Chem. Pharm. Bull. 21(3) 538—545 (1973)

UDC 615.355.011.5 : 577.15.07.08

Antitumor Activity of *Bacillus natto*. IV.[1] Purification and Properties of an Extracellular Protease from *Bacillus natto* KMD 1126[2]

Yukio Kameda, Katsuhiko Matsui, Kazuyoshi Hosoya, Akira Nomura, and Noriko Sugano

Faculty of Pharmaceutical Sciences, Kanazawa University[3]

(Received August 30, 1972)

An alkaline protease (peptidylpeptide hydrolase, EC class 3.4.4.) of *Bacillus natto* KMD 1126 was purified by ammonium sulfate fractionation, DEAE cellulose chromatography, CM cellulose chromatography, and Sephadex G 100 gel filtration. It had a pH optimum over the range of 8.5—9.5 toward casein substrate. It was not inactivated by chelating agents or sulfhydryl reagents, but completely inactivated by incubation with DFP. From these results and the substrate specificity, this enzyme resembles to alkaline protease of *Bacillus natto* Ns. However, the two enzyme differ in specific activity and kinetic properties. This enzyme had not cytolytic activity on Ehrlich ascites carcinoma cells. However, when a mixture of surfactin, the protease, and EDTA was incubated with carcinoma cells, synergetic effect on the cytolysis was observed.

In a previous paper of this series,[4] the authors reported that a strain of *Bacillus natto* (tentatively called KMD 1126), which was newly isolated from "Natto" (fermented beans), has a contact antitumor effect on solid type Ehrlich carcinoma cells. Then, it was reported[1] that there are at least two kinds of cytolytic substances in the culture medium of *Bacillus natto* KMD 1126 by treatment with carbon and Sephadex G 25 gel filtration. That is, one substance was adsorbed on carbon, eluted with EtOH, and large melecule by Sephadex G 25 gel filtration, which constitute approximately 10% of the cytolytic activity in the culture medium. On the other hand, about 50—60% of cytolytic activity in the culture medium was not adsorbed on carbon and this compound was small molecule by Sephadex G 25 gel filtration. The former substance was proved to be identical with surfactin which was potent clotting inhibitor in the thrombin fibrinogen system obtained from culture fluids of *Bacillus subtilis* by Kakinuma, *et al.*[5] It has been found to produce a much amount of protease in the culture medium of *Bacillus natto* KMD 1126. As the continuation of above work, it was now of interest to examine whether the extracellular protease has a cytolytic activity on Ehrlich ascites carcinoma cells, or not. The present paper deals with the purification and some properties of the enzyme and effect on Ehrlich ascites carcinoma cells.

Material and Method

Materials——α-N-Benzoyl-L-arginine amide, α-N-benzoyl-L-arginine ethyl ester, and N-acetyl-L-tyrosine amide were purchased from Protein Research Foundation in the Institute for Protein Research, Osaka University.

1) Part III: Y. Kameda, K. Matsui, H. Kato, T. Yamada, and H. Sagai, *Chem. Pharm. Bull.* (Tokyo), **20**, 1551 (1972).
2) This work was reported at 33rd Meeting of Hokuriku Branch, Pharmaceutical Society of Japan, Toyama, November 1971.
3) Location: *13-1, Takaramachi, Kanazawa.*
4) Y. Kameda, S. Kanatomo, Y. Kameda, and Y. Saito, *Chem. Pharm. Bull.* (Tokyo), **16**, 186 (1968).
5) K. Arima, A. Kakinuma, and G. Tamura, *Biochem. Biophys. Res. Commun.*, **31**, 488 (1968); A. Kakinuma, M. Hori, M. Isono, G. Tamura, and K. Arima, *Agr. Biol. Chem.*, **33**, 971, 973, 1523, 1669 (1969).

Acetyl-L-leucine amide,[6] mp 129—130°, $[\alpha]_D^{20}$ −36.6° (c=3, EtOH), acetyl-L-tryptophan amide,[7] mp 192°, $[\alpha]_D^{23}$ +20° (c=2, MeOH), L-leucine amide acetate,[8] mp 125°, $[\alpha]_D^{23}$ +8.3° (c=2, H_2O), acetyl-L-phenylalanine ethyl ester,[9] mp 90.5°, $[\alpha]_D^{26}$ +13° (c=3, MeOH), carbobenzoxy-glycyl-L-leucine,[10] mp 105—106°, $[\alpha]_D^{23}$ −15.6° (c=2, N NaOH), carbobenzoxy-glycyl-L-phenylalanine,[11] mp 124—125°, $[\alpha]_D^{23}$ +39° (c=2, EtOH), and carbobenzoxy-glycyl-L-leucine amide,[12] mp 125—126°, $[\alpha]_D^{13}$ −13.5° (c=2, MeOH) were prepared as described in the literatures. Surfactin was obtained from culture medium of *Bacillus natto* KMD 1126 by the method of Kameda, *et al.*[1] Sephadex G 25 and Sephadex G 100 were products of Pharmacia Fine Chemicals, and DEAE cellulose and CM cellulose were products of Braun Company. Diisopropyl fluorophosphate was purchased from BDH Chemicals Ltd.

All of other chemicals and salts were of reagent grade and used without further purification.

Bacterial Strain and Culture Condition——*Bacillus natto* KMD 1126 is the stock strain kept in this laboratory on nutrient agar slants. Culture was carried out by the following manner. To 3 liters shaking flasks, 800 ml of bouillon (pH 7.2) were added, sterilized in an autoclave under 1.0 kg/cm^2 pressure for 20 min, and inoculated with 50 ml of seed culture of the bacteria, which was prepared by shaking culture of the organisms at 37° for 15 hr in the same medium. The culture was carried out at 37° with reciprocal shaking (130 c/min, stroke 7 cm) for 24 hr.

Assay of Enzyme Activity——1) Protease Activity: Protease activity was measured at pH 8.0 with casein substrate by the method of Hagihara.[13] A mixture of 2.5 ml of 0.6% casein in 0.02M Tris buffer (pH 8.0) and 0.5 ml of appropriately diluted enzyme solution was incubated in water bath at 30° for 10 or 20 min. High molecular weight protein was precipitated with 2.5 ml of TCA reagent (0.11M Trichloroacetic acid, 0.22M sodium acetate, and 0.33M acetic acid in H_2O) and filtered off. A mixture of 1 ml of the filtrate, 2.5 ml of 0.55M Na_2CO_3, and 0.5 ml of Folin reagent was incubated at 30° for 30 min and absorbance was measured at 655 nm (HIRAMA RIKA KENKYUJO Photoelectric Spectro Photometer Type 6B). One unit of activity was defined as that amount of enzyme which gives an absorbance equivalent to 1 μg of tyrosine per ml per min in the original enzyme solution at 655 nm.

2) Amidase Activity: The rate of enzyme action on the peptide amides was followed by measurement of the extent of ammonia liberation in Conway microdiffusion vessels. The reaction mixture, containing 1 ml of 0.01M substrate in 0.02M phosphate buffer (pH 8.0) and 1 ml of appropriately diluted enzyme solution was incubated at 30°. After 0, 10, and 20 min, (or 0, 2, and 4 hr), 0.2 ml of the reaction mixture were placed in duplicate Conway vessels, which contained 1 ml of 0.005N H_2SO_4 in the center well. The ammonia was liberated by mixing the sample with 1 ml of saturated potassium carbonate solution. The vessels were kept at 37° for 2 hr. The liberated ammonia was determined by the method of Lubochinsky, *et al.*[14] A mixture of 0.5 ml of the content of the center wells, 0.5 ml of phenol reagent (5% phenol in 2% NaOH), 0.25 ml of 0.05% sodium nitroprusside, 3.5 ml of 0.1M phosphate buffer (pH 12.0), and 0.25 ml of sodium hypochlorite (containing 4% of active chlorine) was incubated at 30° for 30 min and absorbance of the incubation mixture was measured at 610 nm.

3) Esterase Activity: The rate of disappearance of the peptide esters was followed by a modification of the hydroxamic acid method of Izumiya, *et al.*[15] The reaction mixture, containing 1 ml of 0.025M substrate in 0.01M Tris buffer (pH 8.0) and 1 ml of appropriately diluted enzyme solution, was incubated at 30° for 0, 10, and 20 min. To 0.2 ml of the reaction mixture, 2 ml of 2M hydroxylamine·HCl and 2 ml of 3.5N NaOH were added. After at least 1 min, the mixture was acidified with 2 ml of 3.5N HCl and added 1 ml of iron solution (0.37M $FeCl_3$ in 0.1N HCl). The optical density of the purple-brown color is determined at 540 nm.

4) Carboxypeptidase Activity: The reaction mixture, containing 1 ml of 0.01M acyl peptide, 0.5 ml of 0.1M Tris buffer (pH 8.0), and 0.5 ml of appropriately diluted enzyme solution was incubated at 30°. The liberated amino acid was measured by Moore and Stein's colorimetric ninhydrin method.[16] After 0, 2, and 4 hr, 0.2 ml of incubated mixture were added to a series of test tubes which was preheated in boiling water and held at this temperature for 2 min. To each of test tubes, 1 ml of ninhydrin reagent (dissolve 0.08 g of $SnCl_2 \cdot 2H_2O$ in 50 ml of 0.1M citrate buffer, pH 5.0. Add this solution to 2 g of ninhydrin dissolved

6) Y. Kameda and S. Kanatomo, *Chem. Pharm. Bull.* (Tokyo), **19**, 2423 (1971).
7) H.T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **73**, 1541 (1951).
8) E.L. Smith and W.J. Polglase, *J. Biol. Chem.*, **180**, 1209 (1949).
9) E. Robert and J. Parks, *J. Biol. Chem.*, **203**, 755 (1953).
10) S. Goldschmidt and H. Lantenschlager, *Ann.*, **580**, 68 (1958).
11) N. Izumiya, *Tanpakushitsu Kakusan Kōso*, **9**, 599 (1964).
12) E.L. Smith and N.B. Slomin, *J. Biol. Chem.*, **176**, 835 (1948).
13) B. Hagihara, H. Matsubara, M. Nakai, T. Yonetani, T. Komaki, and K. Okunuki, *J. Biochem.* (Tokyo), **45**, 251 (1958).
14) B. Lubochinsky and J. Zalta, *Bull. Soc. Chim. Biol.*, **36**, 1363 (1954).
15) N. Izumiya and J.S. Fruton, *J. Biol. Chem.*, **218**, 59 (1956).
16) S. Moore and W.H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

in 50 ml of methyl cellosolve) was added and the mixture was immersed in boiling water for 20 min. The solution was diluted with 5 ml of 60% EtOH, and the absorption intensity was measured at 570 nm, giving a measure of the free amino acid produced by enzyme hydrolysis.

Protein Determination——Protein concentration was determined by the method of Warburg, *et al.*[17] with absorbance at 260 and 280 nm.

Purification of the Extracellular Protease——Buffer A (0.01M Tris-HCl buffer, pH 8.0, containing 0.002M $Ca(OAc)_2$) was used for enzyme purification. *Bacillus natto* KMD 1126 was grown in bouillon at 37° for 24 hr on a reciprocal shaker. The cells were removed by centrifugation for 20 min at 10000 rpm. All subsequent purification steps were carried out at 4°. To 1 liter of the supernatant, 608 g of solid ammonium sulfate (0.8 saturation) was added and pH was adjusted to 8.0 with NH_4OH. The mixture was stirred for 2 hr and centrifuged at 10000 rpm for 20 min. The precipitate was dissolved in a minimum volume of the buffer. After removal of insoluble impurities by centrifugation, the clear supernatant was applied to a column of Sephadex G 25 (4×50 cm), which had been equilibrated with buffer A. The same buffer was used to elute the enzyme. Fractions of 20 ml volume were collected at a rate of 200 ml/hr. The active fraction was applied to a column of DEAE cellulose (3×40 cm), which had been equilibrated with buffer A. The same buffer was used for elution. Fractions of 20 ml volume were collected at a rate of 120 ml/hr. Most of color impurities were adsorbed onto the column. In order to concentrate the protein, solid ammonium sulfate was added to the active fractions to a concentration of 0.8 saturation at pH 8.0. The precipitate was collected by centrifugation at 15000 rpm for 15 min, dissolved in buffer A, and applied to a column of Sephadex G 25 (3×40 cm). The resulting desalted solution was charged on a column of CM cellulose (3×30 cm), which had been equilibrated with buffer A. After the column was washed with the buffer, the adsorbed protease was eluted by a gradient system of NaCl concentration. The reservoir and mixing chamber contained 400 ml of the buffer with and without 0.3M NaCl, respectively. To the active fraction, solid ammonium sulfate was added to a concentration of 0.8 saturation at pH 8.0. The resulting precipitate was collected by centrifugation at 15000 rpm for 15 min, dissolved in a minimum amount of buffer A, and applied to a column of Sephadex G 100 (2.5×40 cm), which had been equilibrated with buffer A. Fractions of 10 ml volume were collected at a rate of 15 ml/hr.

Ultracentrifugal Analysis——Sedimentation velocity analysis was carried out with a Spinco Model E Analytical Ultracentrifuge operating at 996 rps at 20°.

Disc Electrophoresis——Polyacryl amide disc gel electrophoresis was carried out according to the method of Nagai[18] using pH 2.3 and 8.6 gels. Samples containing 50—150 μg of protein in 20—50 μl in 40% sucrose were loaded on gels and a current of 2 to 5 mA per tube was applied for 0.5 to 2 hr. Gels were stained with Amido schwartz dye and destained by gently shaking in frequent changes of 7% acetic acid over 24 hr.

Tumor and Preparation of Tumor Cell Suspension——Ehrlich ascites carcinoma was maintained by weekly intraperitoneal transplantation in ICR-SLC mice. Buffer B, containing 8 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, and 0.4 g of KH_2PO_4 in 1000 ml of H_2O and adjusted to pH 7.2, was used for determination of cytolytic activity. The ascites fluid, aspirated from mice, bearing 6—9 day old Ehrlich ascites carcinoma by sterile syringe, was suspended in chilled buffer B and centrifuged at 700 rpm for 3 min. The sedimented tumor cells were washed twice with chilled buffer B and resuspended in appropriate volume of the buffer and then the tumor cell count was made in a hemocytometer by the standard method for blood leucocytes. The tumor cell suspension was further diluted, if necessary, to give a desired cell concentration (usually $2.5—3.5 \times 10^7$ cells/ml).

Cytolytic Activity——The reaction mixtures, containing 0.2 ml of tumor cell suspension and 0.3 ml of appropriately diluted samples, were incubated in water bath at 37° for 2 hr. After incubation, they were diluted with 3.5 ml of the buffer, and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 260 nm after 10 fold dilution (HITACHI Model 101 Spectrophotometer). At the same time, 2 control experiments were run using following 2 mixtures and absorbance of the controls were deducted from that of sample. 1) A mixture of 0.2 ml of tumor cell suspension and 0.3 ml of the buffer. 2) A mixture of 0.3 ml of the sample and 0.2 ml of the buffer. One unit of the cytolytic activity was defined as the amount of cytolytic substance which gave an E_{260} reading of 1 under above conditions.

Result

Effect of Culture Time on the Production of Extracellular Protease

Bacillus natto KMD 1126 was cultured in bouillon medium at 37° with shaking and protease activity of the culture broth were measured at different culture time. As shown in Fig. 1, Production of the enzyme was maximum at 24 hr culture. Then, we used 24 hr culture broth in the following experiment.

17) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1941).
18) Y. Nagai, *Tanpakushitsu Kakusan Kōso*, **11**, 744, 818 (1966).

Purification of the Extracellular Protease

The extracellular protease was purified by ammonium sulfate fractionation, DEAE cellulose chromatography, CM cellulose chromatography, and Sephadex G 100 gel filtration.

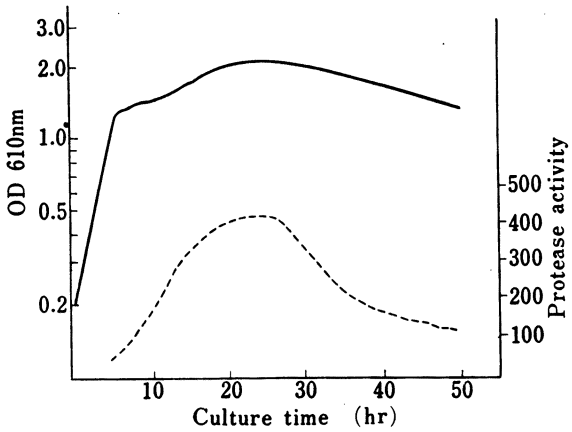

Fig. 1. Growth Curve of *Bacillus natto* KMD 1126 and Protease Production

—: growth curve (OD 610 nm)
-----: protease activity (U/ml)

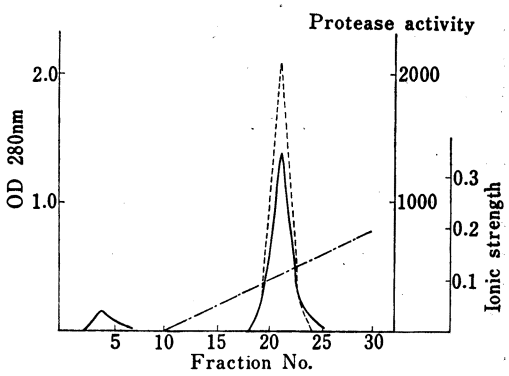

Fig. 2. Chromatography on CM Cellulose

Enzyme solution was loaded on CM cellulose column (3×30 cm). After washing with buffer A, linear gradient elution was carried out at a flow rate of 25 ml/hr.
———: absorbance at 280 nm
———: protease activity
—·—·—: NaCl concentration

TABLE I. Purification of the Extracellular Protease of *Bacillus natto* KMD 1126

Step	Volume (ml)	Protein (mg)	Activity (u)	Specific act. (u/mg)	Recovery (%)
Culture broth	1000	6910	350000	50.7	100
$(NH_4)_2SO_4$0.8 ppt	190	458	233000	508	66.7
DEAE cellulose	545	204	218000	1065	62.3
CM cellulose	157	112	156000	1392	44.6
Sephadex G 100	93	78.3	109500	1398	38.4

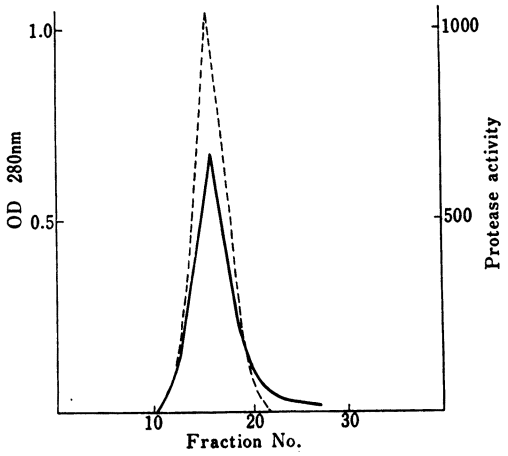

Fig. 3. Sephadex G 100 Gel Filtration

10 ml of enzyme solution was applied to Sephadex G 100 column (2.5×40 cm). The flow rate was 15 ml/hr and 10 ml fractions were collected.
———: absorbance at 280 nm
———: protease activity

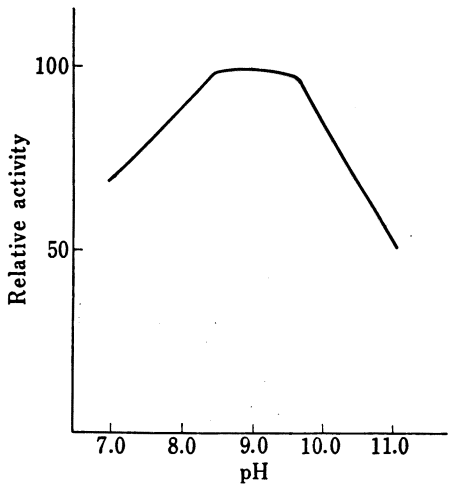

Fig. 4. Effect of pH on the Activity of Protease

The elution patterns of CM cellulose chromatography and Sephadex G 100 gel filtration were shown in Fig. 2 and Fig. 3. A summary of a typical purification is presented in Table I. The protease activity in the fraction 12 to 19 of Sephadex G 100 gel filtration were 77% of the added activity. These fractions were combined and used as "purified enzyme" in all experiments for the examination of enzymatic properties. The specific activity of the enzyme solution was 1400 u/min/mg and represented about 25 fold purification over the original culture broth. This protease was labile and the purification was carried out as rapidly as possible, in order to minimize the loss of activity.

Purity

Purity of the purified enzyme preparation was examined by ultracentrifugal and disc electrophoretic analysis. A single peak was observed throughout the ultracentrifugal run and the sedimentation coefficient was 2.9 S. A single protein band was also obtained in disc electrophoretic analysis at pH 2.3 and pH 8.6.

Enzymatic Properties of the Extracellular Protease. Effect of pH on Enzyme Activity and Stability

Effect of pH on the protease activity against casein was measured over a pH range of 7.0—11.0 with Tris-HCl and carbonate-bicarbonate buffers. As shown in Fig. 4, optimal pH is found at the neighborhood of 8.5—9.5, and the activity is considerably low below 8.0 or above 9.5. Then, the effect of pH on stability of the protease was studied. Mixture of 1 ml of the enzyme solution and 4 ml of 0.05M buffers of various pH values were allowed to stand at 4° for 48 hr and the enzyme activities were measured at pH 8.0. As shown in Fig. 5, the protease was relatively stable at pH 7.0—8.5 but it was unstable at below 7.0 or above 8.5. However, the enzyme was stabilized in the presence of calcium ion, especially at acid pH region.

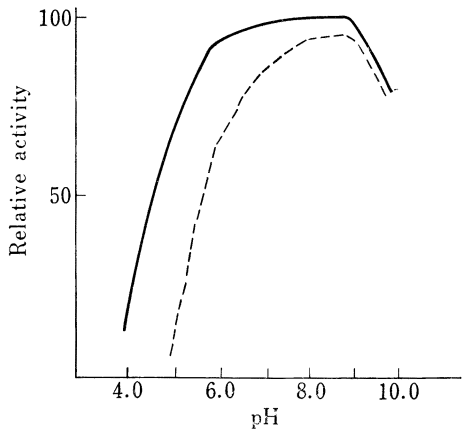

Fig. 5. Effect of pH on the Stability of the Protease

Mixture of 1 ml of the enzyme solution and 4 ml of 0.05M buffers of various pH values (4.0—5.0, acetate; 6.0—7.0, Tris-maleate; 7.0—9.0, Tris-HCl; 10.0, glycine-NaOH) were allowed to stand at 4° for 48 hr and the remaining enzyme activity were measured under standard condition.

———: with 0.005M Ca $(OAc)_2$
- - - - - - - -: without $Ca(OAc)_2$

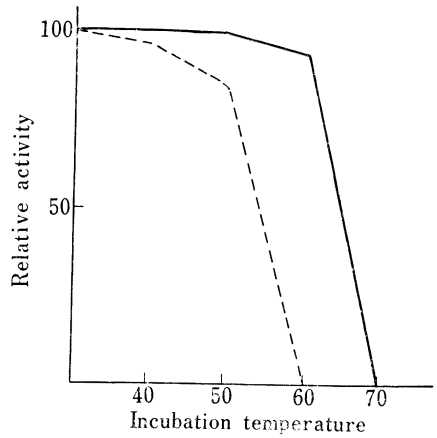

Fig. 6. Thermal Stability of the Protease

The protease solution were heated at indicated temperature for 15 min and the remaining activities were measured under standard condition.

———: with 0.005M $Ca(OAc)_2$
- - - - - - - -: without $Ca(OAc)_2$

Thermal Stability of the Enzyme

A mixture of 1 ml of the enzyme solution and 1 ml of 0.01M Tris buffer (pH 8.0) with or without calcium ion, was heated at the indicated temperature. After 15 min, the mixture

was rapidly cooled and the remaining activity was measured under the standard condition. The result is shown in Fig. 6. In the presence of 0.005 M calcium ion, the enzyme was stable up to 50° and even at 60°, about 90% of activity remained after 15 min. However, under the same condition, complete inactivation of the enzyme was found in the absence of calcium ion.

Effect of some Divalent Metal Salts on the Enzyme Stability

The results of pH- and temperature–stability experiments showed that calcium ion has markedly stabilizing effect on the enzyme. Then, effect of some divalent metal salts on the enzyme stability was examined. As shown in Table II, calcium ion was most effective and manganese ion also stabilized the enzyme.

TABLE II. Effect of Metal Ions on the Stability of the Protease

Metal ion	Remaining activity (%) after several days			
	0	1	2	6 (day)
None	100	85	75	63
$Ca(OAc)_2$	100	100	85	75
$MnCl_2$	100	100	81	73
$MgCl_2$	100	96	81	65
$ZnSO_4$	100	92	81	68

Effect of Metal Ions, Chelating Agents and Sulfhydryl Reagents on the Protease Activity

Protease activity with casein substrate was measured in the presence of metal ion, chelating agents or sulfhydryl reagents. As shown in Table III, Ca^{++}, Mn^{++}, Zn^{++}, or Co^{++} did not inhibit or activate the enzyme at 10^{-3} M. On the other hand, the protease was inactivated by Fe^{+++}, Hg^{++}, or Cu^{++}. It was observed that chelating agents such as EDTA and *o*-phenanthroline or sulfhydryl reagents such as PCMB and As_2O_3 did not inhibit the protease activity at 10^{-3} M.

TABLE III. Effect of Metal Ions, Chelating Agents, and Sulfhydryl Reagents on the Activity of the Protease

Add	Relative activity (%)	Add	Relative activity (%)
None	100	$CuSO_4$	53.9
$Ca(OAc)_2$	102	$NiSO_4$	70.7
$MnCl_2$	102	$Pb(NO_3)_2$	77.3
$MgCl_2$	103	$HgCl_2$	34.9
$ZnSO_4$	101	EDTA	106
$CoCl_2$	93.2	*o*-Phenanthroline	85.5
$FeSO_4$	83.7	PCMB	103
$FeCl_3$	0	As_2O_3	100

Effect of Diisopropylfluorophosphate (DFP) on the Protease Activity

Subtilisin are strongly inhibited by DFP which is an inhibitor of trypsin and chymotrypsin. Then, effect of DFP on the enzyme was measured. As shown in Fig. 7, decrease of activity was observed when enzyme solution was incubated in 0.01 M Tris buffer (pH 8.0) with 10^{-3} or 10^{-4} M DFP at 37° for several min.

Substrate Specificity toward Synthetic Substrate

The hydrolytic activity of the enzyme on synthetic substrate was examined. The results were presented in Table IV. Carbobenzoxy-glycyl-L-leucine and carbobenzoxy-glycyl-L-

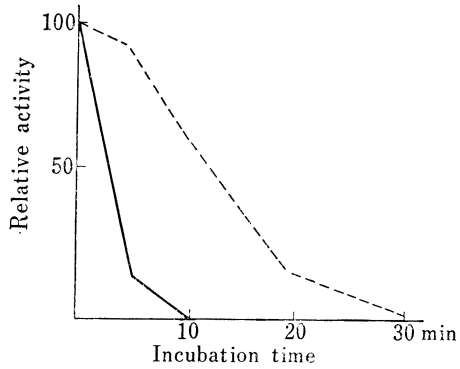

Fig. 7. Effect of DFP on the Activity of the Protease

A mixture of 2.5 ml of the protease solution, 2.0 ml of 0.01M Tris buffer (pH 8.0), and 0.5 ml of DFP (10^{-3}M or 10^{-4}M in isopropanol) was incubated at 37° for several min. 0.5 ml of the incubation mixture was taken out and the remaining activity was measured under standard condition.
———: 10^{-3}M DFP, --------:10^{-4}M DFP

Table IV. Substrate Specificity

Substrate	Acttivty (μM/hr/mg)
Cbz-Gly-L-Leu	0
Cbz-Gly-L-Phe	0
L-Leu-NH_2	0
Ac-L-Leu-NH_2	0.02
Ac-L-Tyr-NH_2	0.16
Ac-L-Trp-NH_2	0.12
Bz-L-Arg-NH_2	0
Cbz-Gly-L-Leu-NH_2	17.0
Ac-L-Phe-OEt	290
Bz-L-Arg-OEt	35.3

phenylalanine, which are specific substrate for carboxypeptidase, were not hydrolyzed at measurable rate. L-Leucine amide was not hydrolyzed by the enzyme. Carbobenzoxy-glycyl-L-leucine amide was the best substrate among the amide type compounds tested here. Acetyl-L-tyrosine amide and acetyl-L-tryptophan amide were also hydrolyzed. Esterase activity was found in the enzyme and acetyl-L-phenylalanine ethyl ester was more susceptible than benzoyl-L-arginine ethyl ester. From these results, it was assumed that blocking of amino and carboxyl group was need for susceptibility. That is, it was suggested that the enzyme had subtilisin like endopeptidase function.

Michaelis Constant

The initial reaction velocity was determined as a function of substrate concentration for carbobenzoxy-glycyl-L-leucine amide. The value of Km was evaluated by the Lineweaver–Burk plots. As the result, the Km value was 2×10^{-2}M for carbobenzoxy-glycyl-L-leucine amide.

Table V. Cytolytic Activity

Sample	Cytolytic	Activity (u/ml)
Protease	0.10	0.15
Surfactin	1.70	1.76
EDTA	0.00	0.00
Surfactin+protease	1.12	1.16
Surfactin+EDTA	1.41	1.48
Protease+EDTA	0.00	0.00
Surfactin+protease+EDTA	3.26	3.29

Data represent two experimental results.
protease: 1200 U/ml, surfactin: 200 μg/ml, EDTA: 50 μM/ml

Cytolytic Activity of the Protease and Surfactin

In order to examine the cytolytic activity of the enzyme, Ehrlich ascites carcinoma cells were incubated at 37° for 2 hr with surfactin, the protease, EDTA, surfactin+protease, surfactin+EDTA, protease+EDTA, or surfactin+protease+EDTA and the amounts of absorbing substance at 260 nm from carcinoma cells were measured. The results were shown in

Table V. The protease had not cytolytic activity and a mixture of surfactin and the enzyme had almost same lebel of cytolytic activity with surfactin only. However, when a mixture of surfactin, the enzyme, and EDTA was incubated with Ehrlich ascites carcinoma cells, it was observed that increasing amounts of 260 nm absorbing substance was obtained from carcinoma cells. That is, a mixture of surfactin, the enzyme, and EDTA had synergetic effect on the cytolysis of carcinoma cells. The mechanism of this effect is under investigation.

Discussion

Various strains of *Bacillus subtilis* and the related bacteria have been known to produce proteolytic enzymes that have usually been classified into two groups, neutral and alkaline protease, from their pH activity profile. In connection with the protease of *Bacillus natto*, which is closely related taxonomically to *Bacillus subtilis*, there are a few reports. That is, Miyake, *et al.*[19] purified a protease from *Bacillus natto* Sawamura and Yoshimoto, *et al.*[20] reported the purification procedure and some enzymatic properties of an alkaline protease obtained from culture broth of *Bacillus natto* Ns. The authors purified a protease from culture broth of *Bacillus natto* KMD 1126 and compared its enzymatic properties with that of other protease of *Bacillus natto*. Optimal pH of the protease was found at the neighborhood of 8.5—9.5. Yoshimoto, *et al.*[20] reported that *Bacillus natto* mainly secretes the alkaline protease. In the case of *Bacillus natto* KMD 1126, alkaline protease was found in the culture broth. This enzyme was not inhibited with chelating agents, and sulfhydryl reagents, but completely inactivated by incubation with DFP. Calcium ion stabilize the enzyme. The sedimentation coefficient was also similar to other alkaline protease. From these results and the substrate specificity, this enzyme resembles to alkaline protease of *Bacillus natto* Ns. However, the two enzymes differ in the specific activity and kinetic properties. That is, specific activity of this enzyme was 1400 u/mg and it was lower than 6600 u/mg of alkaline protease of *Bacillus natto* Ns. Also, this enzyme has a 10 fold lower affinity for carbobenzoxy-glycyl-L-leucine amide (*Km*, 2×10^{-2}M versus 3×10^{-3}M for alkaline protease of *Bacillus natto* Ns).

Then, the authors studied the cytolytic effect of the enzyme on Ehrlich ascites carcinoma cells. As the results, it was observed that the enzyme had not cytolytic effect on the cells and a mixture of surfactin and the enzyme had also cytolytic activity to similar surfactin only. Kakinuma, *et al.*[21] reported that a mixture of surfactin, pronase, and EDTA has a synergetic effect on the lysis of *Alcaligenes faecalis*. The authors also studied the effect of a mixture of surfactin, the enzyme, and EDTA on Ehrlich ascites carcinoma cells and observed synergetic effect on the cytolysis of carcinoma cells. The mechanism of this effect is now under investigation.

Acknowledgement The authors are grateful to Miss H. Kawakami of Cancer Research Institute, Kanazawa University for ultracentrifugal analysis.

19) S. Miyake, J. Shimizu, K. Watanabe, Y. Nonoguchi, T. Saeki, N. Kitayama, and I. Matsushita, *Hyōgo Nōka Daigaku Kenkyu Hōkoku*, **1**, 11 (1953); *idem*, *ibid.*, **3**, 97 (1958); *idem*, *ibid.*, **4**, 69 (1959).
20) T. Yoshimoto, J. Fukumoto, and D. Tsuru, *International J. Protein Res.*, 3, 285 (1971).
21) A. Kakinuma and K. Arima, *Kagaku to Seibutsu*, **6**, 606 (1968), *Takeda Kenkyusho Nempō*, **28**, 140 (1969).