Production of Alkaline Enzymes by Alkalophilic Microorganisms

Part I. Alkaline Protease Produced by *Bacillus* No. 221

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A crystalline alkaline protease was prepared from *Bacillus* No. 221 isolated from soil. The characteristic point of this microorganism is especially good growth in alkaline media. The enzyme was most active at pH 11.5~12 towards casein and was stable at pH values from 4 to 11 on 10 min incubation at 60°C. Calcium ion was effective to stabilize the enzyme especially at higher temperatures. The enzyme was completely inactivated by DFP and urea, but not affected by sulphhydryl reagent, EDTA, SLS, and DBS. The specific activity of the enzyme towards casein was about 18,000 unit/mg, and the isoelectric point was higher than pH 9.4. The molecular weight and sedimentation constant was approximately 30,000 and 3.5*S* respectively, and N-terminal of the enzyme was identified to be alanine. The results indicate that the No. 221 alkaline protease is different from those of alkaline proteases of *Bacillus subtilis*.

It has been reported that a certain species of bacteria can grow in high alkaline media containing high concentration of sodium carbonate or sodium bicarbonate.¹⁾ Some of these bacteria produce a large amount of alkaline protease, and neither neutral nor acid protease can be detected in the culture broth.

Bacillus No. 221 isolated from soil accumulates a large amount of alkaline protease which is entirely different from *Bacillus subtilis* alkaline proteases.^{2~4)} The newly isolated enzyme of *Bacillus* No. 221 is most active at pH 11~12. This paper deals with isolation of the strain *Bacillus* No. 221 and some properties of the crystalline alkaline protease.

MATERIALS AND METHODS

Medium. An isolation medium (I-medium) contained: glucose, 10 g; polypeptone, 5 g; Difco yeast extract, 5 g; K₂HPO₄, 1 g; MgSO₄ 7 H₂O, 0.2 g; Na₂CO₃, 10 g and 1 liter of distilled water. Sodium carbonate was sterilized separately and added to the medium. The I-medium was solidified by the addition of agar (1.5% w/v), if necessary.

Isolation method of alkalophilic bacteria. A small amount of soil was suspended in sterilized water and spread on I-medium agar plates. The plates were incubated at 37°C for 24 to 48 hr. *Bacillus* No. 221 producing large amount of alkaline protease was isolated from about 400 colonies.

Characterization and identification of bacteria. Microbiological properties were investigated according to the methods described in "Aerobic Sporeforming Bacteria"⁵⁾ and "Bergey's Manual of Determinative Bacteriology."⁶⁾ Unless stated otherwise, media used

1) K. Horikoshi, *Proc. Agr. Chem. Soc. Japan*, p. 201 (1971).

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

3) B. Hagihara, "The Enzyme," Vol. 4, ed. by Boyer, Lardy, and Myrback, Academic Press Inc., New York, 1958, p. 193.

4) D. Tsuru, H. Kira, T. Yamamoto and J. Fukumoto, *J. Agr. Biol. Chem.*, **30**, 1261 (1966).

5) N. R. Smith, R. E. Gordon and F. E. Clark, "Aerobic Sporeforming Bacteria," U.S. Dept. of Agric., 1952.

6) R. S. Breed, E. G. D. Murray and N. R. Smith, "Bergey's Manual of Determinative Bacteriology," Williams and Wilkins Co., Baltimore, 1957.

for the identification were supplemented with 1% Na_2CO_3 .

Preparation of crude enzyme solution. *Bacillus* No. 221 was grown aerobically at 37°C in I-medium. After 3 days of incubation with shaking, cell-free enzyme solutions were prepared by centrifugation at $6,000 \times g$ for 10 min.

Assay of enzyme activity. An enzyme solution (1 ml) suitably diluted was mixed with 5 ml of 0.6% Hammersten casein solution (pH 11.5, made up with $2 \times 10^{-2} \text{ M}$ $\text{Na}_2\text{HPO}_4\text{-NaOH}$ buffer) at 30°C. After 10 min incubation, 5 ml of trichloroacetic acid (TCA) solution (consisting of 0.11 M TCA, 0.22 M sodium acetate and 0.33 M acetic acid) was added to the reaction mixture. The mixture was further incubated at 30°C for 30 min and then filtered. An absorbance of the filtrate was measured at 275 m μ . One unit of the protease activity is defined as the amount of the enzyme to produce the digest which is not precipitated by TCA solution and which gives absorbance value equivalent to 1 μg of tyrosine per min at 30°C.

Protein concentration. Purified enzyme concentration was determined spectrophotometrically on the basis that $E_{1\text{cm}}^{1\%}$ at 280 m μ of the alkaline protease of *Bacillus* No. 221 (No. 221 protease) was 12.5, which was determined by the weight of the crystallized No. 221 protease. Protein in a crude solution was measured by the method of Lowry *et al.*⁷⁾

Electrophoresis and ultracentrifugal analysis. Electrophoretic experiments were carried out by using a Hitachi Tiselius Electrophoresis Apparatus HT-B. A Hitachi Analytical Ultracentrifuge UCA-1 was used for ultracentrifugal analysis, and the sedimentation constant was calculated by the method of Schachman.⁸⁾

Amino acid analysis. The crystalline enzyme preparation was dissolved in water and precipitated with acetone (80%). The protein was collected by centrifugation, washed three times with acetone and ether, and then dried *in vacuo* at 100°C. After acid hydrolysis, the hydrolyzate was analyzed with a Hitachi Amino Acid Analyzer KLA-3B.

N-Terminal analysis by dinitrophenyl method. The procedure of Frankel-Conrat, Harris, and Levy as applied by Waller⁹⁾ was used for the identification of N-terminal. The reaction with 2-fluorodinitrobenzene was performed in 8 M urea at 40°C in the presence of 0.1 M sodium bicarbonate. The reaction product was successively washed with HCl, acetone, and ether. The dinitrophenyl (DNP-) protein (about 100 mg) was hydrolyzed in an evacuated sealed tube with constant boiling HCl at 110°C for 12 hr. DNP-amino acid was extracted with ether from the hydrolyzate and chromatographed on silica gel thin-layer chromatography plates. Two dimensional chromatography was performed with toluene-pyridine-ethyl-enchlorohydrin-0.8 N NH_4OH (100 : 30 : 60 : 60 v/v) system in the first direction and a chloroform-methanol-glacial acetic acid (95 : 5 : 1 v/v) system in the second direction.

RESULTS

Characters of Bacillus No. 221

The isolated strain No. 221 was an aerobic, sporeforming, gram positive, motile, and rod-shaped bacterium. It is clear that the bacterium should belong to the genus *Bacillus*. The characteristic point of the microorganism was that the growth was especially good in alkaline media, the optimal pH for growth being about 10. Table I summarizes the morphological and cultural characteristics of the strain No. 221.

Purification of alkaline protease

To 1 liter of the culture fluid (6,300 u/ml), 5 liters of cold acetone were added, the precipitate was collected by centrifugation, and washed with cold acetone. About 25 g of brownish powder were obtained. The acetone dried powder was washed three times with 80% saturated ammonium sulfate, dissolved in water, and dialyzed against 0.01 M phosphate buffer (pH 7.5) for 24 hr. The dialyzate (100 ml) was passed through a DEAE-cellulose

7) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 277 (1951).

8) H. K. Schachman, "Method in Enzymology," ed. by C. P. Clowick and N. O. Koplan, Academic Press Inc. New York, Vol. IV, 1957, p. 32.

9) J. I. Waller and J. I. Harris, *Proc. Natl. Acad. Sci. U.S.A.*, **47**, 18 (1961).

TABLE I. MORPHOLOGICAL, CULTURAL, AND BIO-CHEMICAL CHARACTERISTICS OF STRAIN No. 221

Morphological characteristics			
Form	Rods		
Size	0.6~0.8 $\mu \times 2 \sim 3 \mu$		
Motility	Motile		
Gram stain	Positive		
Sporangia	Slightly swollen		
Spores	0.8~1 $\mu \times 1.3 \sim 1.4 \mu$; oval; central to subterminal		
Cultural and biochemical characteristics			
	pH 7	pH 10.3	
Nutrient broth	±	+	
Glucose-nutrient broth	±	++	
Glucose-nutrient agar slants	—	++	
Potato	±	++	
I-medium	±	++	
Glucose-nitrate agar slants	—	±	
Glucose-asparagine agar slants	—	+	
Anaerobic growth in glucose broth	—	—	
Anaerobic production of gas from nitrate	—	—	
Utilization of citrate		Utilized	
Hydrolysis of starch		Hydrolyzed	
Hydrolysis of gelatin		Hydrolyzed	
Reduction of nitrate to nitrite		Reduced	
Voges-Proskauer test		Negative	
Temperature for growth in I-medium	up to 55°C		
pH for growth in I-medium ^{a)}	pH 7 to pH 11		

^{a)} pH in the I-medium was adjusted by the addition of HCl or NaOH.

— indicates no growth; ±, poor growth; + normal growth; ++, growth abundant.

column (4 $\times$ 30 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.5). The No. 221 protease was not adsorbed with the DEAE-cellulose column, but passed through the column. The effluent (about 150 ml) was loaded on a CM-cellulose column (3 $\times$ 30 cm) equilibrated with 0.01 M phosphate buffer (pH 7.5), by which the enzyme was completely adsorbed. The column was washed with 0.01 M phosphate buffer (pH 7.5) and the enzyme was eluted with 0.01 M phosphate buffer (pH 7.5) containing 0.15 M NaCl. The enzyme was precipitated with 50% saturation of ammonium sulfate, and dissolved in 9 ml of 5 mM CaCl₂. The enzyme solution was brought to 30% saturation with ammonium sulfate. The co-

($\times$ 1600)

FIG. 1. Micrograph of Crystalline Alkaline Protease.

TABLE II. PURIFICATION OF ALKALINE PROTEASE

Steps	Volume (ml)	Unit/ml	Protein (mg)	Specific activity (unit/mg protein)	Recovery (%)
Culture fluid	1,000	6,300	2,000 ^{a)}	3,150	100
Acetone-dried	21g				95
DEAE-cellulose	140	40,000	320	17,000	87
CM-cellulose	50	80,000	220	18,000	62
Crystals (1st)			165	19,000	50
Crystals (2nd)			142	18,500	42

^{a)} The culture fluid was neutralized with HCl and an equal volume of 10% perchloric acid was added. The precipitates were dissolved in 0.5 N NaOH and protein was determined by the method of Lawry *et al.*⁸⁾

lumbar crystals were observed after a few hours. Figure 1 shows the crystals of No. 221 protease. Table II summarizes the results of the purification of the enzyme.

Effect of pH on activity

Figure 2 shows the effect of pH on the protease activity. The pH was adjusted with the following buffer systems: 0.02 M Tris-HCl (pH 7~9); 0.02 M Carbonate-Bicarbonate (pH 9~10.5); 0.02 M Borax-NaOH (pH 9~11); 0.02 M Na_2HPO_4 -NaOH (pH 10~11), and 0.02 M KCl-NaOH (pH 12~13). Other conditions were the same as those of the standard assay method. The enzyme was most active at a pH range between 11 and 12 toward casein.

FIG. 2. Effect of pH on Enzyme Activity.

The following buffer systems were used: 0.02 M Tris-HCl (pH 7~9); 0.02 M carbonate-bicarbonate (pH 9~10.5); 0.02 M Borax-NaOH (pH 9~11); 0.02 M Na_2HPO_4 -NaOH (pH 10~11); 0.02 M KCl-NaOH (pH 12~13). Other conditions were the same as those of the standard assay method.

Effect of pH on stability of the enzyme

Stability of the enzyme was investigated in buffer solutions of various pH's containing 3 mM of Ca^{2+} . The reaction mixtures were incubated at 60°C for 10 min and residual

FIG. 3. Effect of pH on Stability.

Buffer systems used were acetate (0.02 M, pH 4~6); Tris-HCl (0.02 M, pH 7~9), and Borax-NaOH-NaCl (0.02 M, pH 9~12). The reaction mixture were incubated at 60°C for 10 min and the remaining activity was measured at pH 11.5.

activity was measured at pH 11.5. The following buffer systems were used: Acetate (0.02 M, pH 4~6); Tris-HCl (0.02 M, pH 7~9), and Borax-NaOH-NaCl (0.02 M, pH 9~12). As shown in Fig. 3, the enzyme was most stable at pH 7~8.

Temperature optimum for the enzyme action and thermal stability

The optimum temperature was determined by varying the incubation temperature. The measurement was carried out in the presence or absence of 5 mM CaCl_2 . In Fig. 4, the activity measured at 30°C is expressed as 100%. The activity at 60~65°C in the presence of Ca^{2+} was about 700% and that at 60°C in the absence of Ca^{2+} was about 400%.

The enzyme was dissolved in 0.05 M Borax-NaON (pH 10.0) in the presence or absence of 5 mM Ca^{2+} , incubated at various temperatures for 10 min, and residual activity was measured at pH 11.5. As shown in Fig. 5, the residual activity was 100% when heated at 30~60°C in the presence of Ca^{2+} , and 50% at 65°C.

FIG. 4. Optimum Temperature for Enzyme Action.

The reaction was carried out at the temperature indicated for 10 min at pH 11.5. Open circle, in the presence of Ca^{2+} ; closed circle, in the absence of Ca^{2+} .

FIG. 5. Effect of Temperature on the Stability of the Enzyme.

The enzyme was dissolved in Borax-NaOH buffer (pH 10.0), incubated at the temperatures indicated for 10 min, and the residual activities were measured.

A

B

FIG. 6. Ultracentrifuge Pattern and Archibald Run of Alkaline Protease.

A: The sedimentation velocity run. The photographs were taken from right to left at 10 min intervals after reaching the full speed, 55,430 rpm. The enzyme concentration used was 0.7% in 0.04 M Tris-HCl buffer, pH 8.0, containing 0.005 M CaCl_2 .

B: Archibald run. The photographs were taken at 10 min intervals after reaching the full speed, 16,890 rpm. The enzyme concentration used was 0.4% in 0.04 M Tris-HCl buffer, pH 8.0, containing 0.005 M CaCl_2 .

Sedimentation constant and molecular weight

The crystalline enzyme showed only one peak throughout the duration of a run of 120 min at 55,430 rpm. The sedimentation constant of the enzyme at pH 7.2 was about 3.5S. Molecular weight determined by the method of Archibald⁸⁾ was about 30,000, (Fig. 6).

Ultraviolet absorption spectra

Ultraviolet absorption spectra of the enzyme are shown in Fig. 7. Absorbance of 1% enzyme solution (Tris-HCl buffer pH 8.0, 0.02M) at 280 m μ was about 12.5 and the ratio of absorption at 280 m μ to 260 m μ was about 2.1, which indicated the absence of nucleic acid.

FIG. 7. Ultraviolet Absorption Spectra of the Enzyme.

The enzyme concentration was 590 μ g/ml.

Solid line: In 0.1 M Tris-HCl buffer (pH 7.0).

Dotted line: In 0.2 M KCl-NaOH buffer (pH 12).

Isoelectric point of the enzyme

Result of the Tiselius electrophoresis at pH 9.4¹⁰⁾ showed that the isoelectric point of the enzyme was higher than pH 9.4 and that

FIG. 8. Electrophoretic Pattern of the Enzyme.

The electrophoresis was carried out at 6 mA and 15°C. The enzyme concentration was 0.7% in glycine-NaOH-NaCl buffer (pH 9.4, $I=0.1$).¹⁰⁾

the enzyme was homogenous (Fig. 8). Electrophoresis on cellulose acetate plates at pH 4.0¹⁰⁾ and pH 9.0¹⁰⁾ exhibited that the mobilities of the enzyme was faster than those of Bovine pancreatic RNase A (isoelectric point=9.6) and slower than those of lysozyme (isoelectric point=11). From these results, the isoelectric point of the enzyme should be about 10.

Effect of protease inhibitor, surface active reagents, and sulfhydryl reagents

The enzyme was completely inactivated by DFP (diisopropyl fluorophosphate), but not inactivated by surface active reagents such as DBS (sodium dodecylbenzene sulfonate) and SLS (sodium lauryl sulfate). Moreover, EDTA (ethylenediaminetetra acetic acid) and PCMB (*p*-chloromercuribenzoate) showed no inhibitory effect on the enzyme (Table III).

Amino acid composition and N-terminal

The amino acid analysis was carried out with the samples hydrolyzed with constant

10) "Data for Biochemical Research," ed. by R. M. C. Dawson, *et al.*, Oxford at Clarendon Press, 1969, p. 500.

boiling HCl for 24, 48 and 72 hr at 110°C. The values for threonine and serine were those obtained by extrapolating to zero time of hydrolysis. Tryptophane content was calcu-

TABLE III. EFFECT OF VARIOUS INHIBITORS ON ENZYME ACTION

The enzyme was incubated with the reagent in 0.02 M Tris-HCl buffer (pH 7.0) at 25°C for 30 min and residual activity was measured.

Inhibitor	Concentration	Relative activity
None	—	100%
DBS	0.2%	100
SLS	0.2%	100
EDTA	5×10^{-3} M	103
PCMB	10^{-3} M	110
DFP	10^{-3} M	1
Urea	6M	1

TABLE IV. AMINO ACID COMPOSITIONS OF ALKALINE PROTEASES

Amino acid	No. 221	<i>B. subtilis</i> ¹¹⁾	BPN ¹²⁾	<i>B. sub. Carlsberg</i> ¹³⁾
Tryptophane	5	3	3	1
Lysine	6	6	11	9
Histidine	8	5	6	5
Arginine	8	3	2	4
Aspartic acid	29	20	28	28
Threonine	18	14	13	19
Serine	23	37	37	32
Glutamic acid	16	12	15	12
Proline	16	10	14	9
Glycine	39	25	33	35
Alanine	45	27	37	41
Valine	27	20	30	31
Methionine	4	3	5	5
Isoleucine	9	12	13	10
Leucine	22	12	15	16
Tyrosine	7	9	10	13
Phenylalanine	2	2	3	4
Half cystine	0	0	0	0

11) D. Tsuru, H. Kira, T. Yamamoto and J. Fukumoto, *J. Agr. Biol. Chem.*, **31**, 330 (1967).

12) F. S. Markland and E. L. Smith, *J. Biol. Chem.*, **242**, 5198 (1967).

13) E. L. Smith, R. J. Delange, W. H. Evans, M. London and F. S. Markland, *J. Biol. Chem.*, **243**, 2184 (1968).

TABLE V. PHYSICAL AND CHEMICAL PROPERTIES OF ALKALINE PROTEASES

	No. 221	<i>B. subtilis</i> ¹¹⁾	BNP ¹²⁾
$E_{1cm}^{1\%}$ at 280 m μ	12.5	11.9	11.7
S_{20}	3.5	2.89	2.77
Molecular weight	30,000	22,700	27,700
Isoelectric point	<9.4	7.5~8	7.8
Optimum pH	11.5	10.5	10.5
Specific activity unit/mg protein	18,000	2,300	2,200
N-Terminal	alanine	alanine	alanine
C, %	48.04	50.86	49.50
H, %	6.62	7.18	7.5
N, %	16.07	15.9	16.88
S, %	0.82	0.44	0.76

lated from the ultraviolet absorption of the enzyme dissolved in 0.1N NaOH solution. Analysis of the oxidized preparation by performic acid showed that the enzyme did not contain cystine. Table IV shows amino acid composition of the alkaline protease. N-Terminal analysis showed that N-terminal of the enzyme was alanine. No other DNP-amino acid was detected. For the confirmation of DNP-alanine, hydrolysis by ammonium hydroxide was applied to the DNP-amino acid fraction and the regenerated amino acid was also identified as alanine by an amino acid analyzer. Table V summarizes physical and chemical properties of the enzyme.

DISCUSSION

The strain of *Bacillus* No. 221 isolated from soil produces large amount of new alkaline protease in alkaline media. This strain can grow slowly in neutral media, but no alkaline protease was detected. Taxonomic position of this strain will be reported elsewhere together with other Bacilli which grow preferentially or obligatorily in alkaline media. In this series of work, I would like to call these

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

microorganisms "alkalophilic microorganisms."

The alkaline protease of *Bacillus* No. 221 was purified by passing through a DEAE-cellulose column followed by CM-cellulose column chromatography. The crystalline enzyme is a homogenous protein under the tested conditions: ultracentrifugal analysis, Tiselius electrophoresis, cellulose acetate electrophoresis, and gel filtration. The enzyme has optimum pH at 11.5 toward casein. This value is higher than those of alkaline proteases of *Bacillus subtilis*, which are about pH 10~10.5. The No. 221 protease was completely inactivated by DFP, therefore this enzyme can be classified as a serine enzyme. The *B. subtilis* alkaline proteases are also inactivated by DFP. It is noteworthy that the specific activity toward casein was about 18,000 units/mg. Under the same assay method, α -chymotrypsin was 2,500 and trypsin 1,700 units/mg. Specific

activities of alkaline proteases of *B. subtilis* were 2,000~3,000 units/mg. Molecular weight of the enzyme estimated by Archibald method was approximately 3×10^4 and the gel filtration method gave 1.5×10^4 . The gel filtration method may not be applied to the No. 221 enzyme due to the high isoelectric point. For example, lysozyme (Ip=11) deviated from the standard curve of proteins and gel filtration method gave molecular weight of 8,000.

As the results, I wish to conclude that the No. 221 protease is entirely different from known alkaline proteases of *B. subtilis* such as BPN' *etc.* in their properties except N-terminal of the enzyme protein.

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