ISOLATION AND PROPERTIES OF A PURE YEAST POLYPEPTIDASE*

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A yeast polypeptidase has been described by Grassmann and his collaborators (2, 3), who have obtained a highly active preparation of the enzyme (4). In the present investigation a yeast polypeptidase, not identical with the enzyme described by Grassmann, was isolated as an apparently homogeneous, highly active protein.

Analytical Methods

Enzyme Activity—The activity of all enzyme preparations was determined by measurement of leucyldiglycine hydrolysis. The stock substrate solution contained *dl*-leucyldiglycine (0.1 M), Na_2HPO_4 (0.05 M), NaCl (0.15 M), and $ZnCl_2$ (0.00015 M). To 1 cc. of this solution was added 0.5 cc. of suitably diluted enzyme. The pH of the mixture was approximately 7.05. One 0.5 cc. aliquot of the mixture was titrated immediately and another after 30 minutes incubation at 40.0°. The titrations were made by the acetone-HCl method of Linderstrøm-Lang (5). M/30 HCl being used, a titration increase of 0.5 cc. corresponded to complete hydrolysis of one linkage of the *l* component of the peptide. Duplicate determinations almost always agreed within less than 0.01 cc. The peptidase unit employed, which is identical with the unit used in previous papers, may be defined as the amount of enzyme which will bring about 50 per cent hydrolysis of 0.02 mole of *l*-peptide in 30 minutes. In the determination as described a

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titration difference of 0.25 cc. corresponds to 0.0025 enzyme unit. Since the degree of hydrolysis is not strictly proportional to the quantity of enzyme present, an empirical curve must be constructed.

Nitrogen—The nitrogen content of enzyme preparations was determined by a colorimetric micromethod. A sample containing 10 to 40 γ of N was pipetted into an 18 × 150 mm. Pyrex test-tube, 1 cc. of 2 N H_2SO_4 containing 0.2 gm. per liter of $CuSeO_3$ was added, and the tube was covered by a loose glass cap. The contents of the tube were then digested overnight in a digestion rack kept in a 105° oven. The rack was provided with an electric heat source such that, after evaporation of the water, the H_2SO_4 condensed within 5 cm. of the bottom of the tube. To the tube, after digestion, were added in order 2 cc. of water, 2 cc. of color reagent, and 3 cc. of 2 N NaOH. The color reagent contained per liter 4 gm. of KI, 4 gm. of HgI_2, and 1.75 gm. of gum ghatti. After standing 15 minutes, the tube was placed in a photoelectric colorimeter and a reading taken. A 490 mμ filter was used. Blanks and nitrogen standards were always run with each series of samples. Only matched groups of tubes were used. The extinction coefficient was found to be proportional to the quantity of nitrogen for samples containing less than 45 γ of nitrogen. Known samples could be recovered with an error of 3 per cent or less.

Specific Activity—The specific activity of enzyme preparations was expressed as the number of enzyme units present per gm. of nitrogen in the preparation. The most active preparations thus far obtained have had specific activities in the neighborhood of 30,000. 1 gm. of such a preparation will hydrolyze 1.4 moles of *l*-leucyldiglycine per minute at 40°.

Hydrolysis Conditions—In all the hydrolysis experiments reported in this paper, the substrate concentration was M/30 with respect to available substrate; that is, M/15 in the case of *dl* mixtures. Since the acidic dissociation constant of the di- and tripeptides used is approximately 10^{-8}, in the neighborhood of pH 8 the substrates themselves are efficient buffers, and no further buffer need be added. The incubation temperature in all cases was 40° and the pH was 8.0 unless otherwise stated. All hydrolysis figures are expressed as per cent hydrolysis of one linkage of one optical component.

Preparation of Peptidase Protein

Autolysis—The yeast used was brewers' bottom yeast from a local brewery. Many types of yeast were investigated, and were found to vary greatly in their autolysis behavior. The one selected gave the best yield of enzyme. It was found that the yield was greatly improved if the pressed yeast (75 per cent moisture) was kept frozen in a cold room (−6° to −10°) for a few months before being autolyzed. In the procedure finally adopted, the frozen pressed yeast was treated with an equal weight of water, covered with toluene, and allowed to autolyze, with occasional shaking, at room temperature (22–25°). The suspension always maintained itself at pH 6.1 without adjustment. Samples were removed at intervals for peptidase assay, and when there was no further increase in activity (3 to 5 days), the mixture was filtered with suction after the addition of Hyflo super-cel (a diatomaceous filter aid sold by Johns-Manville). The filter cake was washed with water and the washings added to the filtrate.

Acetone Precipitation—The procedure for isolation of the enzyme protein from the crude autolysate consists of a series of fractional acetone precipitations. The number of precipitations necessary, and the optimum pH and acetone concentration for each step, vary somewhat with each preparation. The precipitations are carried out as rapidly as possible, since the enzyme is not stable at high acetone concentrations. The general procedure is illustrated by the example of Table I. Often further fractional acetone precipitation is necessary to produce a preparation of high activity. During the latter stages of purification, a salt of a divalent metal is added to facilitate flocculation of the protein precipitate.

Various procedures involving adsorption, ammonium sulfate fractionation, acid precipitation, and other fractionation methods have not in general produced preparations any more active than can be obtained by repeated fractional acetone precipitation. Ammonium sulfate fractionation of fairly pure material, however, often is advantageous. The most active preparations thus far obtained have been prepared in this manner.

Fractions ranging in specific activity from 15,000 to 30,000 have been found to behave as homogeneous substances when tested in the Svedberg ultracentrifuge or the Tiselius electrophoresis ap-

paratus. When preparations, originally very active, which have decreased in activity during long storage have been fractionated, it has never been found possible to separate active from inactive material. All present data indicate the presence in all preparations of varying proportions of inactivated enzyme or of some other protein inseparable from the enzyme protein.

TABLE I

Isolation of Peptidase Protein

Step No.	Treatment	Volume	Total enzyme	Specific activity
		cc.	*units*	
I	1 kilo pressed brewers' yeast, frozen 3 mos., + 1 liter H_2O, autolyzed 4 days at pH 6.1; filtered; cell residue discarded; solution analyzed	2020	550	25
II	Solution adjusted to pH 5.7, acetone added to 28 volumes %; centrifuged; supernatant discarded; ppt. taken up in water; centrifuged; ppt. washed with water, washings added to supernatant; combined solution analyzed	246	440	1,060
III	Solution adjusted to pH 5.8, acetone added to 30 volumes %; centrifuged; ppt. dissolved in water, solution clarified in centrifuge before analysis	16.7	410	16,700
IV	Solution treated with $MgCl_2$ to 0.001 M, adjusted to pH 5.6, treated with acetone to 13.5 volumes %; ppt. centrifuged off, discarded; acetone added to 18 volumes %; ppt. centrifuged off, dissolved in water, analyzed	5.9	163	20,800
V	Residual 18% acetone solution from Step IV made to 26 volumes % acetone; ppt. centrifuged off, dissolved in water, analyzed	5.9	179	23,000

Properties of Peptidase Protein

Sedimentation, Diffusion, and Mobility[1]—A 0.2 per cent solution of the enzyme protein (specific activity 15,000) in 0.05 M (pH

[1] The author wishes to thank Dr. H. P. Lundgren and Professor J. W. Williams of the Department of Physical Chemistry for their cooperation in studying the physical properties of the protein.

7.05) phosphate buffer containing 0.1 M NaCl was sedimented in the Svedberg velocity ultracentrifuge. The sedimentation diagrams, obtained by the scale method, indicated that the material was homogeneous. The sedimentation constant at 20° was found to be 21.3×10^{-13} cm. sec.$^{-1}$ dyne^{-1}. A diffusion experiment performed at the same protein and buffer concentration showed the diffusion constant at 20° to be 3.09×10^{-7} cm.^{2} sec.$^{-1}$. On the basis of these two values, a provisional value of 670,000 for the molecular weight is obtained. Whether the enzyme exists at physiological concentrations as molecules of this size has not been determined.

As has been mentioned, samples of peptidase which do not possess maximal activity behave as homogeneous proteins. In order to obtain further information on this seeming anomaly, two preparations, one having a specific activity of 30,000 and the other a specific activity of 16,000, were compared on the Tiselius apparatus. The protein concentration in both cases was 0.2 per cent. A phosphate-NaCl buffer (pH 6.95 and ionic strength 0.07) was used. Both preparations were found to migrate as homogeneous substances. The more active sample had a mobility of 6.1×10^{-5} cm.^{2}sec.$^{-1}$, while the mobility of the less active preparation was 7.4×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

Since preparations which undoubtedly contain a large percentage of inactive material are homogeneous with regard to both sedimentation and electrophoretic migration, it seems likely that the inactive material present is a constituent part of the macromolecule. If this were true, the presence of the inactive material might affect the mobility, but not the homogeneity of the sample.

Herriott, Desreux, and Northrop (6) have reported that pepsin preparations containing inactive protein are electrophoretically homogeneous, although their solubility behavior indicates inhomogeneity.

Other Properties—The enzyme is soluble in water at pH values above 5.2 to 5.3. It may be precipitated from aqueous solution by acidification to pH 4.5 to 4.8. At pH values below 4.0 it is soluble, but is very rapidly inactivated. In dilute salt solutions it is soluble at all pH values. It is soluble in 0.6 saturated ammonium sulfate solution, but relatively insoluble in 0.8 saturated ammonium sulfate solution.

Neutral aqueous solutions of the enzyme containing a few gm. of protein per liter are stable for weeks at 2°. In very dilute aqueous solution, the enzyme is rather unstable. Its stability is a function of pH. In Fig. 1 is shown the effect of pH on the stability of the enzyme in a solution containing about 2.4 mg. of protein per liter. The enzyme is unstable in neutral solution, but is relatively stable at higher pH values. It has been found that in the presence of 0.01 M NaCl enzyme solutions are perfectly stable for 2 hours at pH 7, but not at pH 5.6. Because of this instability of the protein in dilute aqueous solution, enzyme solutions were always diluted for assay sampling with 0.001 M borate buffer of pH 9.2.

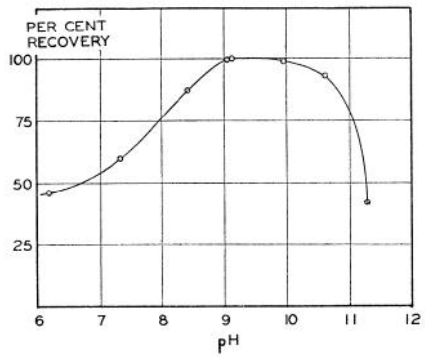

FIG. 1. Stability of a dilute solution of peptidase protein held 3 hours at 22° in 0.001 M borate plus 0.001 M phosphate. Preparation 878, specific activity 17,200; 0.3 γ of enzyme N per cc.

The chemical composition of the enzyme protein has not yet been studied. Orientation experiments have shown it to contain about 13.5 per cent N and 0.3 per cent P. Acid hydrolysis liberates approximately 5 per cent of sugar as determined by copper reduction and orcinol color methods.

Enzymatic Activity of Protein

Specificity—Table II summarizes data on the hydrolysis of various substrates by the enzyme protein. Tripeptides are hydrolyzed more rapidly than corresponding dipeptides. *dl*-Leucylmethylamine (leucyldecarboxyglycine) is split, showing that a

carboxyl group is not essential for hydrolysis. The slow hydrolysis of *dl*-prolyldiglycine indicates that an intact amino group is not necessary. *dl*-N-Methylleucyldiglycine, however, is not split. The data of Table III show the enzyme to have a high degree of optical specificity. *l*-Leucyldiglycine is hydrolyzed 5000 times as rapidly as the unnatural *d*-leucyldiglycine. If the

TABLE II

Hydrolysis of Various Substrates by Yeast Polypeptidase

Preparation 840, specific activity 16,300; 0.1 M NaCl present.

Substrate	Enzyme N	Incubation time	Hydrolysis
	γ per cc.	*hrs.*	*per cent*
dl-Leucyldiglycine	0.091	0.5	55
dl-Alanyldiglycine	0.253	0.5	53
Triglycine	0.253	8	56
dl-Leucylglycine	0.253	2	64
dl-Alanylglycine	0.253	8	39
Diglycine	25.3	2	30
dl-Leucylmethylamine	0.253	8	36
dl-Prolyldiglycine	25.3	2	73
dl-N-Methylleucyldiglycine	25.3	8	0

TABLE III

Optical Specificity of Yeast Polypeptidase

Preparation 913, specific activity 21,000; 0.1 M NaCl and 0.0001 M $ZnSO_4$ present.

Substrate	Enzyme N	Incubation time	Hydrolysis
	γ per cc.	*hrs.*	*per cent*
l-Leucyldiglycine	0.107	0.5	80
d-Leucyldiglycine	13.4	0.5	1
		5	20
		22	76

d-leucyldiglycine used had been contaminated with any of the *l* form, a greater degree of hydrolysis would have occurred during the first half hour. It should also be mentioned that the enzyme hydrolyzes the peptide linkage adjacent to the amino group. When leucyldiglycine is hydrolyzed by a large amount of enzyme, hydrolysis proceeds rapidly until one linkage has been split.

Further hydrolysis is relatively slow. If leucylglycine instead of diglycine were a primary hydrolysis product, further hydrolysis would be much more rapid. When the experiment is performed, the rate of secondary hydrolysis corresponds to the rate at which diglycine is split by the enzyme and not to the more rapid rate at which leucylglycine is split. Since leucylglycine is split 300 times as fast as diglycine, the difference is accurately observable.

Activation—The activity of the peptidase is greatly affected by the presence of chloride ions and zinc ions. The data of Table IV show that its activity in the presence of both zinc and chloride is much greater than the activity in the presence of either alone. Such behavior indicates that both ions are in some way necessary

TABLE IV

Effect of NaCl and $ZnSO_4$ on Enzyme Activity

Preparation 913, specific activity 21,000.

Substrate	Added NaCl	Added $ZnSO_4$	Enzyme N	Incubation time	Hydrolysis
	M	*M*	*γ per cc.*	*hrs.*	*per cent*
dl-Alanyldiglycine	0.0	0.0	0.053	1.0	2
	0.1	0.0	0.053	1.0	12
	0.0	0.0001	0.053	1.0	8
	0.1	0.0001	0.053	1.0	58
dl-Leucyldiglycine	0.0	0.0	0.067	0.5	5
	0.1	0.0	0.067	0.5	14
	0.0	0.0001	0.067	0.5	10
	0.1	0.0001	0.067	0.5	44

in the hydrolysis process. The zinc concentration necessary for maximal activation is rather low. The experiment summarized in Table V shows 10^{-4} M to be approximately optimal. The optimal NaCl concentration is high, as may be seen from Table VI. While 0.0001 M NaCl gives a readily detectable effect, greatest activity was obtained in the presence of 0.1 M NaCl. At concentrations much greater than this, precipitation occurs during the acetone titration.

Salts of Al‴, Ca″, Cd″, Co″, Cu″, Fe″, Hg″, Mg″, Mn″, Ni″, Pb″, and Zn″ were tested for activating effect on alanyldiglycine hydrolysis in the presence of 0.1 M NaCl. The metals were tested at 10^{-5} M, 10^{-4} M, and 10^{-3} M. Only Zn″ gave good activation.

Co″, however, gave at 10^{-4} M a definite and reproducible, though relatively small (60 per cent) activation.

The activation by sodium chloride is due to the chloride ion rather than the sodium ion; $MgCl_2$ is an effective activator, while

TABLE V

Effect of $ZnSO_4$ Concentration on Enzyme Activity

Preparation 913, specific activity 21,000; 0.067 γ of N per cc.; incubation time 30 minutes; substrate *dl*-leucyldiglycine.

Added NaCl	Added $ZnSO_4$	Hydrolysis
M	*M*	*per cent*
0.1	0	14
0.1	10^{-6}	16
0.1	10^{-5}	28
0.1	10^{-4}	46
0.1	10^{-3}	43
0.0	0	5
0.0	10^{-4}	10

TABLE VI

Effect of NaCl Concentration on Enzyme Activity

Preparation 959, specific activity 16,300; 0.091 γ of N per cc.; substrate *dl*-alanyldiglycine; pH 7.2; incubation time 30 minutes; 0.0001 M $ZnSO_4$ present.

Added NaCl	Hydrolysis
M	*per cent*
0	19
10^{-5}	20
10^{-4}	26
10^{-3}	44
10^{-2}	56
10^{-1}	65
10^{-2}	12*

* No $ZnSO_4$ added.

sodium sulfate and sodium acetate are not (Table VII). Moreover the sodium hydroxide necessary to adjust the peptide substrates to pH 8 brings the sodium ion concentration to approximately 0.033 M even in the absence of added activator. Yet the

addition of 0.001 M NaCl to such a substrate more than doubles the activity of the enzyme (Table VI).

Ordinary preparations of leucyldiglycine prepared by the method of Fischer (7) were found to be hydrolyzed as rapidly in the absence of added zinc as in its presence. Repeated recrystallization was necessary to obtain preparations which would give maximal zinc activation. It was found that the trace of ash present in ordinary preparations would, if added to recrystallized preparations, greatly promote hydrolysis. Since peptides prepared from the corresponding α-halogen acyl compounds usually contain traces of ammonium halide, recrystallization was often necessary to obtain maximal NaCl activation. It seems probable

TABLE VII

Effect of Anions on Enzyme Activity

Preparation 878, specific activity 17,200; 0.105 γ of N per cc.; substrate *dl*-leucyldiglycine.

Salt added	Concentration	Hydrolysis
	M	*per cent*
None	0	32
NaCl	0.01	60
Na_2SO_4	0.005	35
$NaOOCCH_3$	0.02	26
NaBr	0.01	58
KI	0.01	52
$MgCl_2$	0.005	56
$NaNO_3$	0.01	44

that the activity observed in the absence of added activators is due to the presence of traces of zinc and halogens in the peptide preparation and in the NaOH used for pH adjustment.

pH Optimum—In Fig. 2 pH-activity curves for the hydrolysis of leucyldiglycine and alanyldiglycine are given. It will be seen that leucyldiglycine is attacked most rapidly at pH 7.9, while alanyldiglycine is most rapidly split at pH 7.0. The leucyldiglycine substrate used did not contain added zinc, but addition of zinc salts had no effect at either pH 7 or 8. The enzyme is stable at pH 10, as may be seen from Fig. 1, but no enzymatic activity was evident at this pH. In the acid pH region, however, it is possible that instability of the enzyme may have affected the shape of the curves of Fig. 2. The leucyldiglycine substrate used

for routine peptidase assay has a pH of 7.05. This pH value was originally adopted because it is the optimal pH for leucyldiglycine hydrolysis by crude yeast autolysate.

Relation of Yeast Polypeptidase to Other Peptidases

The enzyme described above is not identical with the yeast aminopolypeptidase described by Grassmann and his associates. Their enzyme hydrolyzed leucyldiglycine 500 times as rapidly as

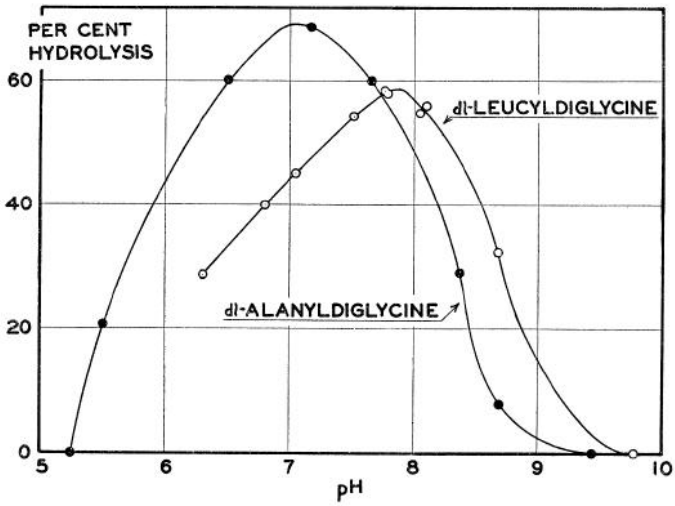

FIG. 2. pH-activity curves for peptide hydrolysis. *dl*-Leucyldiglycine, enzyme Preparation 840, specific activity 16,300; 0.091 γ of N per cc.; 0.1 M NaCl present; incubation time 30 minutes. *dl*-Alanyldiglycine, enzyme Preparation 913, specific activity 21,000; 0.08 γ of N per cc.; 0.1 M NaCl and 0.0001 M $ZnSO_4$ present; incubation time 60 minutes.

leucylglycine (4), while the present peptidase hydrolyzes the tripeptide only 8 times as rapidly as the dipeptide when substrates of the same composition as those used by Grassmann are employed. Moreover, Grassmann's enzyme splits triglycine 40 per cent as fast as leucyldiglycine (8), whereas the enzyme described in this paper splits triglycine only 3 per cent as rapidly as leucyldiglycine. Grassmann (9) has stated that yeast aminopolypeptidase acquires dipeptidase activity in the presence of chlorides. Such behavior seems to indicate the presence in his preparations of a chloride-activated peptidase.

In a previous publication from this laboratory (10) the resemblance of a previously studied mold polypeptidase (11) to yeast polypeptidase was shown. Both enzymes are activated by zinc, both enzymes split tripeptides rapidly and dipeptides slowly, and both enzymes split leucyl peptides much more rapidly than glycyl peptides.

Many molds possess a similar peptidase. Although the mold peptidases were not found to be activated by chloride ions, the many similarities seem to warrant a provisional conclusion that the zinc-activated yeast polypeptidase is one of a group of similar peptidases more or less widely distributed in nature.

SUMMARY

A protein having high polypeptidase activity has been isolated from brewers' yeast. The protein behaves as a homogeneous substance on electrophoresis and ultracentrifugal sedimentation. It has a molecular weight in the neighborhood of 700,000. It has not been obtained in a crystalline state. The polypeptidase splits tripeptides much more rapidly than dipeptides. It hydrolyzes the peptide linkage adjacent to the free amino group of the peptide. It does not require a carboxyl group. The presence of zinc ions and halide ions appears to be necessary for its activity.

A convenient micro-Kjeldahl nitrogen method is described.

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