Phytochemistry, 1967, Vol. 6, pp. 1215 to 1225. Pergamon Press Ltd. Printed in England

PURIFICATION AND CHARACTERIZATION OF A DIPEPTIDASE FROM *CUCURBITA MAXIMA* COTYLEDONS

FLOYD M. ASHTON and WAYNE J. DAHMEN

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

(*Received* 14 *December* 1966)

Abstract—A dipeptidase has been isolated from *Cucurbita maxima* Duch, var. Hubbard cotyledons and purified nearly 2000-fold. The enzyme appeared to be specific for L-dipeptide hydrolysis. Although L-leucylglycine was hydrolyzed more rapidly than any other L-dipeptide investigated, all twelve L-dipeptides tested were hydrolyzed by this enzyme. D-dipeptides, tripeptides, aminoacid amides, chloroacetyldipeptides, tripeptide amides, and proteins were not hydrolyzed by this dipeptidase. There was some indication of activation of L-leucylglycine hydrolysis by Mg^{++} and Mn^{++} and inhibition by EDTA, Zn^{++}, and Co^{++}. The pH optima was a broad one between 8·0 and 8·5. The dipeptidase activity with L-leucylglycine was stable to temperatures up to 85° and the activity changed very little between 25–50°. The Km value was $3{\cdot}34 \times 10^{-1}$ M/l with L-leucylglycine as the substrate at pH 7·6. This dipeptidase was different from the two aminopeptidases studied in our previous paper,[1] and these aminopeptidases were removed during the purification.

INTRODUCTION

PROTEIN is a usual reserve material in seeds. This reserve protein is broken down during germination with a concomitant rise in amino acids and amides.[2,3] 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 new protein.[4] A lesser portion of these degradation products is utilized in the storage organ for the synthesis of other essential proteins, e.g. α-amylase.[5]

Although it appears that proteases are active in seeds during germination, detailed information on the nature of these enzymes is lacking. Numerous workers have demonstrated the presence of proteinase activity in extracts of germinating seeds using casein, gelatin, or edestin as substrates. 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.

Pett[7] demonstrated protease activity with edestin as substrate and dipeptidase activity with D,L-alanylglycine as substrate in both dormant and germinating wheat seeds. These activities were further studied by Engel and Heins[8] in 1947. Irving and Fontaine[9] extracted an enzyme from peanuts, *Arachia hypogaea*, which hydrolyzed benzoyl-L-arginine amide but was completely inactive toward carbobenzoxyglycylphenylalanine, carbobenzoxyglycyltyrosine, and carbobenzoxyglutamyltyrosine. Tazawa and Hirokawa[10] reported that an

[1] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* **6**, 641 (1967).
[2] E. SCHULZE and B. UMLOUFT, *Landwirtsch. Jahrb.* **5**, 819 (1876).
[3] E. SCHULZE, *Z. Physiol. Chem.* **20**, 327 (1895).
[4] Y. OOTA, R. FUJII and S. OSAWA, *J. Biochem.* (*Tokyo*) **40**, 649 (1953).
[5] J. E. VARNER and G. R. CHANDRA, *Proc. Natl Acad. Sci. U.S.* **52**, 100 (1964).
[6] J. B. MOUNFIELD, *Biochem. J.* **30**, 549, (1930).
[7] L. B. PETT, *Biochem. J.* **29**, 1898 (1935).
[8] C. ENGEL and J. HEINS, *Biochem. Biophys. Acta* **1**, 190 (1947).
[9] G. W. IRVING, JR. and T. D. FONTAINE, *Arch. Biochem.* **6**, 351 (1945).
[10] Y. TAZAWA and T. HIROKAWA, *J. Biochem.* (*Tokyo*) **43**, 785 (1956).

extract from germinating soybean, *Glycine Soja*, seeds was able to hydrolyze gelatin but not benzoylglycine, benzoylglycylglycine, or leucylglycylglycine. Berger and Johnson[11] obtained crude extracts from dried green barley malt which hydrolyzed D,L-leucylglycine and D,L-leucylglycylglycine at a high rate and to a lesser extent D,L-alanylglycine, glycylglycine, D,L-alanylglycylglycine, and glycylglycylglycylglycine. Guitton[12] 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 those with N-terminal glycine.

There appear to be no reports in the literature on the purification of proteolytic enzymes from germinating seeds, except our recent paper reporting two aminopeptidases from *Cucurbita maxima* Duch. cotyledons.[1] However, there are two reports on the purification of proteolytic enzymes from dormant seeds.[10,13] The proteolytic enzyme which Soedigo and Gruber[13] 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[14] purified a proteinase from azuki seeds 40-fold using casein as a substrate, but did not determine its substrate specificity.

Recently, Shain and Mayer[15] 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 dipeptidase from germinating squash, *Cucurbita maxima* Duch., seeds. Evidence is present that there are at least three peptidases present. The purification and characterization of one of these peptidases, a dipeptidase, is given.

RESULTS AND DISCUSSION

The assay used to detect the activity with the various substrates was performed by an incubation and formol titration as described in detail in the experimental section. Various experiments were conducted to establish the validity of the assay. The activity with time for the crude enzyme (fraction I) and the purified enzyme (fraction IV) with L-leucylglycine as the substrate was found to be linear within the times used. The activity with various concentrations of the crude enzyme and the purified enzyme was also linear within the range used. The amount of L-leucylglycine required to saturate the purified enzyme under the conditions used was 0·10 M.

Purification

The flow sheet of the purification procedure is presented in Fig. 1. The quantitative data for the preparation of the enzyme are given in Table 1. The data from a representative purification indicate a 1962-fold purification of this dipeptidase enzyme with a 35·6 per cent recovery. The increased activity of fraction IV (35·6 per cent) relative to fraction III (28·3 per cent), as well as the increase in activity with storage for fraction IV enzyme (Table 2) suggests that an inhibitor of the enzyme was removed during these treatments.

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, hippuryl-D,L-phenyllactic acid, L-leucylglycylglycine, glycylglycylglycine, L-leucyl-L-leucine, L-phenyl-

[11] J. BERGER and M. J. JOHNSON, *J. Biol. Chem.* **130**, 641, 655 (1939).
[12] Y. GUITTON, *Bull. Soc. Franc. Physiol. Veg.* **9**, 125 (1963).
[13] R. SOEDIGO and M. GRUBER, *Biochem. Biophys. Acta* **44**, 315 (1960).
[14] S. AKUNE and S. TAKAGI, *J. Agr. Chem. (Japan)* **36**, 63 (1962).
[15] Y. SHAIN and A. M. MAYER, *Physiol. Plantarum* **18**, 853 (1965).

alanine amide, L-tyrosine amide, and glycine amide. 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 the four purification steps were measured. This was done using L-leucylglycine, glycylglycylglycine and L-leucine amide at all four levels of the purification procedure

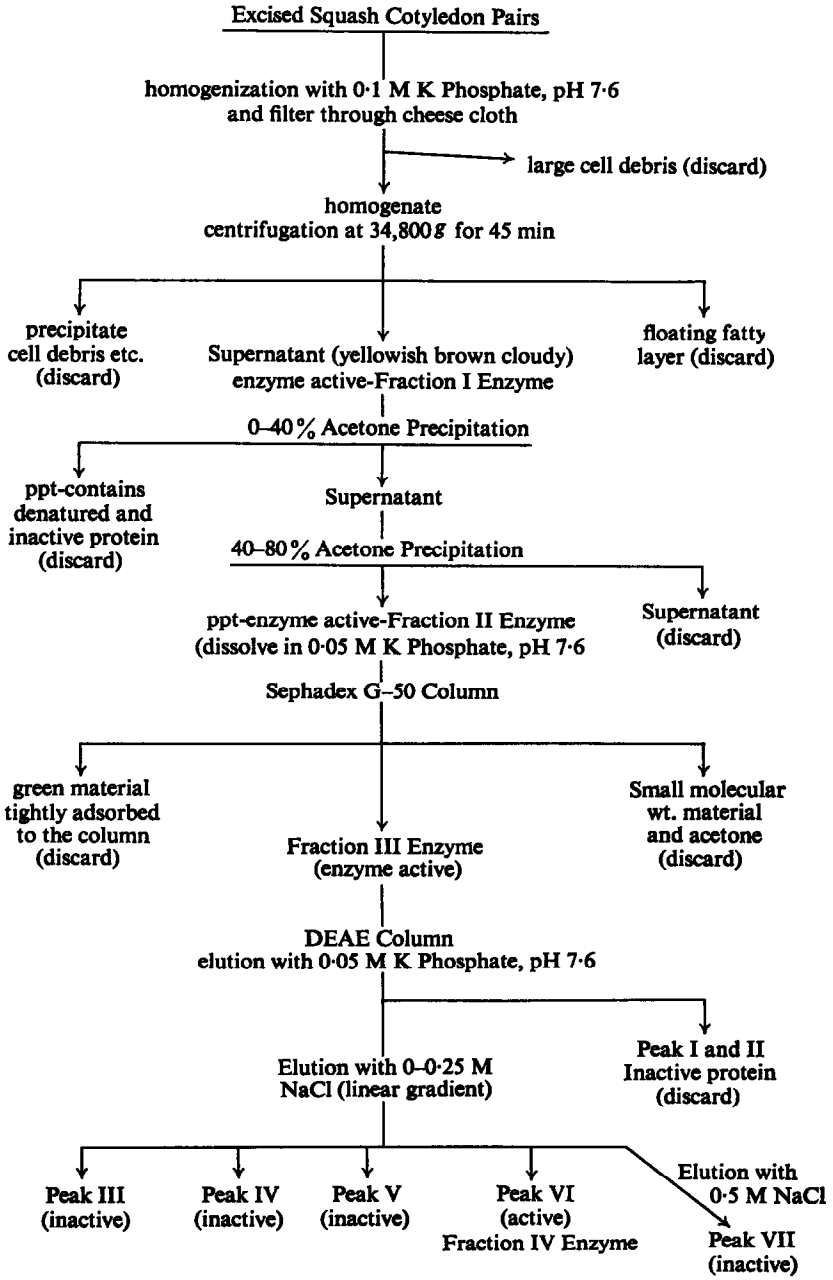

FIG. 1. FLOW CHART OF ENZYME PURIFICATION.

(Table 3). The ratios of L-leucylglycine activity to glycylglycylglycine activity varied from 9·8 in fraction I to 1·8 in fraction III. The ratios of L-leucylglycine activity to L-leucine amide activity varied from 8·1 in fraction I to 17·3 in fraction III. The activities for both glycylglycylglycine and L-leucine amide dropped to zero in the purified fraction IV enzyme, while the L-leucylglycine activity remained high. These data indicate that at least three different enzymes are present in the crude fraction according to the criteria mentioned by Irving *et al.*[16] This agrees with the data presented in our previous paper.[1]

TABLE 1. PURIFICATION OF DIPEPTIDASE

Fraction	Total volume (ml)	Protein activity		Specific activity (units/mg)	Recovery (%)	Purification (Fold over crude)
		Total (mg)	Total (units)*			
I (Crude)	245	16,790	722·0	0·0431	100	1·0
II (Acetone ppt)	36·5	181·2	208·5	1·152	28·9	26·7
III (Sephadex)	45·8	132·9	196·0	1·476	28·3	34·3
IV (DEAE)	86	2·9	240·0†	84·6	35·6†	1,962

* Unit = 1 ml of 0·1 M KOH/60 min.
† Greater than 100 per cent recovery suggests that an inhibitor was removed.

TABLE 2. STABILITY OF THE PURIFIED ENZYME TO DIALYSIS AND STORAGE AT LOW TEMPERATURE

Storage time (days) treatment	Per cent activity	Dialysis treatment (pH = 7·6)	Per cent activity
Ice			
2	99·5	6 hr with 1·5 ml enzyme against 1l. of 0·05 M K phosphate	94·9
10	104·0		
−20°			
5	112·0	8 hr with 6·4 ml enzyme against 2l. of 0·05 M K phosphate	107·9
6	113·0		
8	117·0		
13	123·0	2-8 hr and 20 min dialysis periods with 10 ml of enzyme against fresh 4l. volumes of 0·05 M tris	95·7

The Sephadex enzyme, fraction III, was placed on a DEAE cellulose column, eluted with a linear NaCl gradient, and the elutate monitored at 280 nm. All eluted fractions were analyzed for activity and only one activity peak was detected. This is referred to as Fraction IV enzyme or the purified enzyme (peak VI, Fig. 2).

The four enzyme fractions were analyzed by vertical acrylamide gel electrophoresis and the data are given in Fig. 3. These data indicate that substantial amounts of the protein impurities were removed at each stage in the purification procedure. The data indicate that fraction IV contains only one protein.

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

TABLE 3. CHANGE IN ACTIVITY RATIOS OF SUBSTRATE DURING PURIFICATION

Enzyme fraction	Substrate 0·05 M	Activity (units/ml of enzyme)	Ratio of activities substrate to L-leucylglycine
I (Crude)	L-Leucylglycine	3·375	1·000
	Glycylglycylglycine	0·344	0·102
	L-Leucine amide	0·415	0·123
II (Acetone ppt)	L-Leucylglycine	4·255	1·000
	Glycylglycylglycine	0·447	0·106
	L-Leucine amide	0·100	0·024
III (Sephadex)	L-Leucylglycine	3·605	1·000
	Glycylglycylglycine	1·992	0·552
	L-Leucine amide	0·208	0·058
IV (DEAE)	L-Leucylglycine	3·308	1·000
	Glycylglycylglycine	0·000	0·000
	L-Leucine amide	0·000	0·000

The reaction mixture for L-leucylglycine activity contained 0·75 ml of enzyme fraction, 0·15 mmole potassium phosphate, pH 7·6 and 0·15 mmole of L-leucylglycine in a total volume of 3·0 ml. These reaction mixtures were incubated at 34·5° for 60 min. The reaction mixtures for L-leucine amide and glycylglycylglycine activity contained 2 ml of enzyme fraction, 0·2 mmole potassium phosphate, pH 7·6 and 0·2 mmole of substrate in a 4 ml total volume. These reaction mixtures were incubated at 34·5° for 100 min. These samples were assayed using the modified formal titration method and all rates were corrected to 100 min. One unit = 1 ml of 0·1 M KOH per 100 min.

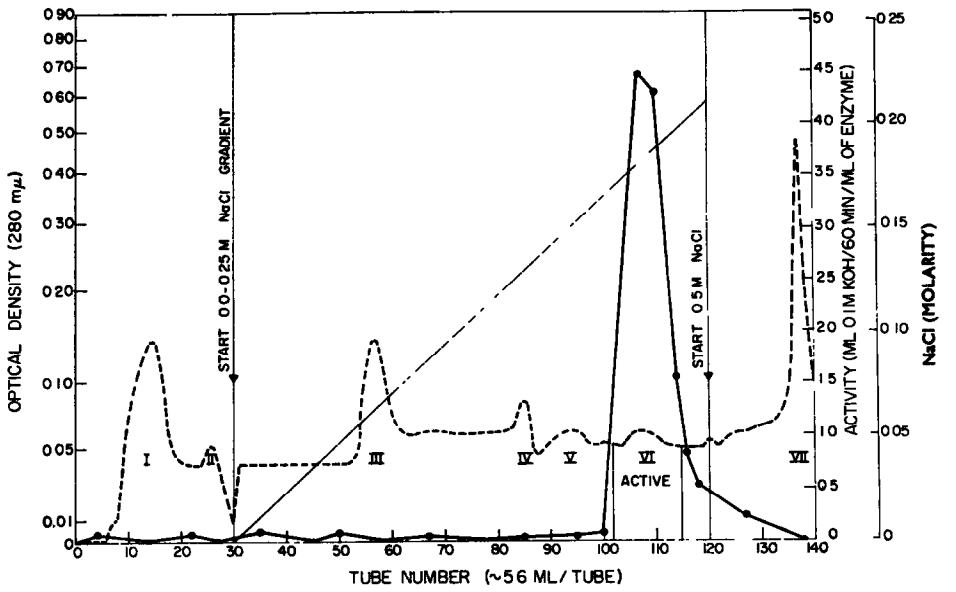

FIG. 2. COPY OF TYPICAL U.V. RECORDING OF DEAE COLUMN WITH ACTIVITY FOR L-LEUCYLGLYCINE PLOTTED AS WELL AS ABSORPTIVITY AT 280 NM. THE CONDITIONS FOR THIS COLUMN ARE GIVEN IN THE EXPERIMENTAL SECTION FOR DEAE COLUMN. THE TOTAL TIME ELAPSED FOR THE RUN WAS APPROXIMATELY 26 HR.

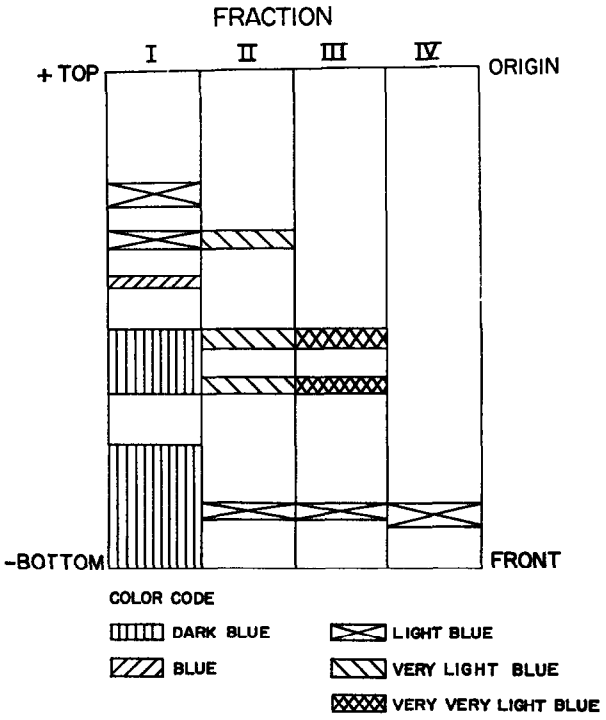

FIG. 3. VERTICAL GEL ELECTROPHORESIS OF FRACTIONS I, II, III, AND IV.

Fraction IV enzyme was subjected to ultracentrifugation and the pattern is presented in Fig. 4. The data indicates that this fraction contains only one protein.

Enzyme Properties

The fraction IV preparation showed a substrate specificity quite different than those attributed to the dipeptidase by Smith[17] in that it was capable of hydrolyzing many kinds of L-dipeptides. This fraction IV enzyme was tested for activity with the dipeptidase substrates L-leucylglycine, L-leucyl-L-alanine, L-leucyl-L-leucine, L-leucyl-L-phenylalanine, glycylglycine, glycyl-L-alanine, glycyl-L-leucine, glycyl-L-phenylalanine, glycyl-L-valine, glycyl-L-tyrosine, D,L-leucylglycine, glycyl-D-leucine and D,L-leucyl-D,L-leucine (Table 4). All twelve of the L-dipeptides were hydrolyzed to some extent, but not the D-dipeptide. The dipeptidases which have been reported in detail in the literature are all from animal systems. The glycylglycine dipeptidase of skeletal muscle and human uterus was specific for glycylglycine with a slight activity on sarcosylglycine and had a pH optima of 8·0. It was activated by Co^{++} and Mn^{++} and inhibited by Zn^{++}.[17] It did not attack benzoylglycine amide, benzoylglycylglycine, carbobenzoxyglycylglycine, benzoylglycylglycylglycine or glycine amide. Glycyl-L-leucine dipeptidase of human uterus was quite specific for glycyl-L-leucine, and sarcosyl-L-leucine was acted on slightly. It did not attack carbobenzoxyglycyl-L-leucine, carbobenzoxyglycyl-L-leucine amide or glycyl-L-leucine amide.[18] Prolidase from swine kidney (purified 12,000-fold) was a Mn^{++} activated enzyme inhibited by Co^{++} and Zn^{++} and had a pH optima of 8·0. It

[17] E. L. SMITH, *J. Biol. Chem.* **173**, 571 (1948).
[18] E. L. SMITH, *J. Biol. Chem.* **176**, 9 (1948).

was specific for dipeptides bearing the special property of having a free amino group of L-proline or hydroxy-L-proline.[19] Prolidase did not hydrolyze L-leucylglycine, glycyl-L-leucine, glycylglycine, glycylglycylglycine, D,L-alanylglycine, glycyl-D,L-alanine, glycyl-D,L-valine, glycyl-L-tyrosine, or L-leucine amide. A free amino group on the dipeptides was

TABLE 4. SUBSTRATE SPECIFICITY STUDIES ON THE PURIFIED DIPEPTIDASE

Substrate type and molarity	Relative hydrolysis L-leucylglycine = 100	
	0·1 M (%)	0·05 M (%)
L-Leucylglycine	100·0	100·0
L-Leucyl-L-alanine (0·05 M)	15·3	19·4
L-Leucyl-L-leucine (0·042 M)	8·3	10·6
L-Leucyl-L-phenylalanine (½ sat.)	4·2	5·3
Glycyl-L-glycine (0·050 M)	24·2	30·6
Glycyl-L-alanine (0·050 M)	25·9	32·7
Glycyl-L-leucine (0·050 M)	23·9	30·3
Glycyl-L-phenylalanine (0·050 M)	23·1	29·3
Glycyl-L-valine (0·050 M)	18·3	23·2
Glycyl-L-tyrosine (0·050 M)	21·5	27·3
D,L-Leucylglycine (0·050 M)	14·6	18·5
Glycyl-D-leucine (0·050 M)	0·0	0·0
D,L-Leucyl-D,L-leucine (½ sat.)	0·0	0·0
L-Leucyl-leucyl-L-leucine (½ sat.)	0·0	0·0
L-Leucyl-glycylglycine (0·050 M)	0·0	0·0
L-Leucyl-glycyl-L-leucine (½ sat.)	0·0	0·0
Glycylglycylglycine (0·0375 M)	0·0	0·0
L-Leucine amide (0·0375 M)	0·0	0·0
L-Phenylalanine amide (0·050 M)	0·0	0·0
L-Tyrosine amide (0·05 M)	0·0	0·0
Glycine amide (0·05 M)	0·0	0·0
L-Serine amide (0·050 M)	0·0	0·0
Glycyl-L-phenylalanine amide acetate (0·009 M)	0·0	0·0
Chloroacetylglycyl-L-leucine (0·050 M)	0·0	0·0
Chloroacetyl-glycyl-L-glycine (0·050 M)	0·0	0·0
Hippuryl-L-leucine amide (½ sat.)	0·0	0·0
Hippuryl-D,L-phenyllactic acid (0·05 M)	0·0	0·0
Casein (0·7%)	0·0	0·0

The complete reaction mixture for the substrates not described below contained 0·2 mmole potassium phosphate, pH 7·6, 2 ml of enzyme (fraction IV), and substrate to make a total of 4·0 ml. The complete system for the substrate L-leucylglycine contained 0·75 ml of the above enzyme, 0·15 mmole potassium phosphate, pH 7·6, and substrate in a total volume of 3·0 ml. The reaction mixtures were incubated at 34·5° for 100 min (60 min for L-leucylglycine) and assayed by the modified formal titration method. The enzyme was also tested for activity with 0·05 M hippuryl phenyllactic acid using a pH stat[21] and 0·7% casein using the standard protease assay.[22]

essential as well as the L-histidine residue on the free carboxyl end for carnosinase (swine kidney) activity.[20] Thus, the reported dipeptidases appear to be limited to a quite specific

[19] N. C. DAVIS and E. L. SMITH, *J. Biol. Chem.* **224**, 261 (1957).
[20] N. C. DAVIS, *J. Biol. Chem.* **223**, 935 (1956).
[21] M. O. HENSEN, *Acta Chem. Scand.* **10**, 151 (1956).
[22] N. C. DAVIS and E. L. SMITH, *Methods Biochem. Analy.* **2**, 215 (1955).

TABLE 5. ACTIVATION AND INHIBITION STUDIES WITH METAL IONS AND EDTA

Type of additive	Dialyzed enzyme	
	Units/mg protein	Per cent change
No addition	46·0	0·0
EDTA	45·0	−0·02
Mg^{++}	52·2	+13·4
Mg^{++}+EDTA	39·2	−14·7
Mn^{++}	50·6	+10·1
Mn^{++}+EDTA	42·0	−8·6
Zn^{++}	40·0	−12·9
Zn^{++}+EDTA	40·4	−12·2
Co^{++}	38·3	−16·8
Co^{++}+EDTA	39·6	−13·7
MoO_4^-	49·0	+6·6
MoO_4^-	48·5	+5·4

The complete system contained 0·051 mg of fraction IV (0·068 mg/ml), 0·3 mmole of L-leucylglycine, 0·15 mmole Tris, pH 7·6 and metal ion ion (0·005 M) or EDTA (0·005 M) or both plus H_2O to make a total of 3 ml. These reaction mixtures were incubated at 34·5° for 60 min and assayed by the modified formol titration method. One unit = 1 ml 0·1 M KOH/60 min.

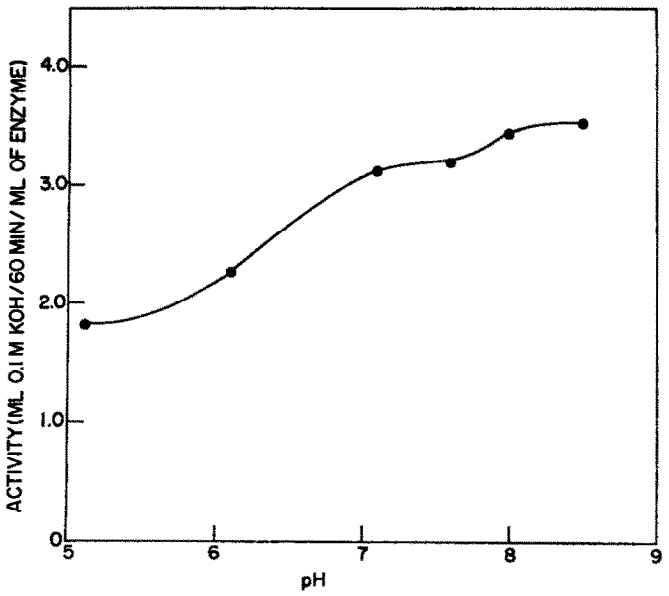

FIG. 5. pH OPTIMA OF FRACTION IV ENZYME. THE COMPLETE SYSTEM CONTAINED 51 μG OF FRACTION IV ENZYME (0·068 MG PROTEIN/ML), 0·3 MMOLE L-LEUCYLGLYCINE AND 0·15 MMOLE OF THE BUFFER AT THE INDICATED pH TO GIVE A FINAL VOLUME OF 3 ML. THESE REACTION MIXTURES WERE INCUBATED AT 34·5° FOR 60 MIN AND ASSAYED BY THE MODIFIED FORMOL TITRATION METHOD.

range of dipeptides, but our dipeptidase attacks all L-dipeptides tested. The following amino-peptide and tripeptide substrates were not hydrolyzed by the fraction IV enzyme: glycyl-D-leucine, L-leucyl-L-leucyl-L-leucine, L-leucylglycylglycine, L-leucine amide, L-phenylalanine amide, L-tyrosine amide, glycine amide and L-serine amide. The endopeptidase substrates chloroacetylglycyl-L-leucine, chloroacetylglycyl-L-leucine, chloroacetylglycylglycine, hippuryl-L-leucine amide and casein were not attacked by the fraction IV enzyme. The carboxypeptidase substrate hippuryl-D,L-phenyllactic acid was not hydrolyzed by our dipeptidase fraction IV enzyme.

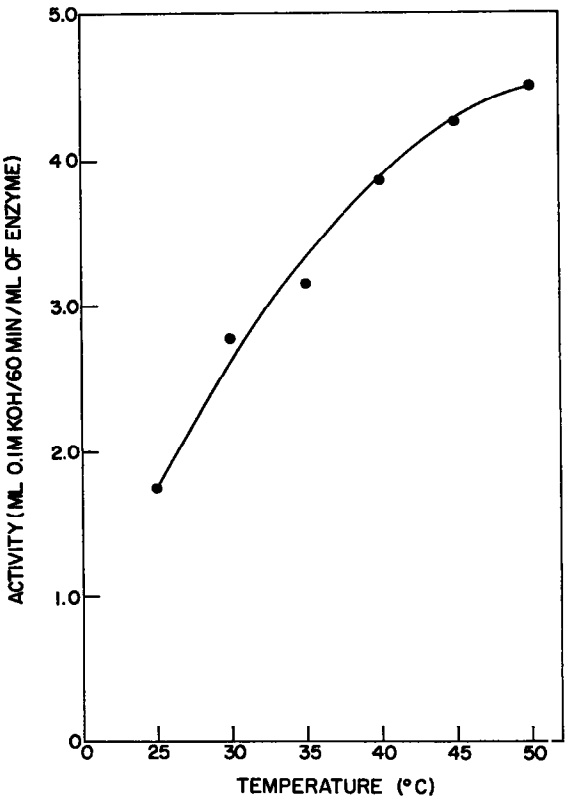

FIG. 6. TEMPERATURE OPTIMA OF FRACTION IV ENZYME. THE COMPLETE SYSTEM CONTAINED 51 μG OF FRACTION IV ENZYME (0·068 MG PROTEIN/ML), 0·3 MMOLE L-LEUCYLGLYCINE AND 0·15 MMOLE POTASSIUM PHOSPHATE, pH 7·6 TO GIVE A FINAL VOLUME OF 3 ML. THESE REACTION MIXTURES WERE INCUBATED AT THE VARIOUS TEMPERATURES FOR 60 MIN AND ASSAYED BY THE MODIFIED FORMOL TITRATION METHOD.

The effects of selected metal ions and EDTA on the activity of the purified-dialyzed enzyme is given in Table 5. This data showed that Mg^{++} and Mn^{++} ions increased the activity of this preparation and that EDTA nullified this activation. EDTA alone had little effect on the activity. Zn^{++} and Co^{++} inhibited the activity. Mo^{++} had some stimulatory effect which was not nulified by EDTA.

The pH optima experiment indicated a rather broad pH optima at pH 8·0 to 8·5, (Fig. 5). Fraction IV appeared to have the high temperature optima characteristic of many proteolytic enzymes. The range covered was between 25–50°, (Fig. 6). The heat stability of fraction IV was very high and the L-leucylglycine activity declined rapidly at 85° and above (Fig. 7). The

Km determined for the purified enzyme (fraction IV) with L-leucylglycine as the substrate was $3{\cdot}34 \times 10^{-1}$ M/l. The enzyme was stable over long periods when stored at $-2°$ in 0·05 M potassium phosphate, pH 7·6 (Table 2). The enzyme was also stable to dialysis against 0·05 M potassium phosphate, pH 7·6 (Table 2). This dipeptidase (fraction IV) was considered to be highly purified from the substrate specificity for dipeptides (Table 4), the high degree of purification (Table 1) and the changes in activity ratios (Table 3).

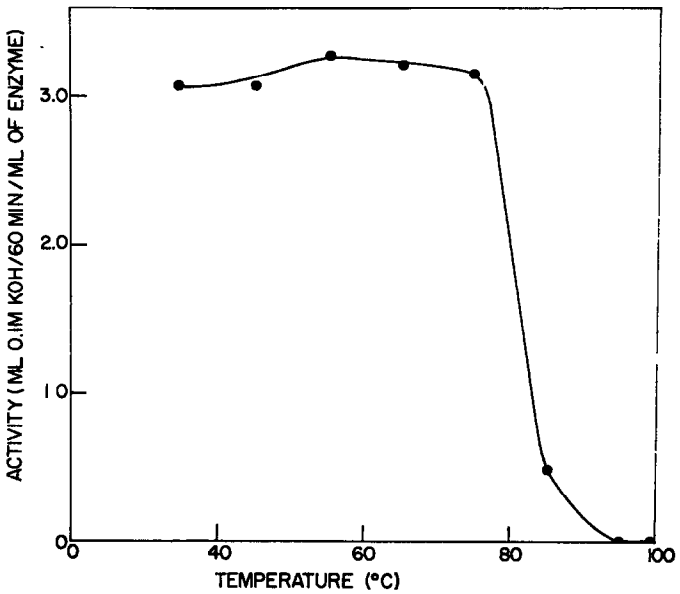

FIG. 7. TEMPERATURE OF INACTIVATION FOR FRACTION IV ENZYME. THE COMPLETE SYSTEM CONTAINED 51 μG OF FRACTION IV ENZYME (0·068 MG PROTEIN/ML), 0·3 MMOLE L-LEUCYLGLYCINE AND 0·15 MMOLE OF POTASSIUM PHOSPHATE, pH 7·6, TO GIVE A FINAL VOLUME OF 3 ML. THE ENZYME FRACTION IV USED AT EACH TEMPERATURE WAS EXPOSED TO THE GIVEN TEMPERATURE FOR 5 MIN. THE REACTION MIXTURES WERE INCUBATED AT 34·5° FOR 60 MIN AND ASSAYED BY THE MODIFIED FORMOL TITRATION METHOD.

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 three days in the dark at 30°.

Assay Methods

Protein analysis was accomplished by use of the Lowry Method.[23]

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 0·3 mmole of L-leucylglycine, 0·15 mmole potassium phosphate, pH 7·6, and enzyme to make a total volume of 3 ml. The enzyme was added at 0 time and a sample was removed immediately and placed in ice until assayed. The reaction mixtures were incubated at 34·5° for 60 min in a shaking water bath. A sample was removed at 60 min and placed in ice until assayed. The analysis of samples was accomplished by a modification of the formol titration method of Sorensen.[24] A 0·5 ml volume of reaction mixture was added to 0·5 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 60-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/60 min).

[23] O. N. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[24] S. P. L. SORENSEN, *Compt. Rend. Trav. Lab. Carlesberg, Ser. Chim.* **7**, 1 (1907).

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 0·1 M potassium phosphate, pH 7·6. 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 or fraction I. All subsequent manipulations were carried out at near 0° by use of ice buckets or working in a cold room.

Acetone ppt. enzyme (fraction II). A total of 245 ml crude enzyme was placed in a beaker in a salt-ice bath −4° and constantly mixed with a magnetic stirrer. A total of 162·8 ml of reagent grade acetone at −10° was added slowly to the above enzyme which gave a 40% acetone solution. After a 10-min period of equilibration, this suspension was centrifuged at 34,800 *g* for 15 min at −5° in a refrigerated centrifuge. The supernatant fluid was removed and placed in a beaker in a −4° salt-ice bath, while this fluid was constantly mixed by a magnetic stirrer, 817·7 ml of reagent grade acetone at −10° was added which gave an 80% acetone solution. After an equilibration period and centrifugation under the conditions indicated above, the precipitate was dissolved in 33·5 ml of 0·05 M potassium phosphate, pH 7·6 yielding a final volume of 36·5 ml. This clear brownish solution is referred to as the acetone ppt. enzyme or fraction II.

Sephadex enzyme (fraction III). A total of 35 ml of fraction II enzyme was passed through a Sephadex G-50 column in the 0·05 M potassium phosphate, pH 7·6, form giving a final volume of 45·8 ml. This almost colorless clear solution is referred to as the Sephadex enzyme or fraction III.

DEAE enzyme (fraction IV). A column of 0·75 meq/g DEAE cellulose 30 × 2 cm, was prepared using 0·05 M potassium phosphate buffer, pH 7·6, according to the methods of Peterson and Sober.[25] Approximately 44·3 ml of fraction III enzyme (128·5 mg of protein) was added to the column at 0·5 ml/min using a constant flow pump. The column was then eluted with about 240 ml of 0·05 M potassium phosphate, pH 7·6 and the inactive fractions displaced were discarded. The column was then eluted with a linear gradient of 0–0·25 M NaCl in 0·05 M potassium phosphate, pH 7·6, at a flow rate of about 0·56 ml/min using a constant flow pump. The fraction collection was 5–5·6 ml per tube and a recording u.v. absorption analyzer was used to detect peaks. The active fraction was present in tubes 102–115 or peak VI on the u.v. absorption recording at 280 mμ. This fraction is referred to as DEAE enzyme or fraction IV. The volume of this fraction was 86 ml.

Vertical Gel Electrophoresis

Enzyme fractions I, II, III, IV were submitted to vertical gel electrophoresis under the conditions given in our previous paper.[1] The dialyzed samples which were added to the top of the gel contained the following amounts of protein fraction I −4·0 mg, fraction II −3·9 mg, fraction III −3·4 mg, fraction IV −5·2 mg.

Ultracentrifuge Pattern

A sample of fraction IV was subjected to ultracentrifugation. The sample contained 13·1 mg of protein per ml in 0·1 M potassium phosphate, pH 7·6. The sample had been concentrated by lyophilization and treated with Sephadex and dialysis.

Acknowledgements—This research was supported in part by a grant from USPHS, GM-11736. We are grateful to Professor Richard S. Criddle for the ultracentrifuge analyses.

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