Intracellular Peptidase of *Bac. subtilis*

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Received September 1, 1965

A peptidase was isolated from the cells of amylase-producing *Bac. subtilis* by means of cell lysis with egg white lysozyme, followed by freezing and thawing, salting out, dialysis and ion-exchanger column chromatography. The enzyme required manganese ion to show the enzyme activity. Also the enzyme was stable in the presence of magnesium ion. The enzyme hydrolyzed various synthetic peptides by stepwise removal of the amino terminal amino acid of peptides and thus the peptidase was found to be aminopeptidase.

It is well known that aminopeptidase is widely distributed in various organisms. However, there have been only a few papers¹⁾ on the nature of the enzyme with exception of the peptidase derived from animal kidney. Recently, a certain strain of *Bac. subtilis*, which has been reported to secrete a large quantity of proteinase²⁾ as well as amylase³⁾ into media, was found to contain aminopeptidase in a substantial quantity in the cells.

The present paper deals with the method of purification and some properties of intracellular peptidase of the bacteria.

MATERIALS AND METHODS

As the source of enzyme, an amylase-producing bacterium, *Bac. subtilis* var. *amyloliquefaciens* FUKUMOTO, was used throughout the work. The bacteria were surface-cultured in a Fernbach's flask containing one l of medium consisted of 5% soybean cake extract, 3.5% dextrin, 0.8% $(\text{NH}_4)_2\text{HPO}_4$, 0.05% CaCl_2 and 0.02% each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KCl in tap water (pH 7.0). After a forty hours' culture at 37°C, the cells were collected by centrifuge and washed four times with ten volumes of 0.9% NaCl

solution. The cells thus washed were immediately subjected to lysis with egg white lysozyme in order to extract the peptidase as described later.

The peptidase activity was assayed on DL-Leu-Gly-DL-Phe as substrate. To a mixture of 0.5 ml of M/50 of the peptide, 0.2 ml of M/5 tris(hydroxymethyl)aminomethane-HCl buffer (Tris buffer, pH 7.5) and 0.1 ml of M/40 MnCl_2 was added 0.1 ml of enzyme at 25°C. After 2.5 min., 0.1 ml of N/2 HCl was added to the mixture and 0.1 ml aliquot of the mixture was taken into 1.4 ml of citrate buffer of pH 5.0 and subjected to the ninhydrin reaction test according to the Yemm and Cocking's method.⁴⁾ One unit of enzyme activity was defined as the enzyme quantity that produced 1.0 μ mole of amino acid per min. under the condition. In Fig. 1 is shown the relationship between the enzyme quantities and the degree of liberation of amino acids in the mixture under the condition. The specific activity was expressed arbitrarily as the enzyme activity per one unit of absorbancy of enzyme protein at 280 m μ using the silica cuvette of one cm light path.

In the test of the effect of metal ions on the enzyme, a peptide isolated from polypepton (Daigo Eiyo Kagaku Co., Ltd., Japan) was used as the substrate. The polypepton was dissolved in deionized water and, after adjusting the pH to 2.5 with HCl, dialyzed using a cellulose tubing (Visking Company) against frequently changing solution of dilute hydrochloric acid (pH 2.5) for 5 days in the cold. The two dimensional paperchromatography of the dialyzed

1) E.L. Smith and R.L. Hill, *The enzyme* (Academic Press Inc.), 4, 37 (1960).

2) J. Fukumoto, et al., *J. Agr. Chem. Soc. Japan*, 31, 331 (1957), 32, 230, 233 (1958), 33, 6, 9 (1959).

3) J. Fukumoto, *Annual Report of the Institute of Osaka Municipal Res. Tech.*, 1944, No. 9.

4) E. M. Yemm and E. C. Cocking, *Analyst*, 80, 209 (1955).

FIG. 1. Enzyme Concentration and Amino Acid Liberation.

polypepton showed to contain only two peptides, their R_F being very close each other and giving nine kinds of amino acid on acid hydrolysis.

The crystalline egg white lysozyme ($[E]_{1\%}^{280m\mu} = 24.5$) used here was prepared according to the modified method of Alderton.⁵⁾

RESULTS

1. Extraction and purification of the enzyme

The washed bacterial cells were suspended in $M/50$ phosphate buffer of pH 7.2 containing $M/200$ ethylenediaminetetraacetic acid sodium salt (EDTA) and incubated with an appropriate amount of lysozyme (usually 2~3 mg of lysozyme for one g of the cells as dry weight) at 37°C for one hr. The lyzed cell mixture was frozen and stored at -20°C for two days. The frozen preparation was then thawed at room temperature and centrifuged at 67,000 g for 30 min. (0°C). To the supernatant solution was added solid ammonium sulfate to a 0.5 saturation and the mixture was centrifuged at 15,000 g, whereby very viscous material was precipitated. The clear supernatant was then salted out by supplemental addition of solid ammonium sulfate to a 0.8 saturation. The precipitate there occurred was collected by centrifugation at

15,000 g and dialyzed under stirring against changing $M/20$ acetate buffer of pH 5.8 containing $M/400$ magnesium acetate for two days. The dialyzed enzyme was then subjected to diethylaminoethyl(DEAE)-cellulose column chromatography.

To the DEAE-cellulose column (1.8×42 cm), which had been equilibrated with the buffer of the same constituents as those used in the dialysis, the enzyme was applied and developed by linear gradient elution with a mixing chamber containing 0.5 l of 0.05 M NaCl and a reservoir containing 0.5 l of 0.5 M NaCl in the buffer at a flow rate of 80 ml per hr. at 4°C. The chromatogram is shown in Fig. 2.

The active enzyme fractions obtained in the column chromatography were combined together and dialyzed against buffer solution of the same constituents as those used in the dialysis before. The dialyzed enzyme was again chromatographed using DEAE-cellulose column, the chromatographic pattern showing almost only one peak in regard either to protein or enzyme activity (Fig. 3).

FIG. 2. Chromatograms of *Bac. subtilis* Peptidase on a DEAE-Cellulose Column (1.8×42 cm; enzyme applied, 85 ml (11,000 U)).

—, $[E]_{1cm}^{280m\mu}$; ---, enzyme activity,
- - -, Conc. of NaCl

5) G. Alderton, *Biochemical Preparation*, 1, 67 (1950).

FIG. 3. Rechromatography of the Peptidase on a DEAE-Cellulose Column (1.2×30 cm; enzyme applied, 167 ml (8,900 U)).

FIG. 4. Zone Electrophoresis of the Peptidase on a Starch Block.

—, $[E]_{1cm}^{280cm}$; ---, Enzyme activity, — · —, Specific activity, Starch block, 1.5×3.0×20 cm; M/20 acetate buffer (pH 5.6) containing M/400 Mg-Acetate; 300 V, 20 mA; 6 hr at 4°C, Enzyme charged, 220 U of rechromatographed preparation.

The zone electrophoresis on a starch block revealed that the rechromatographed enzyme preparation was almost homogeneous, as shown in Fig. 4. However, the purified peptidase still showed a slight activity of proteinase when assayed by the modified Folin's method,²⁾ but this proteinase activity was nullified by treating the preparation with EDTA or potato proteinase inhibitor, giving no effect on the peptidase activity of the enzyme preparation (Table I). The purification procedure described above is summarized in Table II.

TABLE I. EFFECT OF EDTA AND POTATO PROTEINASE INHIBITOR (PPI) ON THE PURIFIED PEPTIDASE

Substrate	Enzyme activity		
	Leu. Gly. Phe*	Polypeptide* from polypepton	Casein**
Treatment (25°C, 30 min.)			
No	55 U/ml	5.0 U/ml	3.2 U/ml
EDTA	67	4.2	0
PPI	51	4.1	0
EDTA (M/250) +PPI	70	4.2	0

* Ninhydrin method.

** Determined by the modified Folin's method, but reacted for 30 min. at 30°C.

2. Some properties of the enzyme

The purified peptidase was found to act best at pH 7.5, as shown in Fig. 5. In the presence of Mg^{++} , the enzyme was stable at pH values between 5.5 and 8.0 (25°C, 16 hr, Fig. 6). However, the enzyme was sensitive to heat and lost a half of its activity even at 60°C (pH 7.2) in 10 min. (Fig. 7).

TABLE II. SUMMARY OF PURIFICATION PROCEDURE OF *Bac. subtilis*' PEPTIDASE

Step	Activity U/ml	Total volume ml	Total activity U	Specific activity	Recovery %
Cell lysis	15 (6.4)	750	11,000 (4,800)	0.48 (0.21)	100 (100)
Salting out and dialysis	130 (46.0)	85	11,000 (3,900)	7.1 (2.6)	100 (80)
Chromatography (DEAE-cellulose)	53 (6.0)	167	8,900 (1,000)	68 (7.7)	81 (21)
Rechromatography (DEAE-cellulose)	73 (8.1)	60	4,400 (490)	81 (9.0)	41 (10)

The number in parenthesis indicates the enzyme activity on the polypeptide. See the text.

FIG. 5. pH-Activity Curve.
(DL-Leu-Gly-DL-Phe, M/360 MnCl₂)

FIG. 6. pH-Stability.
(Mg⁺⁺, M/500)

The enzyme, purified as above and finally dialyzed against Tris buffer, showed no enzyme activity but, was activated by the addition of divalent metal ions. The best reactivation was observed by the addition of Mn⁺⁺ to the reaction mixture as given in Table III. The effect of Mn⁺⁺ or Co⁺⁺ was significantly distinct with the enzyme which had been pretreated with EDTA. The EDTA-treated enzyme preparation showed no reactivation by any metal ion other than Mn⁺⁺ or Co⁺⁺

FIG. 7. Thermal Stability.
(pH 7.2, M/50 Tris buffer, M/400 MgCl₂, 10 min.)

TABLE III. EFFECT OF VARIOUS METAL IONS ON ENZYME ACTIVITY*

Pretreatment of enzyme	Metal added (Acetate, M/250)	Enzyme activity U	Relative activity %
No	None	0	0
	Mg	0.3	5.6
	Ca	0.3	5.6
	Co	3.2	63
	Mn	5.0	100
	Zn	0	0
	Ni	0.4	7.8
Preincubated with M/500 EDTA (pH 7.6, 30°C, 1 hr)	None	0	0
	Mg	0	0
	Ca	0	0
	Co	1.5	30
	Mn	4.1	83
	Zn	0	0
	Ni	0	0

* Assayed on the peptides isolated from polypepton.

In the test of the relationship between the concentration of Mn⁺⁺ and the enzyme activity was used, as substrate, the peptide that was isolated from polypepton. The peptidase purified as above showed activity on these peptides only in the presence of Mn⁺⁺ or Co⁺⁺. The enzyme activity as a function of

the concentration of Mn^{++} is shown in Fig. 8. However, the synthetic peptides used here were contaminated by certain metals and thus no clear result was found with them on the requirement of metal ions of the enzyme.

On the other hand, the stability of the enzyme, especially during dialysis, was greatly affected by the sort of divalent metal ions present in the outer solution, as given in Table IV. The best stability of the enzyme was bestowed by Mg^{++} whereas the presence of Mn^{++} or Co^{++} rather caused a considerable loss of enzyme activity during dialysis.

The ultraviolet absorption spectra of the purified peptidase are shown in Fig. 9.

FIG. 8. Enzyme Activity as a Function of Concentration of Mn^{++} .

TABLE IV. EFFECT OF VARIOUS DIVALENT METAL IONS ON ENZYME STABILITY DURING DIALYSIS (4°C, 40 hr.)

Outer solution	Survival activity %
Deionized water	72
M/200 Tris buffer, pH 7.2	96
" + M/400 Mn-acetate	42
" + M/400 Mg-acetate	100
" + M/400 Ca-acetate	77
" + M/400 Co-acetate	50
" + M/400 Zn-acetate	0

FIG. 9. Ultraviolet Absorption Spectra of *Bac. subtilis* Peptidase.

(0.11 mgN/ml, 60 U of activity/ml)

3. Specificity of the enzyme

The specificity of the peptidase was investigated using the enzyme preparation treated with EDTA and making to react with various synthetic peptides under the following condition: A 0.1 ml of enzyme ($[E]_{cm}^{280m\mu} = 0.35$) was added to a mixture of 0.05 ml of peptide solution (approx. 1 μ mole) and 0.05 ml of M/25 Tris buffer (pH 8.0) containing M/100 $MnCl_2$. After incubation for 20, 120 min. and 24 hr. at 25°C, a 0.02 ml aliquot of the reaction mixture was spotted on a filter paper of Toyo No. 50 and developed at 25°C by the descending method using a mixture of butanol, acetic acid and water (4:1:2) as the solvent. The paper was then dried in a current air and sprayed by a 0.2% solution of ninhydrin dissolved in butanol saturated with water. As given in Table V, the enzyme hydrolyzed various peptides, excepting DL-Ala-DL-Val, although the rate of hydrolysis differed according to the kind of peptide.

The peptidase also hydrolyzed D-amino acid peptides, but the hydrolysis rate was much slower than that of L-amino acid as presented in Fig. 10. However, no hydrolysis was found

TABLE 5. ACTION ON VARIOUS SYNTHETIC PEPTIDES (Part 1)

Peptide	Activity	Peptide	Activity
DL-Ala·Gly	++	Gly·L-Try	+
DL-Ala·Gly·Gly	+	Gly·L-Tyr	++
DL-Ala·DL-Val	—	Gly·L-Val	+
α -amino-Buty·Gly	+++	L-Leu-NH ₂	++
Gly·DL-Ala	+	L-Leu·Gly	+++
Gly·L-Asp	++	L-Leu·L-Tyr	+++
Gly·Gly	+	Cbz-Gly·Gly-NH ₂	—
Gly·Gly·Gly	+	Cbz-Gly·L-Phe	—
Gly·Gly·Gly·Gly	+	Cbz-Gly·L-Phe-NH ₂	—
Gly·L-Leu	++	Cbz-L-Leu·Gly	—
Gly·DL-Phe	++	Hippuryl-L-Leu-NH ₂	—
Gly·DL-Phe-NH ₂	+		

+++ Completely hydrolyzed in 2 hr.

++ Completely hydrolyzed in 24 hr.

+ Hydrolyzed but not completely in 24 hr.

— No hydrolysis.

FIG. 10. Action on Various Synthetic Peptides (Part II).

with any peptide whose terminal amino groups were blocked.

In Fig. 11 is shown the progressive curve of hydrolysis of DL-Leu·Gly·DL-Phe by the peptidase. To a mixture consisting of 0.1 ml of M/50 DL-Leu·Gly·DL-Phe, 0.1 ml of M/40 MnCl_2 and 0.1 ml of M/50 Tris buffer (pH 8.0) was added 0.1 ml of the peptidase ($[E]_{\text{cm}}^{280\text{m}\mu} = 0.9$) and, at varied intervals of time at 25°C, 0.01 ml aliquots were taken to spot on a filter paper of Tōyō No. 50 and paperchromatographed as described above. After color development by spraying ninhydrin solution, the color density was measured using a densitometer (Tōyō Densitol) to determine the amount of liberated amino acid, comparing with the color derived from a given amount of amino acid applied as a marker. The result shown in Fig. 11 indicates that on hydrolysis of DL-Leu·Gly·DL-Phe by the peptidase a rapid liberation of leucine occurred, leaving behind Gly·DL-Phe peptide there. However, the latter was gradually hydrolyzed to its constituting amino acids in the subsequent

incubation. In the experiment, the rate of hydrolysis of leucyl peptide linkage was calculated to be about one hundred times faster than that of glycyl peptide.

DISCUSSION

The peptidase isolated from the cells of *Bac. subtilis* was found to hydrolyze various peptides. The facts, that the enzyme hydrolyzed only the peptides, of which amino group of the terminal amino acid was free, and that the hydrolysis progressed to liberate amino acid from the amino terminal amino acid of the peptides, clearly indicate that the enzyme is an aminopeptidase. This enzyme might be classified into leucyl aminopeptidase, because it acted best on leucyl peptides.

However, in contrast to leucine aminopeptidase isolated from swine kidney by Smith and Spackman,⁶⁾ the enzyme in the present paper hydrolyzed leucyl peptide much faster than leucine amide (The C_1 of the peptidase which was determined according to the method of Smith⁶⁾ was 14.3 on DL-Leu·Gly·DL-Phe whereas it was 0.22 on Leu-NH₂) and showed activity only in the presence of Mn^{++} and Co^{++} . No enzyme activity was found in the presence of Mg^{++} alone, although the enzyme was stabilized by the metal ion.

In other experiments, the peptidase was found to hydrolyze polypeptides or denaturated proteins deeply but the hydrolysis ceased at certain points. This reason and the detailed nature of the peptidase are now under investigation.

Acknowledgement. The authors are indebted to Mr. Y. Sasaki and Y. Matsumura for their assistance in part of the experiment.

6) E. L. Smith and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).

FIG. 11. Progressive Curve of Hydrolysis of DL-Leu·Gly·DL-Phe.

—○—, DL-Leu·Gly·DL-Phe; ---○---, L-Leu,
---○---, Gly