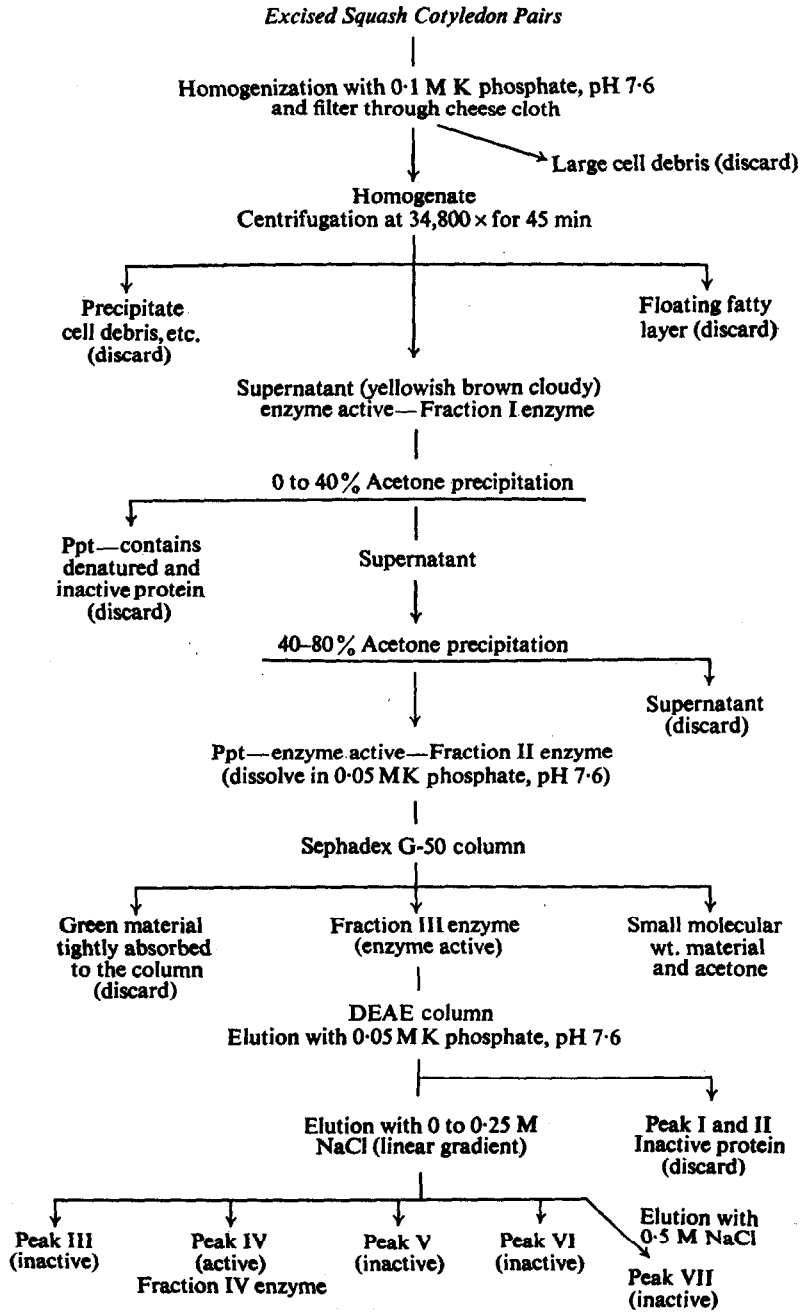

FIG. 1. ENZYME PURIFICATION—FLOW CHART FORM.

tion indicated a 500-fold purification of this aminopeptidase enzyme with a 26·4 per cent recovery. The increased activity of fraction III (198·8 per cent) relative to fraction I (100·0 per cent) suggested that an inhibitor of this enzyme was removed during these treatments.

TABLE 1. PURIFICATION OF AMINOPEPTIDASE FROM *Cucurbita maxima*

Fraction	Total volume	Protein (mg)		Activity		Specific activity (units/mg)	Recovery (%)	Purification (fold)
		Per ml	Total	Per ml (units*)	Total (units)			
I (crude)	320	49·2	15,700	0·371	118·8	0·0075	100·0	1·0
II (acetone ppt.)	43·4	—	—	—	—	—	—	10·2†
III (Sephadex)	55·7	5·09	283·4	4·210	234·2	0·826	198·8‡	110
IV (DEAE)	68·5	0·12	8·1	0·449	30·8	3·803	26·4	507

* 1 unit = 1 ml of 0·1 M KOH/100 min.
† Data of previous paper.[2]
‡ Greater than 100 per cent recovery suggests that an inhibitor was removed.

The presence of proteolytic activities in the crude enzyme extract has been detected in preliminary work using such substrates as L-leucine amide, L-leucylglycine, hippuryl-DL-phenyllactic acid, L-leucylglycylglycine, glycylglycylglycine, L-leucyl-L-leucine, L-phenylalanine amide, L-tyrosine amide, and glycine amide. To determine if certain of these activities were catalyzed by one or more than one enzyme, the change in the ratios of the various activities during the four purification steps were measured. This was done by using L-leucylglycine, glycylglycylglycine, and L-leucine amide at all four levels of the purification procedure (Table 2). The ratios of glycylglycylglycine activity to L-leucylglycine activity varied from 13·48 in fraction I to 5·45 in fraction III and to 0·0 in fraction IV. The ratios of glycylglycylglycine activity to L-leucine amide activity varied from 0·919 in fraction I to 0·111 in fraction III and 0·142 in fraction IV. This data indicated that at least three different enzymes were present in the crude fraction according to the criteria mentioned by Irving *et al.*[4] These values indicated a complete removal of dipeptidase activity from the fraction IV enzyme preparation. The hydrolysis of the various substrates by fractions I, II, and III indicated a drastic removal of the activity of L-leucine amide hydrolysis compared to glycylglycylglycine, the variation in ratios was a factor of 9. The purification of fractions III to fraction IV gave a similar ratio of glycylglycylglycine hydrolysis to L-leucine amide for fraction III and IV, which indicated that the residual L-leucine amide activity of fraction IV was caused by the aminopeptidase enzyme rather than a leucine aminopeptidase contaminant. This was further supported by the fact that the activities for the two amides, L-phenylalanine amide and L-tyrosine amide, were very close to the activity with L-leucine amide for fraction IV (Table 3). However, purified leucine aminopeptidase animal source or plant source,[1] hydrolyzed other amino acid amides at a lower rate than L-leucine amide (20 per cent of the L-leucine amide activity). The fact that L-leucyl-L-leucine was not hydrolyzed by our purified aminopeptidase (Table 3), whereas leucine aminopeptidase showed almost identical affinities for both L-leucine amide and L-leucyl-L-leucine, further supported the purity of our preparation. The lack of activation of activity of our aminopeptidase preparation by either Mn^{2+} or Mg^{2+} (Table 4) while leucine aminopeptidase is highly activated by both ions was another

[4] G. W. IRVING, JR., J. S. FRUTON and M. BERGMANN, *J. Biol. Chem.* **138**, 231 (1941).

TABLE 2. CHANGE IN ACTIVITY RATIOS OF SUBSTRATE DURING PURIFICATION

Enzyme fraction	Substrate	Activity (units/ml enzyme)	Ratio of activities substrate to Triglycine
I (crude)	glycylglycylglycine	0·432	1·000
	L-leucylglycine	5·820	13·480
	L-leucine amide	0·397	0·919
II (acetone* ppt.)	glycylglycylglycine	0·447	1·000
	L-leucylglycine	4·255	9·520
	L-leucine amide	0·100	0·224
III (Sephadex)	glycylglycylglycine	3·943	1·000
	L-leucylglycine	21·500	5·450
	L-leucine amide	0·440	0·111
IV (DEAE)	glycylglycylglycine	0·449	1·000
	L-leucylglycine	0·000	0·000
	L-leucine amide	0·064	0·142

The reaction mixture for L-leucylglycine contained enzyme fraction, 0·2 mmole potassium phosphate, pH 7·6 and 0·4 mmole of substrate in a 4 ml total volume. The reaction mixture for glycylglycylglycine contained enzyme fraction, 0·2 mmole potassium phosphate, pH 7·6 and 0·2 mmole of substrate in a 4 ml total volume. The reaction mixture for L-leucine amide contained enzyme fraction, 0·2 mmole potassium phosphate, pH 7·6 and 0·15 mmole of substrate in a 4 ml total volume. The reaction mixtures were incubated at 34·5° for 60 or 100 min depending on the substrate. These samples were assayed using the modified formol titration method and all rates were corrected to 100 min. One unit = 1 ml of 0·1 M KOH/100 min.

* Data obtained from previous paper.[2]

TABLE 3. SUBSTRATE SPECIFICITY ON AMINOPEPTIDASE

Substrate*	Activity (units/ml enzyme)	Relative hydrolysis (glycylglycylglycine = 100)
Glycylglycylglycine (0·05 M)	0·449	100·0
Tetraglycine (½ sat.)	0·425	94·7
L-Leucylglycylglycine (0·05 M)	0·342	81·0
Pentaglycine (½ sat.)	0·121	26·9
L-Leucylglycyl-L-leucine (0·026 M)	0·080	17·8
L-Leucyl-L-leucyl-L-leucine (½ sat.)	0·077	17·2
L-Leucine amide (0·037 M)	0·064	14·3
L-Phenylalanine amide (0·05 M)	0·049	10·9
L-Tyrosine amide (0·05 M)	0·046	10·2

The complete reaction mixtures for the formol assay contained 236 μg of enzyme (fraction II), 0·2 mmole potassium phosphate, pH 7·6 and substrate plus H_2O in a total of 4·0 ml. The reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formol titration method.

* The following substrates showed no activity: Glycine amide (0·05 M), L-Leucylglycine (0·10 M), L-Leucyl-L-leucine (0·042 M), Glycylglycine (0·05 M), Polyglycine (M.W. = 15,000) (½ sat.), L-Glutamyl-cystenylglycine (0·05 M), Chloroacetyl-glycylglycine (0·05 M), Hippuryl-L-leucine amide (½ sat.), Hippuryl-DL,-phenyllactic acid (0·05 M).

TABLE 4. ACTIVATION AND INHIBITION STUDIES ON AMINOPEPTIDASE

Treatment of enzyme fraction IV	0·005 M additive: EDTA	0·005 M additive: metal ion	Activity (units/ml enzyme)	Per cent change
Untreated enzyme	—	—	0·427	0·0
Untreated enzyme	+	—	0·280	−34·5
Untreated enzyme	—	Mg^{2+}	0·431	+ 1·0
Untreated enzyme	+	Mg^{2+}	0·338	−20·9
Untreated enzyme	+	Mn^{2+}	0·328	−23·2
Untreated enzyme	+	Co^{2+}	0·272	−36·4
Untreated enzyme	+	Zn^{2+}	0·298	−30·2
Dialyzed enzyme*	—	—	0·242	−43·4
Dialyzed enzyme*	+	—	0·136	−68·2
Dialyzed enzyme*	—	Mg^{2+}	0·253	−40·8
Sephadex G-50 treated enzyme	—	—	0·433	+1·0
Sephadex G-50 treated enzyme	—	Mg^{2+}	0·427	0·0
Sephadex G-50 treated enzyme	—	Mn^{2+}	0·376	−12·0
Sephadex G-50 treated enzyme	—	Co^{2+}	0·380	−11·0
Sephadex G-50 treated enzyme	—	Zn^{2+}	0·230	−46·2
Dialyzed enzyme (0·004 μ cysteine present)	—	—	0·274	−35·9

The complete system contained fraction IV enzyme (treated as indicated), 0·2 mmole glycylglycylglycine, 0·2 mmole Tris-HCl, pH 7·6 and metal ion or EDTA plus H_2O in a total of 4 ml. These reaction mixtures were incubated at 34·5° for 100 min and assayed by the modified formol titration method. One unit 1 ml of 0·1 M KOH/100 min.

* Dialysis conditions: against 2 l. 0·05 M potassium phosphate; pH=7·6, 6½ hr.

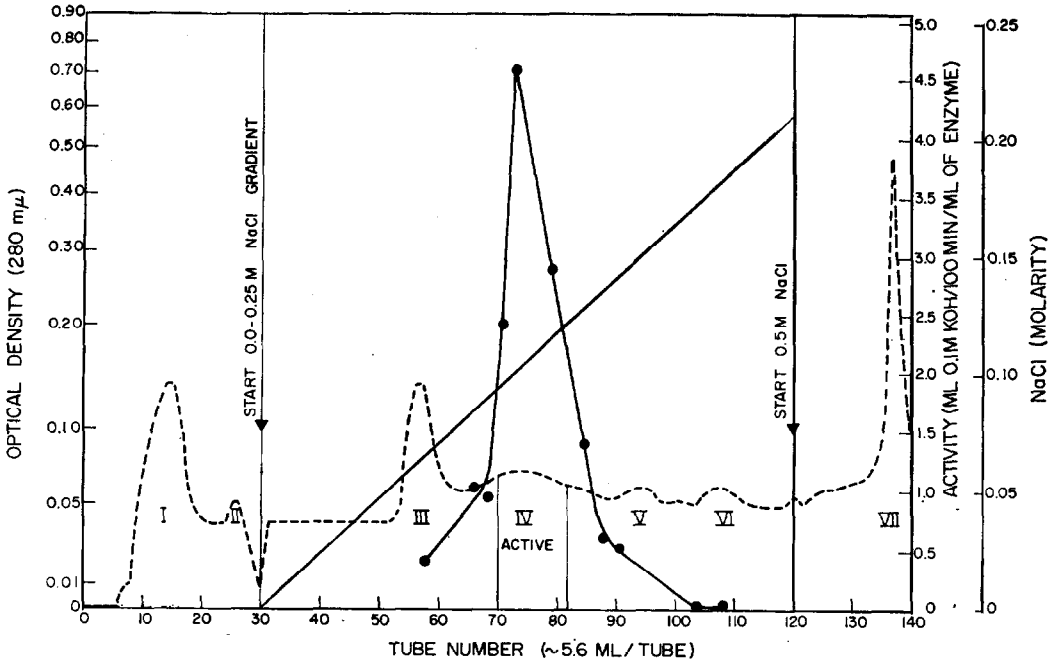

FIG. 2. COPY OF TYPICAL U.V. RECORDING OF DEAE COLUMN WITH ACTIVITY FOR GLYCYLGLYCYL-GLYCINE PLOTTED AS WELL AS O.D. AT 280 nm. THE CONDITIONS FOR THIS COLUMN ARE GIVEN IN THE EXPERIMENTAL SECTION FOR DEAE COLUMN.[2] THE TOTAL TIME ELAPSED FOR THE RUN WAS APPROXIMATELY 26 hr.

supporting factor. The u.v. recording of the aminopeptidase elution from the DEAE column (Fig. 2) shows one peak for the peptidase activity which indicated a homogeneous preparation. The two pH optima, pH 7·0 and one at 8·0, exhibited by our previous peptidase preparation[1] appears to have been caused by two different enzymes both having leucine amide activity. One being leucine aminopeptidase and the other the aminopeptidase described in this paper.

The fraction III was placed on the DEAE cellulose column, eluted with a linear NaCl gradient and the elutate monitored at 280 nm. The eluted fractions were analyzed for activity with both L-leucylglycine and glycylglycylglycine (Fig. 2). The aminopeptidase activity was located in peak IV and the dipeptidase activity in peak VI.

Enzyme Properties

The substrate specificity of fraction IV was similar to several animal peptidases.[5–7] The animal tripeptidases showed a specificity for a free amino group next to the bond cleaved on the tripeptidase substrate, as well as a high specificity for tripeptides and negligible activity on dipeptides. They exhibited no activity on carboxypeptidase substrates or glutathione and exhibited pH optima around pH 8. Generally, they were not inhibited by EDTA or activated by metal ions such as Mn^{2+}, Mg^{2+}, Co^{2+} or Zn^{2+}. There was some inhibition of the animal tripeptidase by cysteine and dialysis and this loss was not recoverable by metal ion addition. Our plant aminopeptidase exhibited similar specificity for the free amino group next to the bond cleaved. The substrate specificity (Table 3) for tripeptides with the free amino group and our analysis of the products of aminopeptidase activity on the substrate L-leucylglycylglycine supported this. The only products of L-leucylglycylglycine hydrolysis by our purified aminopeptidase were L-leucine and glycylglycine, glycine and L-leucylglycine were not formed. Since the glutathione substrate was not hydrolyzed, the presence of a free carboxyl group near the free amino group appeared to interfere with cleavage. This data and the lack of hydrolysis of carboxypeptidase substrates or substrates with the amino group blocked, further supported the concept that our enzyme was an aminopeptidase. Our plant aminopeptidase did not hydrolyze dipeptides, which was also the case for the animal tripeptidase. Our aminopeptidase hydrolyzed tetraglycine almost as well as triglycine but was markedly less effective on pentacylycine and polyglycine (M.W. = 15,000) hydrolysis was not detected. The animal tripeptidase studied by Furton, Smith, and Driscoll[6] had very little activity toward tetraglycine or other tetrapeptides. The other two investigations[5,7] with animal tripeptidases did not utilize tetrapeptides as substrates. Therefore, perhaps an appropriate name for our aminopeptidase would be, aminoolgiopeptidase.

Our purified aminopeptidase (fraction IV) exhibited a pH optima of 8·0 (Fig. 3) and gave no activation of activity by the metal ions Mn^{2+}, Mg^{2+}, Co^{2+} or Zn^{2+} (Table 4). Although our plant enzyme (fraction IV) was inhibited by cysteine, EDTA and dialysis, its activity was not restored by addition of metal ions (Table 4). The animal tripeptidases appear to be quite similar to our aminopeptidase from plants in most of their properties, although they did not exhibit the dialysis inactivation in all cases.

Fraction IV appeared to have the high temperature optima characteristic of several proteolytic enzymes. The range observed was between 4° and 48° (Fig. 4). The heat stability of fraction IV is shown in Fig. 5 for glycylglycylglycine. The enzyme was rapidly inactivated above 50°. The K_m obtained for the purified aminopeptidase with glycylglycylglycine as the

[5] G. AGREN, *Acta Physiol. Scand.* **9**, 255 (1945).
[6] J. S. FRUTON, V. A. SMITH and P. E. DRISCOLL, *J. Biol. Chem.* **173**, 457 (1948).
[7] E. ADAMS, N. C. DAVIS and E. L. SMITH, *J. Biol. Chem.* **199**, 845 (1952).

substrate was $7{\cdot}3 \times 10^{-3}$ M at pH 7·6. The enzyme appeared to be quite stable, 80 per cent of activity after 1 month when stored in the frozen state at − 10° in 0·05 M potassium phosphate, pH 7·6.

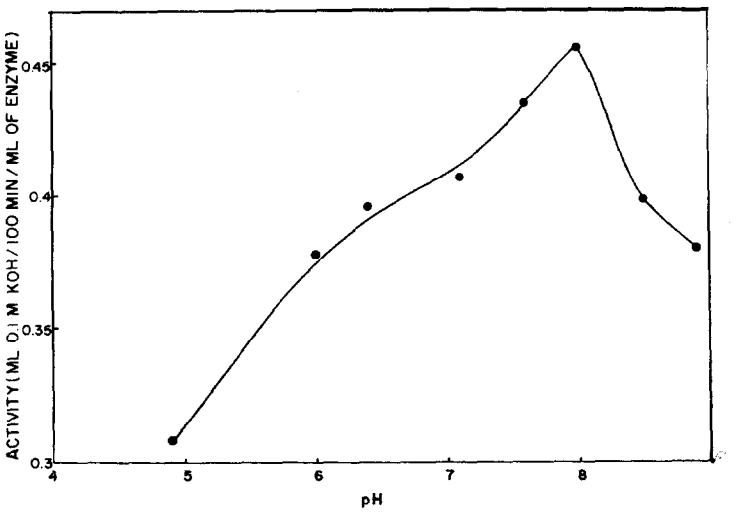

FIG. 3. pH OPTIMA OF FRACTION IV ENZYME. THE COMPLETE SYSTEM CONTAINED 240 μg OF FRACTION IV ENZYME (0·12 mg PROTEIN/ml), 0·20 mmole GLYCYLGLYCYLGLYCINE AND 0·20 mmole OF THE BUFFER AT THE INDICATE pH TO GIVE A FINAL VOLUME OF 4 ml. THESE REACTION MIXTURES WERE INCUBATED AT 34·5° FOR 100 min AND ASSAYED BY THE MODIFIED FORMOL TITRATION METHOD.

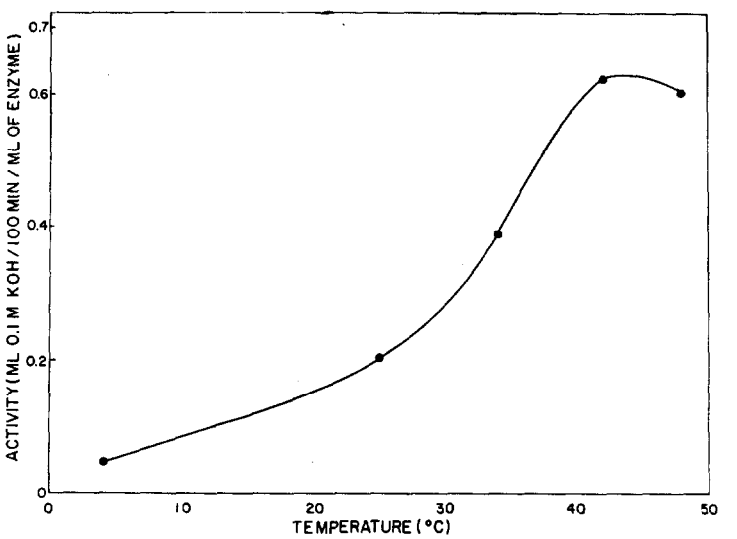

FIG. 4. TEMPERATURE OPTIMA OF FRACTION IV ENZYME. THE COMPLETE SYSTEM CONTAINED 240 μg OF FRACTION IV ENZYME (0·12 mg PROTEIN/ml), 0·20 mmole GLYCYLGLYCYLGLYCINE AND 0·20 mmole POTASSIUM PHOSPHATE, pH 7·6 TO GIVE A FINAL VOLUME OF 4 ml. THESE REACTION MIXTURES WERE INCUBATED AT THE VARIOUS TEMPERATURES FOR 100 min AND ASSAYED BY THE MODIFIED FORMOL TITRATION METHOD.

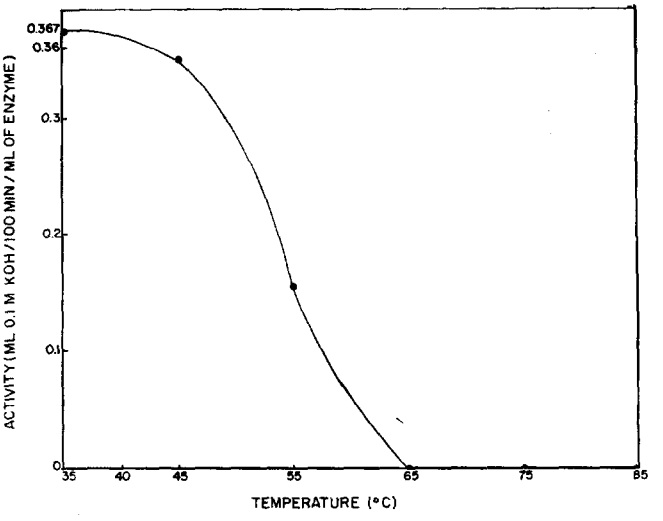

FIG. 5. TEMPERATURE OF INACTIVATION FOR FRACTION IV ENZYME. THE COMPLETE SYSTEM CONTAINED 240 μg OF FRACTION IV ENZYME (0·12 mg PROTEIN/ml), 0·20 mmole GLYCYLGLYCYLGLYCINE AND 0·20 mmole POTASSIUM PHOSPHATE, pH 7·6 TO GIVE A FINAL VOLUME OF 4 ml. THE ENZYME FRACTION IV USED AT EACH TEMPERATURE WAS EXPOSED TO THE TEMPERATURE FOR 5 min. THE REACTION MIXTURES WERE INCUBATED AT 34·5° FOR 100 min AND ASSAYED BY THE MODIFIED FORMOL TITRATION METHOD.

This purified aminopeptidase enzyme (fraction IV) was considered to be identical to the contaminating aminopeptidase activity found to be present in our previous leucine aminopeptidase activity found to be present in our previous leucine aminopeptidase fraction (peak II of enzyme fraction IV of our earlier paper).[1] The present aminopeptidase exhibits a pH optima of 8 as did the previous aminopeptidase. The two fractions also exhibit the much higher specificity for tripeptides over amides.

EXPERIMENTAL

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

Assay Methods

Protein analysis was accomplished by use of the Lowry Method.[8]

Determination of peptidase 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 0·2 mmole glycylglycylglycine, 0·2 mmole potassium phosphate, pH 7·6, and enzyme to make a total of 4 ml. The enzyme was added at O time 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 formol titration method of Sorensen.[9] A 0·75 ml volume of reaction mixture was added to 0·75 ml of 36 per cent formaldehyde solution, pH 7·0, and titrate 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 to O time sample and expressed in units (1 unit = 1 ml of 0·1 M KOH/100 min).

Purification Procedure

The purification procedure was the same as the one given in our previous paper.[2] It varied for this aminopeptidase enzyme (fraction IV) only in that its activity was located in the peak IV of the DEAE recording for the column chromatography step (Fig. 3), where as the dipeptidase activity is located in peak VI.

[8] O. N. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[9] S. P. L. SORENSEN, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.* **7**, 1 (1907).

L-leucylglycylglycine Hydrolytic Products

L-leucylglycylglycine, 0·2 mmole, was subjected to hydrolysis by 236 μg of the purified aminopeptidase for 2 hr at 34·5° in 0·2 mmole potassium phosphate buffer, pH 7·6. The reaction mixture were subjected to paper chromatography using an eluting solvent of butyl alcohol:acetic acid:water (4:1:5). After 16 hr, the chromatograph was dried and sprayed with ninhydrin. Solutions of pure glycine, L-leucine, glycylglycine, and L-leucylglycine were also chromatographed on the same paper for comparison.

Acknowledgements—This research was supported in part by a grant from USPHS, GM-11736.