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PARTIAL PURIFICATION AND CHARACTERIZATION OF BARLEY PEPTIDE HYDROLASES

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Abstract—Two peptidases from germinated barley were partially purified and characterized. Recycling filtration on dextran gel was used in an improved preparative procedure. BAPA-ase (barley peptide hydrolase A) was found to be inactivated by low pH, mildly resistant to heat treatments and activated by relatively high concentrations (10^{-2}–$10^{-1\,3}$ M) of Ca^{2+} and Mg^{2+}. This enzyme had a very low order of substrate specificity when tested with a variety of dipeptides. It did not hydrolyze several possible protein substrates. BAEE-ase (barley peptide hydrolase B) was inactivated at high pH values, was more resistant than BAPA-ase to heat inactivation and was not activated by a variety of metal ions. This enzyme exhibited a relatively high specificity when tested with simple peptide substrates. It also did not hydrolyze proteins. Values for K_m and V with alpha-*N*-benzoyl-L-arginine ethyl ester as substrate are given.

INTRODUCTION

GERMINATED barley contains an acidic and a neutral enzyme each of which hydrolyzes alpha-*N*-benzoyl-L-arginine ethyl ester (BAEE), and an acidic enzyme which hydrolyzes alpha-*N*-benzoyl-DL-arginine p-nitroanilide (BAPA).[1] Their substrates suggest that these enzymes are peptidases. This has been tentatively accepted until information on their ability to hydrolyze other authentic substrates is available. They have, therefore, been referred to as acidic and neutral BAEE-ase, and BAPA-ase. The two acidic enzymes have not been shown to be distinct from one another although they have been purified considerably.[1]

This paper describes: (*a*) an improved procedure for the purification of the enzymes from germinated barley; (*b*) some effects of pH, temperature, and metal ions upon enzyme activity; (*c*) kinetic constants for BAEE-ase activity; and (*d*) tests for hydrolysis of a number of dipeptides and proteins.

RESULTS AND DISCUSSION

Purification of Peptidases

The principal improvement incorporated in the the procedure for purification of the peptidases is the use of recycling gel filtration.[2] Four to five cycles through a 90 cm column of dextran gel, corresponding to 3·6 to 4·5 m of gel, produced substantial increases in the specific activities and a relatively high yield of both enzymes. The data in Table 1 show

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[1] W. C. BURGER, N. PRENTICE, J. KASTENSCHMIDT and J. D. HUDDLE, *Cereal Chem.* **43**, 546 (1966).

[2] J. PORATH and H. BENNICH, *Arch. Biochem. Biophys.* Suppl. **1**, 152, (1962).

TABLE 1. PURIFICATION OF PEPTIDASES FROM GERMINATED BARLEY

Purification step	Vol. (ml)	Protein (mg/ml)	Activity U/ml		Specific activity U/mg protein		Recovery %	
			BAPA-ase	BAEE-ase	BAPA-ase	BAEE-ase	BAPA-ase	BAEE-ase
Dialyzed extract	486	1·9	32	360	17	189	100	100
Acidic peptidase								
CMC supernatant	99	0·81	164	204	200	246	104	11
5th Cycle G-100	24	0·18	223	249	1240	1389	35	3
Neutral peptidase								
CMC eluate	62	17	13	2082	0·8	123	5	74
5th Cycle G-100	42	0·28	0	1998	0	7140	0	48

specific activities of 1240, 1389 and 7140 units per mg protein for BAPA-ase, acidic BAEE-ase and neutral BAEE-ase, respectively. These are to be compared with values of 623, 837, and 2463 obtained with earlier preparations from the same sample of Trophy barley which were filtered through 1·2 m of gel.[1]

From the relative amounts of BAPA-ase and BAEE-ase in the purified acidic peptidase fraction the amount of the latter activity in the dialyzed extract is calculated to be approximately 10 per cent of the esterase activity in the extract. Although this amount of activity is relatively small, it is consistently found in the BAPA-ase fractions and coincides with the elution volume of the BAPA-ase during recycling on dextran gel columns during purification.

The data in Table 1 show little evidence of the inhibitor of BAPA-ase that is normally found in barley or malt extracts and often remains in these after dialysis.[3] This is indicated by the lack of large increase in the recovery of enzyme in the CMC supernatant when compared with the dialyzed extract. The particular experiment summarized in Table 1 included extensive dialysis against 0·005 M acetate buffer, pH 5·5, i.e. five changes of buffer instead of three changes as used in earlier work. This additional dialysis may have removed more of the inhibitory materials and thus reduced the degree of enhancement of BAPA-ase activity that normally occurs upon treatment of the dialyzed extract with carboxymethyl cellulose.

The Effect of pH *on Peptidase Activity*

Table 2 shows the effect of pH and various buffering ions on the barley enzymes. The neutral BAEE-ase is resistant to low pH but is inactivated at pH values of 8 or higher, the rate or degree of inactivation depending upon the particular buffer used. Tris chloride buffer was found to inactivate the neutral BAEE-ase more rapidly at pH 8 than the anionic phosphate and TES buffers. Redialyzing at a lower pH in acetate buffer did not restore the activity to the inactive enzyme that was produced in Tris buffer.

TABLE 2. THE EFFECT OF pH ON PEPTIDASE ACTIVITY

Buffer (0·05 M)	pH	BAPA-ase	Acidic BAEE-ase	Neutral BAEE-ase
		% of original activity		
Tartarate	3·00	0	0	120
	3·50	0	0	96
Acetate	4·00	38	23	98
	5·00	82	92	—
	5·50	110	110	93
Phosphate	8·00	110	95	88
TES	8·00	110	105	40
Tris-chloride	8·00	93	95	8
	8·20	—	100	3
	8·40	—	93	2
	8·60	87	118	0
	8·80	110	105	0
Borate	8·80	110	105	0
	9·20	85	112	0
Glycine	10·00	98	103	0

[3] E.-M. SUOLINNA, J. MIKOLA and T.-M. ENARI, *J. Inst. Brewing* **71**, 519 (1965).

Similar treatments of BAPA-ase revealed a decrease in activity upon exposure to pH values of 5 or below. The pattern of activity obtained with the acidic BAEE-ase with the exception of that treated with borate buffer, pH 9·2, closely followed that of the BAPA-ase and suggests the two activities are associated with the same enzyme.

The Effect of Freezing, Lyophilization and Heating on Peptidase Activity

Neutral BAEE-ase, BAPA-ase and acidic BAEE-ase were unaffected by repeated freezing and thawing of their solutions. Similarly, lyophilization or storage of lyophilized samples for 2 months reduced the activity of the BAPA-ase and neutral BAEE-ase by approximately 10 per cent. Storage of the two enzyme preparations in the gel filtration buffer for 2 months at 4° resulted in about 30 per cent reduction in BAPA-ase or acidic BAEE-ase but no loss in neutral BAEE-ase. Storage of frozen enzyme solution for 2 months resulted in no loss of neutral BAEE-ase. Under the same conditions the acidic peptidase suffered no loss of acidic BAEE-ase activity but its BAPA-ase content was reduced by 23 per cent.

The activities of these enzymes when held at 35, 45, and 55° are given in Table 3. The close agreement between the responses of BAPA-ase and BAEE-ase, as with inactivation by low pH, again suggests that the hydrolysis of the anilide and the ester is catalyzed by the same enzyme. The neutral BAEE-ase is considerably more stable to higher temperature and was unaffected after 1 hr at 45°.

TABLE 3. THE EFFECT OF TEMPERATURE ON PEPTIDASE ACTIVITY

Temperature* (°C)	BAPA-ase	Acidic BAEE-ase	Neutral BAEE-ase
35	70	69	100
45	0	0	100
55	—	—	63

* Samples treated 1 hr at designated temperatures.

Effect of Metal Ions

When Ca, Mg, Mn, Ni and Zn ions were examined for possible stimulation of activity the neutral BAEE-ase preparation was not stimulated at concentrations of 10^{-7} to 10^{-1} M, and all ions were inhibitory at concentrations above 10^{-4} M. The acidic BAEE-ase and the BAPA-ase were stimulated by relatively high concentrations of Ca^{2+}, Mg^{2+} and Mn^{2+}.

Figure 1 shows the effect of metal ion concentration upon BAPA-ase activity. Calcium and magnesium ions were maximally effective in the range of 10^{-2} to $10^{-1\,3}$ M when added to the substrate solution approximately 1 min prior to enzyme addition. At only slightly higher concentrations, $10^{-1\,3}$ M Ca^{2+} or $10^{-1.2}$ M Mg^{2+}, the stimulation decreases precipitously and these ions are inhibitory at 10^{-1} M. The corresponding plot for acidic BAEE-ase (not shown) revealed maximal stimulation (20–30 per cent) near $10^{-1\,6}$ M Ca^{2+} and Mg^{2+} and somewhat higher (46 per cent) for $10^{-2\,3}$ M Mn^{2+}.

Incubation of metal ion with substrate for 1 hr prior to addition of enzyme, or incubation of metal ion with enzyme for the same period prior to addition of substrate, did not cause stimulation of activity at metal ion concentrations less than 10^{-3} M. It therefore appears that time is not a limiting factor in the combining of the metal ion with either the substrate or enzyme individually.

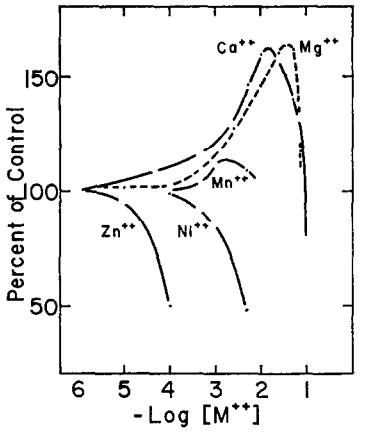

FIG. 1. EFFECT OF METAL IONS UPON BAPA-ASE ACTIVITY.

The requirement for relatively high (10^{-2} M) concentrations of calcium and magnesium for enhancement of activity suggests a weak association between the metal and the enzyme, substrate or enzyme–substrate complex.

The consistent occurrence of low levels of BAEE-ase in the BAPA-ase preparations during purification, the close agreement of rates of denaturation by heat for the two activities as well as the similarities in their response to various pH and metal ion treatments are consistent with the acidic esterase activity being a secondary or quantitatively lesser action of the BAPA-ase. However, the ratio of these two activities varies somewhat depending upon the treatment given the preparations. Dialysis against borate buffer, pH 9·2 (Table 2), or prolonged storage of frozen enzyme solution cause a reduction in BAPA-ase activity but have no effect or a slight stimulatory effect upon the BAEE-ase activity. Such differences might be attributed to slight changes in the conformation of the enzyme which alter its relative abilities to act on the two substrates.

Determination of Kinetic Constants

D-BAPA has been shown to be an effective competitive inhibitor of trypsin in the hydrolysis of DL-BAPA.[4] The rates of hydrolysis obtained in the present study are in all likelihood

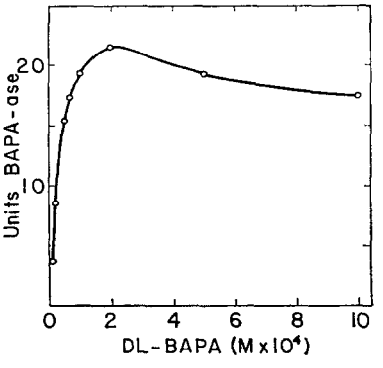

FIG. 2. VARIATION IN RATE OF BAPA-ASE ACTIVITY WITH BAPA CONCENTRATION.

[4] B. F. ERLANGER, N. KOKOWSKY and W. COHEN, *Arch. Biochem. Biophys.* **95**, 271 (1961).

those of the L-isomer in the presence of an initial equimolar concentration of the D-isomer. Figure 2 shows the relation between reaction rate of BAPA-ase and various concentrations of DL-BAPA. A mild inhibition is apparent at DL-BAPA concentrations above 2×10^{-4} M. It is not known if the D-isomer is responsible for the apparent substrate inhibition obtained at the higher levels of DL-BAPA. At $5–10 \times 10^{-4}$ M a linear response to increased enzyme concentration occurs and the requirements for a useful assay procedure are satisfied. In view of the presence of the D-isomer of BAPA and the occurrence of substrate inhibition, a determination of K_m, if possible, was deemed to be meaningless.

Similar experiments with L-BAEE as substrate for the acidic and neutral enzyme preparations were more fruitful since L-BAEE is not inhibitory at suitable working concentrations. Plots for determining K_m and V gave the following values:

acidic BAEE-ase, $K_m = 4{\cdot}4 \times 10^{-5}$ M, $V = 18{\cdot}9$ units;
neutral BAEE-ase, $K_m = 3{\cdot}8 \times 10^{-4}$ M, $V = 80{\cdot}6$ units.

Hydrolysis of Simple Peptides and Esters

A number of simple peptide substrates were used to examine the specificity requirements of the two peptidases. Table 4 summarizes the results obtained after incubation for 16 hr at 35° and pH 6·7.

TABLE 4. HYDROLYSIS OF PEPTIDE AND ESTER SUBSTRATES

Substrate	BAPA-ase	BAEE-ase	Substrate	BAPA-ase	BAEE-ase
Gly-gly	+	−	Ala-phe	+	−
Ala-val	+	−	Gly-phe	+	±
Leu-gly		−	Gly-try	+	±
Gly-thr	+	−	Gly-tyr	+	+ slow
Gly-leu	+	±	Cbz-glu-tyr	?	+
Ala-ser	?	−	Phe-leu	+	+
Gly-gly-gly	+	−	Try-leu	+ slow	+
Ala-gly-gly	+	−	Try-gly	+	+ slow
Leu-gly-gly	+	+ slow	Gly-lys	+ slow	+ complete
Hippuryl-gly	−	+ slow	Gly-glu	+	
Penta-gly	+	−	Glu-asn	?	+ complete
Lys-gly	+	−	Acetyl-tyr-ethyl ester	?	+ complete
Ala-lys	+ slow	−	Tyr ethyl ester	?	+ complete
Lys-ala	+ slow	-	γ-ethyl-glu	?	−
Tyr-gly	+	?			
Ala-asn	+	−			

The data show that the two enzymes differ greatly in their substrate requirements. BAPA-ase hydrolyzed most of the peptides employed; those instances where results were indefinite with this enzyme resulted when hydrolysis occurred in the substrate controls and are indicated in Table 4 by question marks. The only peptide that was definitely not hydrolyzed by BAPA-ase under the conditions employed was hippurylglycine.

Neutral BAEE-ase, on the other hand, appears to have a much higher order of specificity at pH 6·7. This enzyme did not hydrolyze fourteen of the twenty-nine peptides or amino acid esters examined. Of the three tripeptides examined leucylglycylglycine was the only one attacked. Hippuryl glycine was also slowly hydrolyzed. Alanine residues on the carboxyl side of the peptide bond appear to hinder hydrolysis of the bond. alanyllysine was not

hydrolyzed but glycyllysine was completely hydrolyzed; alanylglycylglycine was not attacked but leucylglycylglycine was slowly hydrolyzed.

The presence of an aromatic residue on the amino side of the peptide bond was found to be conducive to very low levels of hydrolysis by BAEE-ase; aromatic residues on the carboxyl side tended to promote hydrolysis. The presence of lysine on the amino side of the peptide link resulted in complete hydrolysis except in alanyllysine where the alanyl residue prevented such promotion. Glutamyl asparagine, acetyl tyrosine ethyl ester and tyrosine ethyl ester were all completely hydrolyzed but the γ-ethyl ester of glutamic acid was not.

Action of Peptidases of Proteins

The two peptidases were examined for their ability to hydrolyze proteins. The results (Table 5), expressed as per cent of total substrate nitrogen that was rendered acid-soluble by the enzymes, indicate that very feeble or no hydrolysis occurred after as long as 16 hr at 35°. By contrast, a relatively crude preparation of acidic proteinase from germinated barley described previously[1] rendered 60 per cent of the nitrogen of purified human hemoglobin acid-soluble in 2 hr at pH 4 and 35°.

TABLE 5. ACTION OF BAPA-ASE AND BAEE-ASE ON PURIFIED PROTEINS

	Per cent available N rendered acid-soluble	
Substrate	BAPA-ase	Neutral BAEE-ase
3 hr, 35°		
Hemoglobin	0·5	0·5
Lysozyme	0·2	0·5
Cytochrome C	0·6	0
16 hr, 35°		
Hemoglobin	0·9	0·9
Lysozyme	0	0·8

Reaction mixture: 5 mg substrate.
1 ml buffer, 0·005 M HPO_4^{2-}, pH 7·0.
0·2 ml enzyme—BAEE-ase, 34·4 U, 0·024 mg protein.
BAPA-ase 5·6 U, 0·060 mg protein.
0·1 ml TCA (100 mg TCA per 0·1 ml) to stop reaction.

The ability of both of the enzymes to hydrolyze peptide substrates and the lack of any significant action by them on protein substrates indicates that these enzymes are peptidases or, in accordance with recommendations of the International Union of Biochemistry, peptide hydrolyases.[5] To differentiate between the acidic and the neutral peptide hydrolase, we suggest that the acidic enzyme be called barley peptide hydrolase A and the neutral enzyme be called barley peptide hydrolase B.

The preference of these enzymes for peptide substrates over protein substrates strongly suggests that these enzymes are not responsible for the hydrolysis of the storage proteins of barley kernel endosperm during the development of the barley seedling. This view is supported by the absence of either peptide hydrolase in the so-called starchy endosperm of barley or

[5] International Union of Biochemistry, *Enzyme Nomenclature*, p. 34. Elsevier, Amsterdam (1965).

wheat during the first 6 days of growth when this body of reserve material undergoes striking physical change.[6] The two hydrolases appear to be intracellular enzymes with no role in the degradation of hordein or hordenin as such.

EXPERIMENTAL

Materials and Methods

Barley. Hordeum vulgare, variety Trophy, 1963 crop from Madison, Wis.

Reagents. Potassium gibberellate, "Gibrel" (Mark 60830), Merck and Co; cytochrome c, from horse heart, type VI, lot 115B-7140, Sigma Chemical Co.; ribonuclease A, from bovine pancreas, protease free, type X11-A, lot 65B-8590, Sigma Chemical Co.; human hemoglobin, 2 × crystallized, lot 12, code HM 2262, Pentex Inc , Kankakee, Illinois.

Peptides and related compounds were obtained from Mann Research Laboratories, Inc., M.A. grade, L-isomers, with the following exceptions: glycylglycine, Sigma Chemical Co.; L-alanyl-L-lysine, L-lysyl-L-alanine, L-phenylalanyl-L-leucine, L-tryptophyl-L-leucine, L-tryptophylglycine and glycyl-L-lysine, Yeda Research and Development Co. Ltd., Rehovoth, Israel; DL-alanyl-DL-asparagine and L-glutamyl-L-asparagine, C.P. grade, Mann Research Laboratories, Inc.; glycyl-DL-threonine, DL-alanyl-DL-serine and γ-ethyl glutamate, Nutritional Biochemicals Corporation; glycyl-L-leucine, glycyl-DL-phenylalanine, glycyl-L-tryptophan, glycyl-L-tyrosine, General Biochemicals Inc., Chagrin Falls, Ohio.

Germination of Barley

The grain was steeped in tap water at 16° until the moisture content was approximately 45 per cent. After steeping, 2·25 mg Gibrel in 10 ml of water was applied to each 225 g original dry weight of barley. The barley was germinated for 6 days at 45 per cent moisture and 16°.

The germinated products were lyophilized directly to approximately 10 per cent moisture, or dried to 30 per cent moisture in a kiln with no heat applied and then lyophilized to 10 per cent moisture. All products were stored at −25°.

Extraction of Germinated Barley

200 g were finely milled in a Labconco Mill and extracted with occasional stirring for 1 hr at 0° with 500 ml of 0·05 M Tris chloride buffer which was 0·005 M with respect to EDTA and contained 12·5 per cent sucrose and 0·5 per cent ascorbic acid, pH 7·5. The extraction mixture was centrifuged for 30 min at 30,000 × *g*. The supernatant was removed for storage at −25° or used immediately.

Purification of Peptidases

The purification procedure used in earlier studies[1] consisted of; (*a*) dialysis of the tissue extract against acetate at pH 5·5 and $\mu = 0{\cdot}005$, and removal of the precipitated extraneous proteins; (*b*) treatment with carboxymethyl cellulose previously equilibrated with this buffer to separate the acidic (unabsorbed) proteins containing BAPA-ase and acidic BAEE-ase from the so-called neutral proteins (adsorbed) containing neutral BAEE-ase which were then eluted with 0·5 M acetate, pH 5·5, and (*c*) gel filtration of the acidic and neutral fractions on a 32 cm column of Sephadex G-100 followed by filtration on a 90 cm column of the same gel. In the present work the gel filtration was carried out at 5° in 0 1 M succinate (Na) containing 0 2 M NaCl and 0 004 M EDTA, pH 6 0, and consisted of 4 or 5 cycles through a 90 cm column of Sephadex G-100 at a flow rate of 16 ml per hr according to the procedure of Porath and Bennich.[2]

Assay of Peptidases

BAPA-ase was assayed by the method of Erlanger *et al.*[4] modified for use at pH 8·6 and 35°. 3 ml of $6{\cdot}67 \times 10^{-4}$ α-*N*-benzoyl-DL-arginine-p-nitroanilide HCl-ide in 0 05 M Tris chloride buffer, pH 8·6, were treated with 0·1 ml of appropriately diluted enzyme and the increase in absorptivity at 410 nm was recorded for the initial 5–6 min with a Beckman DU spectrophotometer equipped with thermo-spacer plates and a Gilford optical density converter. A unit of enzyme is defined as that amount of enzyme which hydrolyzes 1 mμmole of BAPA per minute under the above conditions.

BAEE-ase was assayed by a similar procedure in which 3 ml of 3×10^{-4} M α-*N*-benzoyl-L-arginine ethyl ester HCl-ide in 0·05 M sodium phosphate buffer, pH 7·0, was treated with 0·1 ml of appropriately diluted enzyme and the increase in absorptivity at 252 nm was recorded for the initial 5–6 min at 35°. The rate of hydrolysis of the ester under the above conditions was calculated by the procedure of Cammarata and Cohen[7]

[6] N. PRENTICE, W. C. BURGER, J. KASTENSCHMIDT and J. D. HUDDLE, *Physiol. Plantarum* **20**, 361 (1967).
[7] P. S. CAMMARATA and P. P. COHEN, *J. Biol. Chem.* **193**, 45 (1951).

using micromolar extinction coefficients of $3{\cdot}55 \times 10^{-3}$ for α-*N*-benzoyl-L-arginine and $2{\cdot}47 \times 10^{-3}$ for α-*N*-benzoyl-L-arginine ethyl ester. A unit of BAEE-ase is defined as that amount of enzyme which catalyzes the hydrolysis of 1 mμmole of BAEE per minute under the above conditions. (For work currently in progress a substrate concentration of 8×10^{-4} M is used with measurement of absorption at 259 nm. Under these conditions the micromolar extinction coefficients are $2{\cdot}02 \times 10^{-3}$ for α-*N*-benzoyl-L-arginine and $1{\cdot}21 \times 10^{-3}$ for α-*N*-benzoyl-L-arginine ethyl ester.)

Assay of Protein

Protein was determined by the method of Lowry *et al.*[8] with crystalline bovine plasma albumin (Pentex Inc., Kankakee, Ill.) as calibrating protein.

Treatment of Enzymes with Solutions of Various pH

1 ml of the enzyme solution obtained from gel filtration was dialysed against 2×125 ml of 0·05 M buffers of pH 3–10 for about 18 hr at 0–4°. Activities were compared with the undialyzed sample after allowances for small changes in volume were made.

Treatment of Enzymes at Various Temperatures

1 ml of the enzyme solution as obtained from gel filtration was held at 35°, 45°, or 55° for 1 hr. Activities were compared with the corresponding unheated enzymes.

Low Temperature Treatment of Enzyme Solutions

1 ml of each enzyme solution from gel filtration purification was frozen at −25° and thawed alternately five times over a period of about 4 hr and the activity compared with corresponding enzyme stored at about 4°.

1 ml of the enzyme solutions from gel filtration purification were stored for 2 months at 4°, and at −25°. 1 ml of each was lyophylized and stored at −25°.

Effect of Metal Ions upon Enzyme Activity

2–3-ml portions of BAPA-ase preparations used for determining the effect of metal ions upon BAPA-ase activity were dialyzed against 2×1300 ml of 0·05 M Tris chloride buffer, pH 8·60, for 16 hr to remove residual EDTA. Neutral BAEE-ase preparations were treated in a similar manner with 0·05 M phosphate buffer, pH 7·0. Solutions of BAEE were prepared in 0·05 M N-tris- (hydroxymethyl) methyl-2-amino-ethanesulfonic acid ("TES") buffer, pH 7·0, to avoid precipitation of metal ion. Solutions of metal chlorides were prepared in water.

The effect of metal ions at varied concentrations was measured in the presence of $6{\cdot}7 \times 10^{-4}$ M BAPA or 3×10^{-4} M BAEE by the following three procedures: (*a*) substrate (BAEE or BAPA), enzyme and metal ion were combined and the activity compared immediately to the corresponding reaction mixture without the metal ion; (*b*) substrate and metal ion were equilibrated 1 hr at 35° before addition of enzyme and measurement of reaction rate, controls were treated similarly without metal ion; (*c*) enzyme and metal ion were equilibrated 1 hr at 35° before addition of substrate. In this case enzyme and metal ion were dissolved in the appropriate buffer and after 1 hr at 35° 0·1 ml of $2{\cdot}7 \times 10^{-2}$ M BAEE in 0·05 M TES buffer, pH 7·0, or 0·01 M BAPA in dimethyl sulfoxide was added. The reaction rate was measured as usual and compared to that obtained by the same procedure but without metal ion.

Determination of Kinetic Constants

Initial reaction rates of the acidic and neutral enzymes were determined at pH 7·0 and 35° with 7–10 concentrations of L-BAEE from $3{\cdot}3 \times 10^{-5}$ to 5×10^{-4} M. The data were treated in several different ways[9] and the method providing the best distribution of points (s plotted against s/v) was used for determining K_m and V.

Barley peptide hydrolase B has a pH optimum of 7. Peptide hydrolase A is optimum in the region of pH 7·8–8·5 with BAPA as substrate. With BAEE substrate the optimum cannot be determined because alkaline hydrolysis occurs above pH 8. Therefore, the kinetic constants for this enzyme acting on BAEE were determined at pH 7·0.

Similar determinations with BAPA-ase were not possible owing to the use of DL-BAPA as substrate and the existence of substrate inhibition (see Results and Discussion).

Hydrolysis of Peptide Substrates

The ability of the peptidase preparations to hydrolyze a variety of simple peptides was determined by incubating 0·1 ml of peptide solution (6×10^{-3} M in 0·05 M sodium phosphate buffer, pH 7·0) with 0·1 ml of

[8] O. H. LOWRY, NIRA J. ROSEBROUGH, A. L. FARR and ROSE J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[9] M. DIXON and E. C. WEBB, *Enzymes*, Chapter 2. Academic Press, New York (1958).

enzyme solution for 16 hr at 35°. The enzyme solutions contained from 42 to 97 units BAEE-ase per 0·1 ml and 20–25 units BAPA-ase per 0·1 ml. The pH of the reaction solutions was 6·7. Controls without enzyme were incubated similarly.

10–20 μl of the incubated solution were applied to a 250 μ plate of silica gel G, Brinkman Instruments Co., Westbury, N.Y., which was developed with *n*-butanol: water: glacial acetic acid in the ratio 4:1:1. Controls for substrates and reaction products were also chromatographed. Substrates and products were located with ninhydrin reagent.[10]

Tests for Hydrolysis of Proteins

The reaction mixture consisted of 5 mg protein, 1·0 or 1·2 ml 0 05 M phosphate, pH 7·0, and 0·2 ml enzyme. This was incubated 3 hr and 16 hr at 35°. The blank, containing 5 mg of protein in a corresponding amount of buffer, was incubated similarly and at the end of the incubation 0·2 ml enzyme was added. To both the reaction mixture and the blank 0·1 ml trichloracetic acid solution (equal parts by weight TCA and water) was added. The acidified mixtures were further incubated at 35° for 2 hr and filtered. Nitrogen was determined in the filtrates by the method of Johnson.[11]

[10] K. RANDERATH, *Thin Layer Chromatography*, p. 94. Verlag Chemie, Academic Press, Weinhein, New York (1963).

[11] M. JOHNSON, *J. Biol. Chem.* **137**, 575 (1941).