Studies on Bacterial Protease

Part XVII. Some Physicochemical Properties and Amino Acid Composition of Alkaline Protease of *Bacillus subtilis* var. *amylosacchariticus*

By Daisuke TSURU, Heizo KIRA, Takehiko YAMAMOTO and Juichiro FUKUMOTO

Faculty of Science, Osaka City University, Sumiyoshi-ku, Osaka

Received October 31, 1966

Some physicochemical properties and amino acid composition of the alkaline protease of *B. amylosacchariticus* were determined. The molecular weight and sedimentation coefficient were estimated to be 22,700 and 2.89 s, respectively, and the amino terminal amino acid was identified to be alanine. The enzyme contained 15.9% of nitrogen and was composed of 220 residues of amino acid: lys₆, his₅, arg₃, asp₂₀, thr₁₄, ser₃₇, glu₁₂, pro₁₀, gly₂₅, ala₂₇, val₂₀, met₃, isoleu₁₂, leu₁₂, tyr₉, phe₂, try₃ and amide ammonia₁₆. The results indicate that protein nature and chemical properties of the alkaline protease presented here are distinct from those of alkaline proteases obtained from the other strains of *B. subtilis*, such as subtilopeptidase A, B and BPN*.

INTRODUCTION

In the previous paper,¹⁾ the authors reported the purification method and some enzymatic properties of the alkaline protease isolated from *B. amylosacchariticus* which was a strain of saccharifying α -amylase-producing *Bacillus subtilis*.^{2,3)} The protease showed properties almost similar to those of *B. subtilis* alkaline proteases ever reported such as K₁₃ protease,⁴⁾ subtilopeptidase A,⁵⁾ B,^{6,7)} and BPN*,^{8~11)*} However,

it was found that solubility in neutral solution of the purified preparation of *B. amylosacchariticus* alkaline protease was very low as compared with that of other *B. subtilis* proteases.¹⁾ The former enzyme was easily crystallized out during the dialysis against 2×10^{-3} M calcium acetate solution at pH values between 6.5 and 8.5, if the enzyme concentration was higher than 0.5%.

The present investigation was undertaken to determine some physicochemical properties and amino acid composition of the alkaline protease of *B. amylosacchariticus*, comparing with those of *B. subtilis* alkaline proteases so

1) D. Tsuru, H. Kira, T. Yamamoto and J. Fukumoto, This Journal, **30**, 1261 (1966).

2) J. Fukumoto, T. Yamamoto and K. Ichikawa, *Proc. Japan Acad.*, **27**, 352 (1951).

3) J. Fukumoto and S. Okada, *J. Ferm. Tech.*, **41**, 427 (1963).

* Subtilopeptidase A and B were previously called subtilisin and nore enzyme, respectively. BPN* is commercially available under the trade name of nagarse.

4) J. Fukumoto, T. Yamamoto and K. Ichikawa, *J. Agr. Chem. Soc. Japan*, **33**, 9 (1959).

5) A. V. Guntelburg and M. Ottesen, *Compt. Rend. Trav. Lab. Carlsberg*, **29**, 36 (1954).

6) G. Johansen and M. Ottesen, *ibid.*, **34**, 199 (1964).

7) M. Ottesen and A. Spector, *ibid.*, **33**, 63 (1960).

8) B. Hagihara, "The Enzymes," Vol. **4**, Academic Press Inc., New York, (1958), p. 193.

9) H. Matsubara, B. Hagihara, M. Nakai, T. Komaki, T. Yonetani and K. Ohnuki, *J. Biochem.*, **45**, 251 (1958).

10) H. Matsubara, C. B. Kasper, D. M. Brown and E. L. Smith, *J. Biol. Chem.*, **240**, 1125 (1965).

11) C. B. Kasper, H. Matsubara and E. L. Smith, *ibid.*, **240**, 1135 (1965).

far reported, in order to ascertain whether these enzymes were distinguishable in their protein chemical properties.

MATERIALS AND METHODS

The purification and assay methods of the alkaline protease of *B. amylosacchariticus* were described previously.¹⁾ The enzyme preparations used in this experiment showed a specific activity (activity units per mg of protein) greater than 2200, that indicated the purity of the preparations to be more than 95%. Protein concentration was determined spectrophotometrically at 280 m μ using the factor $E_{1\text{cm}}^{1\%} = 11.9$.¹⁾

Element analysis. Determination of carbon, hydrogen and nitrogen contents of the enzyme was performed at Microanalysis Laboratory of our Institute and nitrogen was also analyzed by the semi-micro Kjeldahl method. Sulfur content was calculated from the data of amino acid analysis.

Ultracentrifugal and electrophoretic analyses. Ultracentrifugal analysis was carried out at Institute for Protein Research, Osaka University, using a Hitachi Analytical Ultracentrifuge UCA-I, as described previously.^{1,12)} The sedimentation coefficient of the enzyme was calculated by the method described by Schachman,¹³⁾ and was corrected to that at 20° in water according to the method of Svedberg.¹⁴⁾ For the determination of molecular weight, approach to sedimentation equilibrium was adopted. The weight average molecular weight of the enzyme was calculated at the meniscus according to the method described by Schachman.¹³⁾ Electrophoretic analysis was made using a Hitachi Tiselius Electrophoresis Apparatus HT-B at Department of Applied Biochemistry, Osaka Municipal Technical Research Institute. The lyophilized enzyme preparation was dissolved in 25 mM buffer solution of various pH values, and, after adjusting the ionic strength to 0.1 with sodium chloride, the enzyme solution was dialyzed against a buffer solution of the same constituent in the cold and then subjected to the electrophoretic analysis. Partial specific volume was measured with a 5-ml pycnometer at enzyme concentrations of 1.0 and 0.7% and at 30°.

Amino terminal amino acid of the enzyme was

identified by the Sangers' DFB method with an inactivated enzyme preparation by TCA.^{12,15)} The details of the experimental procedures were described in the previous paper.¹²⁾

Determination of amino acid composition was kindly carried out at Shionogi Research Laboratory, Shionogi & Co., Ltd., with a Hitachi Automatic Amino Acid Analyzer. The crystalline enzyme preparation lyophilized was dissolved in a minimum volume of water and precipitated by the addition of acetone to a final concentration of 67%(v/v) in the cold. The protein was collected by centrifugation, washed twice with 80% acetone, successively once with acetone and ether, and then dried at 100°C in vacuo to a constant weight.

RESULTS AND DISCUSSION

1) Physical and Chemical Properties.

Sedimentation coefficient and molecular weight of the enzyme. The sedimentation coefficient, $S_{20,w}$ was calculated from the sedimentation velocity run shown in Fig. 1-A, and a value of 2.89×10^{-13} was obtained. The analysis was operated at 55,100 r. p. m. and 18.4° in a standard 12 mm, 4° sector cell with 0.68 to 0.39% enzyme solution. In Fig. 1-B, approach to sedimentation equilibrium run is shown. The experimental conditions were almost the same as those described above with the exception that 13,100 r. p. m. was chosen. The concentration of the original sample was determined in arbitrary units in a 12 mm, 4° sector synthetic boundary cell. The apparent molecular weight was calculated for each photograph at meniscus and the values were plotted against time (Fig. 2). The time dependence of molecular weight with a positive slope was observed and the molecular weight, 22,700, was obtained by extrapolating the apparent molecular weight to zero time by the least squares method.

Amino terminal amino acid. The TCA-inactivated enzyme was used for the identification of the amino terminal amino acid. By the method described in the previous paper,¹²⁾ a

12) D. Tsuru, H. Kira, T. Yamamoto and J. Fukumoto, This Journal, **30**, 1164 (1966).

13) H. K. Schachman, "Methods in Enzymology," IV, Academic Press Inc., New York, (1957), p. 32.

14) T. Svedberg and D. K. Pederson, "The Ultracentrifuge," Clarendon Press Inc., Oxford (1940), p. 445.

15) D. Tsuru, J. D. McConn and K. T. Yasunobu, *J. Biol. Chem.*, **240**, 2415 (1965).

FIG. 1. Ultracentrifuge Pattern and Archibald Run of Alkaline Protease of *B. amylosacchariticus*

- A: The sedimentation velocity run. The photographs were taken from right to left at 7, 18, 33, 43, 53 and 60 minutes after reaching the full speed, 55,100 r.p.m. The enzyme concentration used was 0.68% in 0.01 M acetate buffer, pH 5.8, containing sodium chloride to adjust the ionic strength to 0.1.
- B: Archibald run. The photographs were taken at 15(a), 25(b), 35(c), 45(d) and 55 minutes (e) after reaching the full speed, 13,100 r.p.m. a' indicates a synthetic boundary run performed under the same condition as in b.

FIG. 2. Variation of Apparent Molecular Weight with Time in Archibald Run.

The experimental conditions are described in Fig. 1-B.

considerable quantity of alanine together with a trace amount of aspartic acid were found as dinitrophenylated amino acids. Quantitative analysis indicated that 0.6 to 0.7 moles of DNP-alanine were recovered per mole of enzyme on the basis of molecular weight of 22,700 while the quantity of DNP-aspartic acid obtained was stoichiometrically negligible.

Partial specific volume. Duplicate determinations of partial specific volume of the enzyme

yielded a value of 0.72.

Using the values obtained for molecular weight, $S_{20,w}$, and partial specific volume, the frictional ratio, f/f_0 , of the enzyme was computed to be 1.04.

In Table I physical and chemical properties of the alkaline protease of *B. amylosacchariticus* are summarized and compared with those of *B. subtilis* alkaline proteases so far reported. Table I shows that all these alkaline proteases are possessed of alanine as the amino terminal amino acid. However, there can be seen a considerable difference in the molecular weight between *B. amylosacchariticus* alkaline protease and the other three enzymes.

2) Amino Acid Composition.

In Table II, amino acid composition of *B. amylosacchariticus* alkaline protease is presented. The amino acid analysis was carried out with the samples hydrolyzed for 24, 48 and 72 hours. The values for threonine, serine and amide ammonia were those obtained by extrapolating

TABL I. SOME PHYSICAL AND CHEMICAL PROPERTIES OF ALKALINE PROTEASES
 OBTAINED FROM VARIOUS STRAINS OF *B. subtilis*

	<i>B. amylosacch.</i> protease	Subtilopeptidase ⁵⁻⁸⁾		BPN' ¹⁰⁾
		A	B	
$E_{280m\mu}^{1\%}$ { observed	11.9	8.6	—	11.7
{ calculated*	11.6	7.1	11.0	9.7
$S_{20, w}$	2.89	2.85	2.7	2.77
$\bar{V}$ { experimental	0.72	0.749	—	—
{ calculated*	0.725	—	—	0.73
Isoelectric point	7.5~8.0	9.4	9.1	7.8
Molecular weight $\times 10^{-3}$	22.7	30	28	27.7
f/f_0	1.04	1.14	—	1.18
Specific activity (units/mg protein) $\times 10^{-3}$	2.3	—	2.2	2.2
Amino terminal amino acid	Alanine	Alanine	Alanine	Alanine
C, %	50.86	—	—	49.5
H, %	7.18	—	—	7.5
S, %	0.44	—	—	0.76
N, %	15.9	16.6	16.4	16.88
Ash, %	1.3	—	—	1.3

* Calculated from the amino acid composition shown in Tables II and III and References 6 and 10.

TABLE II. AMINO ACID COMPOSITION OF ALKALINE PROTEASE OF *B. amylosacchariticus*

	Amino acid	Amino acid residue	Nitrogen	Number of residue ^{a)}	
	%	%	%	Calculated	Integral
Lysine	3.62 ^{b)}	3.18	0.694	5.6	6
Histidine	3.19 ^{b)}	2.82	0.863	4.7	5
Arginine	2.15 ^{b)}	1.92	0.690	2.8	3
Aspartic acid	12.04 ^{b)}	10.41	1.270	20.4	20
Threonine	7.21 ^{c)}	6.13	0.851	13.7	14
Serine	17.21 ^{c)}	14.26	2.294	37.0	37
Glutamic acid	7.64 ^{b)}	6.70	0.724	11.7	12
Proline	4.83 ^{b)}	4.07	0.589	9.5	10
Glycine	8.16 ^{b)}	6.20	1.522	24.6	25
Alanine	10.56 ^{b)}	8.42	1.660	26.8	27
Half cystine	0	0	—	—	—
Valine	10.24 ^{d)}	8.66	1.230	19.8	20
Methionine	2.04 ^{d)}	1.79	0.191	3.1	3
Isoleucine	7.05 ^{d)}	6.08	0.755	12.1	12
Leucine	6.99 ^{b)}	6.03	0.747	12.0	12
Tyrosine	7.33 ^{b)}	6.60	0.567	9.2	9
Phenylalanine	1.66 ^{b)}	1.48	0.140	2.3	2
Tryptophan	2.40 ^{c)}	2.18	0.327	2.7	3
Amide-NH ₂	(1.20) ^{e)}	(1.13)	0.989	(16)	(16)
Total		96.93	16.10		220

a) Based on a molecular weight of 22,700.

b) Average values from 24, 48 and 72 hour hydrolyzates.

c) Values extrapolated to zero hydrolysis time.

d) Maximum values adopted.

e) Calculated from ultraviolet absorption.

to zero hydrolysis time. For valine, methionine and isoleucine, the maximum values obtained from 72 hour hydrolyzate were adopted. Analysis on the oxidized preparation by performic acid revealed that the enzyme contained no half cystine. Tryptophan content was calculated from the ultraviolet absorption of the enzyme dissolved in 0.1N sodium hydroxide solution¹⁶⁾ (Fig. 3). The tyrosine content calculated from Fig. 3 was in good

FIG. 3. Ultraviolet Absorption Spectra of *B. amylosacchariticus* Alkaline Protease.

The enzyme concentration was 413 $\mu\text{g/ml}$.
A: In 10^{-2} M Tris-HCl buffer, pH 7.3.
B: In 10^{-1} M NaOH solution.

agreement with that obtained from the analysis by an amino acid analyzer. The absorption spectrum of the enzyme in neutral solution revealed a maximum at 278 $m\mu$ and the ratio of absorption at 280 to 260 $m\mu$ was 2.1, indicating the absence of significant amounts of nucleic acid component.

The sum of total residues of amino acids accounted for about 97% of the dried enzyme preparation, whereas the recovery of nitrogen was shown to exceed 100%. The remaining 3% might be impurities such as ash and non-nitrogenous compounds. Element analysis indicated that the crystalline preparation con-

tained 1.3% of ash. Using the value of 22,700 for the molecular weight, the enzyme was found to be composed of 220 residues of amino acids. The partial specific volume and absorbancy at 280 $m\mu$ computed from the amino acid composition were 0.725 and 11.6, respectively.

In Table III, the amino acid composition of

TABLE III. COMPARISON OF AMINO ACID COMPOSITION OF ALKALINE PROTEASES OBTAINED FROM VARIOUS STRAINS OF *B. subtilis*

	Moles amino acid/ 10^5 g of protein			
	<i>B. amylosacch.</i> protease	Subtilopeptidase ⁶⁾		BPN' ¹⁰⁾
		A	B	
Lysine	27	31	39	39
Histidine	23	17	21	21
Arginine	14	14	7	7
Aspartic acid	91	106	103	100
Threonine	64	68	46	50
Serine	168	126	135	136
Proline	45	31	53	50
Glutamic acid	55	41	53	57
Glycine	114	133	120	121
Alanine	123	147	135	135
Half cystine	0	0	0	0
Valine	91	120	110	107
Methionine	13	20	18	18
Isoleucine	55	38	46	46
Leucine	55	58	53	54
Tyrosine	41	44	35	36
Phenylalanine	9	14	11	11
Tryptophan	14	4	14	11
Amide-NH ₂	73	96	103	100

the proteases obtained from several strains of *B. subtilis* is compared with that of *B. amylosacchariticus* alkaline protease. For the sake of convenience, nearest integral numbers of amino acid residues per 100,000 grams of protein are presented.

It is most likely that subtilopeptidase B and BPN' are the same since they are quite similar not only in their enzymatic properties but also in their amino acid composition, as has been suggested by Johansen and Ottesen⁶⁾ and Matsubara et al.¹⁰⁾ The alkaline protease of

16) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

B. amylosacchariticus also showed enzymatically similar properties with those of subtilopeptidase B(BPN^{*}). However, solubility in neutral solution¹⁾ and molecular weight of both enzymes were somewhat different each other. More appreciable difference between the two proteases was in their amino acid composition, especially in the content of lysine, arginine, threonine, serine, methionine, valine, isoleucine and tyrosine. The results indicate that the alkaline protease of *B. amylosacchariticus* is distinguished in the protein chemical nature from the other *B. subtilis* proteases so far reported,

suggesting the existence of diversity in alkaline proteases of *B. subtilis* group.

Acknowledgements. The authors wish to express their gratitude to Dr. M. Ebata of Shionogi Research Laboratory, Shionogi & Co., Ltd., for the performance of amino acid analysis. Thanks are also due to Dr. K. Kakiuchi of Institute for Protein Research, Osaka University, and to Dr. Y. Tsujisaka of Osaka Municipal Technical Research Institute for their courtesy on ultracentrifugal and electrophoretic analyses.