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Isolation of Neutral Protease Hyperproducing *Bacillus* sp. KN103N and Some Properties of the Enzyme

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中性 Protease 高 生産性 *Bacillus* sp. KN103N의 分離 및 酵素의 特性

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Abstract — A bacterial strain KN, which highly produced a protease, was isolated from several soil samples and identified to belong to the genus *Bacillus*. We selected mutant strain *Bacillus* sp. KN103N, which was hyperproducer of protease and was resistant to D-cycloserine, from the strain KN by several steps of mutagenesis. Neutral protease productivity of mutant strain KN103N was about 55 times as much as that of the original strain KN. The optimum pH and temperature for the enzyme activity were 7.0 and 50℃, respectively and the enzyme was relatively stable at pH 6.0~8.0 and below 40℃. The enzyme was inactivated by EDTA, but not by DFP. These results indicate that the enzyme from *Bacillus* sp. KN103N was a neutral (metallo-) protease.

Microbial proteases have been divided into four groups based on their machanisms of action. These groups are serine, metal, thiol, and acid protease. The extracellular proteases of the genus *Bacillus* are mainly either serine (alkaline) or metal (neutral) enzymes (1, 2).

Neutral proteases are used for bating in tenderis, to hydrolyze barley proteins in breweries, and for proteolysis in food industry and pharmaceutical industry (3-5).

Metallo-proteases are endopeptidase that contain an essential metal ion usually Zn. Their optimum pH for reaction is around pH 7.0, so they are called neutral proteases. Neutral proteases are produced by several *Bacillus* sp., such as *B. subtilis*, *B. cereus*, *B. megaterium* and *B. stearothermophilus* (6-11).

There have been many reports on the isolation of protease hyperproducing mutant from *Bacillus* sp. (12-15). However, they have been commonly focused on the academic research rather than the practical applications. Selection of a hyperproducing microbial strain is an important step in the development of an industrial enzyme product.

Therefore, this paper deals with the selection of hyperproducing mutant strain from *Bacillus* sp. and some properties of the enzyme from the mutant strain *Bacillus* sp. KN103N.

Key words: Neutral protease, *Bacillus* sp. KN103N
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Materials and Methods

Isolation of the bacteria and primarily screening for protease producing strain

Several samples of soil collected from nearby Seoul were used in isolating the proteolytic bacteria. Aproximately 1 g of soil sample was suspended in 100 m*l* of sterile water and then the dilutions were plated on agar medium (Table 1) containing 2% casein and 0.5% skim milk and incubated at 37℃. After 2 days, colonies sorrounded by large

Table 1. Composition of culture media

Slant agar medium		Submerged culture medium	
Glucose	5 g	Corn starch	30 g
Yeast extract	2 g	Defatted soybean meal	40 g
Polypeptone	10 g	Glucose	10 g
NaCl	2 g	Casein	10 g
Agar	15 g	KH_2PO_4	0.5 g
Distilled water	1,000 ml	K_2HPO_4	2 g
pH 7.0		$MgSO_4 \cdot 7H_2O$	0.5 g
		$CaCl_2 \cdot 2H_2O$	0.5 g
		Distilled water	1,000 ml
		pH 7.0	

*Liquid medium for seed culture was identical to slant agar medium except for the omission of agar.

halos were isolated. The isolates were further screened for their capacities producing protease. The cells from agar medium were inoculated into 100 ml of submerged culture media (Table 1) in 1 l Sakaguchi flask. After 2 days of incubation in a reciprocal shaker at 37℃, the proteolytic activities of culture broths were measured and compared.

Characterization and identification of selected bacteria

The morphological and physiological characteristics of selected bacteria were examined by following the procedures described in Bergey's Manual of Determinative Bacteriology (16) and Gerhardt (17).

Partial purification of the enzyme

All procedures were carried out at 4℃. The cultures were centrifuged at 12,000×g for 20 min and then ice-cold ethanol was added to supernatant to be the final concentration of 75%. After standing overnight at 4℃, precipitate was collected by centrifugation and freeze dried. The resulting enzyme powder was used as crude enzyme.

Assay of protease activity

Protease activity was assayed by the modified method of Nakamura (18). One mililiter of 0.6% milk casein in 50 mM phosphate buffer, pH 7.0, was mixed with 1 ml of enzyme solution. After mixture was incubated at 37℃ for 10 min, 2 ml of 1% TCA solution was added to terminate the reaction. The insoluble part of the mixture was filtered through Toyo-roshi paper (No. 2) and, followed by adding 0.5 M Na_2CO_3 and 2 ml of 1/5 Folin-Ciocalteu phenol reagent to 1 ml of the filterate. The mixture was kept at 37℃ for 20 min. The resultant colour was measured with optical density in 1 cm layer at 660 nm.

For the blank test, the precipitation reagent was added to enzyme solution before the addition of casein solution and measurement of colour reaction was carried out as above.

One unit of protease activity was defined as the liberated miliequivalent of tyrosine per minute.

Mutagenesis of the protease producing strain

Exponentially growing cells of bacteria in submerged culture were harvested by centrifugation (18,000×g, 15 min) and washed with 50 mM Tris (hydroxymethyl) aminomethane-malate buffer (pH 6.0). Cells were resuspended in the same buffer, and followed by adding sterile N-methyl-N-nitro-N-nitrosoguanidine (NTG) to be a final concentration of 150 μg/ml and then incubated at 37℃ for 30 min with gentle shaking. Treated cells were collected on a nitrocellulose membrane filter, washed twice with the same buffer and grown at 37℃ for 6 hr in submerged culture medium.

The culture was diluted properly and plated on a agar medium containing 2% casein and 0.5% skim milk. After 2 days, colonies with a large halo around them were selected. The resulting mutants were tested for their resistance against various antibiotics including penicillin (2 μg/ml), ampicillin (5 μg/ml) or D-cycloserine (50 μg/ml).

Cultivation in jar fermenter

Cells grown in liquid medium (Table 1) for 24 hr with shaking were used as the seed and the amount of inoculum was 4% of main cultivation medium. In order to produce the enzyme, cells were cultivated in submerged culture medium by using 30 l Jar Fermenter (Marubishi Model, MSJ-U3 30L).

The temperature was maintained at 37℃, the pH

was kept above 6.0 and the air flow rate was kept 0.5 vvm, the speed of agitation was 300 rpm. The protease activities of culture broths were assayed and cell growth was determined spectrophotometrically by measuring optical density at 625 nm (19). Total sugar was determined using the phenol-sulfuric method described by Dubois (20).

Results and Discussion

Isolation of protease producing bacteria

From soil samples, 56 colonies showing protease activity were isolated on agar medium containing 2% casein and 0.5% skim milk. Each strain was cultivated in submerged culture medium at 37℃ for 48 hr. Strain KN was identified to produce protease at the highest level among them by measuring the protease activity of culture broth.

Table 2. Microbiological characteristics of the isolated strain KN

1. Morphological characteristics	
Cells	Rods, 0.4~0.6×1.0~2.0 μm Short to long
Gram stain	Gram-positive
Spore formation	Positive, Ellipsoidal, Central
Motile	Positive
Sporangium swollen	Negative
2. Physiological characteristics	
Casein hydrolysis	Positive
Gelatin hydrolysis	Positive
Starch hydrolysis	Scant hydrolysis
Catalase	Positive
Voges-proskauer test	Positive
Anaerobic growth	Negative
Gas from Glucose, Fructose, Mannose, Maltose, Mannitol.	Negative
Acid from Glucose, Fructose, Mannose, Maltose, Mannitol, Sucrose, D-Xylose, L-Arabinose	Positive
Utilization of citrate	Positive
pH for growth	5.5~8.0
Optimum temperature for growth	28~40℃

Identification of the selected strain KN

Microbial characteristics of the selected strain KN were shown in Table 2 and Fig. 1. The strain KN was strictly aerobic, gram-positive, catalase-producing, and endospore forming *Bacillus*.

According to Bergery's mannual of Determinative Bacteriology (16), the strain KN was identified as *Bacillus* sp. from morphological and physiological studies.

Improvement of *Bacillus* sp. KN strain for enhancement of enzyme productivity

Mutational experiments were performed to improve the protease productivity of *Bacillus* sp. KN strain. Strain KN cells were treated with NTG and the mutagenized cells were plated on agar medium containing 2% casein and 0.5% skim milk. From approximately 6,500 colonies, 136 colonies showing larger halo than that of strain KN in size were primarily selected (Fig. 2), and they were cultured in submerged culture medium at 37℃ for 48 hr.

The highest protease producing strain KN-5 among them were selected and used in further experiments. The selected strain KN-5 showed enzyme activity about 8.5 times (765 U/m*l* of protease) greater than that produced by the parental strain KN (90 U/m*l*).

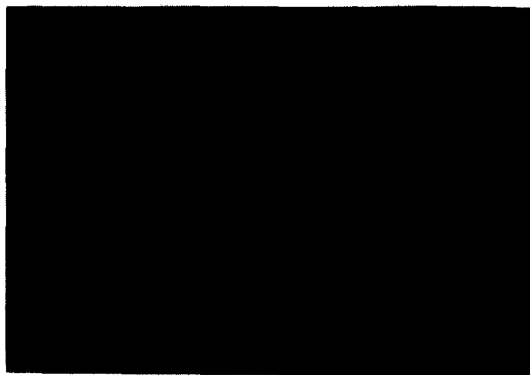

Fig. 1. Microphotograph of *Bacillus* sp.
*Microscopic obserbation showed that the *Bacillus* sp. KN grown on the slant agar medium for 1 day at 37℃ were rods.
*One scale indicates 1.0 μm.

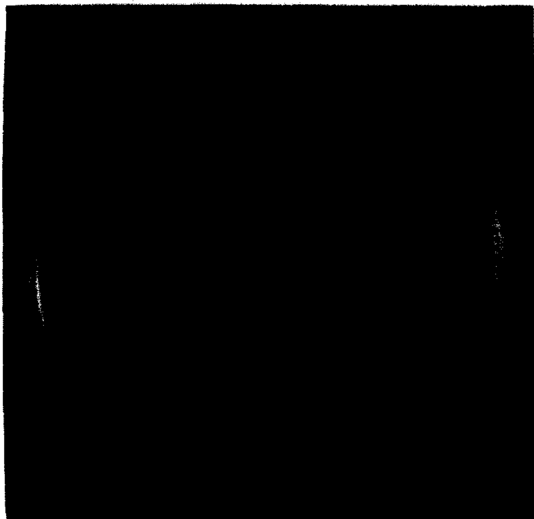

Fig. 2. Two day-old colonies surrounded by halo on agar medium old containing 2% casein and 0.5% skim milk.

Table 3. Effect of reagents on protease activity

Reagents(mM)		Relative activity (%)
None		100.0
EDTA	1 mM	22.0
	5 mM	9.0
	10 mM	3.2
DFP	1 mM	92.0
	5 mM	88.5
	10 mM	83.0
PMSF	1 mM	93.3
	5 mM	91.0
	10 mM	87.1

*EDTA; ethylenediaminetetraacetic acid, DFP; diisopropylfluorophosphate, PMSF; phenylmethylsulfonyl fluoride.
*The mixture consisting of 1 ml of enzyme, 2 ml of 50 mM Tris-HCl buffer (pH 7.0) and 1 ml of chemical reagent was incubated at 25℃ for 30 min and residual activity was measured.

Strain KN-5 was further treated with NTG and resulting mutants, which were resistant to various antibiotics, were selected. They were subjected to culture test for protease productivity. From about 2,500 D-cycloserine resistant mutants, the largest halo producing mutant strains among them were primarily selected (Fig. 2), the selected strains were subjected to culture test for protease productivity. The highest protease producing mutant strain KN 103 among them was selected.

The selected strain KN103 produced protease about 3.5 times (2,680 U/ml) morea than the strain KN-5. From the about 260 ampicillin resistant mutants, the highest protease producing strain KN205 among them was selected. But the strain produced protease about 1.3 times (980 U/ml) more than the strain KN-5. Penicillin resistant mutants were also isolated from the mutagenized cells of strain KN-5, but hyperproducer of protease could not be isolated.

After treating strain KN103 with NTG, we selected mutant strain KN103N which was resistant to 50 μg/ml of D-cycloserine and was hyperproducer of protease. The selected strain KN103N produced about 1.9 times (5,090 U/ml) as much protease as the strain KN103.

Cosequently, protease productivity of the selected strain KN103N was about 55 times more than the original strain KN (Table 4).

The above results indicate that the D-cycloserine resistance as a guide mark was effective in developing protease hyperproducer, agreeing with those of Hitotsuganagi (21).

The process of secretion of the enzyme is considered to be very complicated one affected not only by the transcription and translation efficiency of corresponding gene but also by many kinds of secretion mechanisms, and probably by other yet unknown mechanisms. Mutation of any one of these should effect the synthesis, secretion, and total accumulation of the enzyme.

The final mutant barely shows differance from the original strain KN except the growth rate of the former being a little slower than that of the latter.

Production of extracellular protease

Bacillus sp. KN103N was cultivated in submerged culture medium by using 30 l jar fermenter at 37℃. As show in Fig. 3, the production of protease by strain KN103N has reached a maximum level at 48 hr of cultivation. And pH profiles showed a decrease down to 5.8 until 28 hr and turned rapidly upward thereafter.

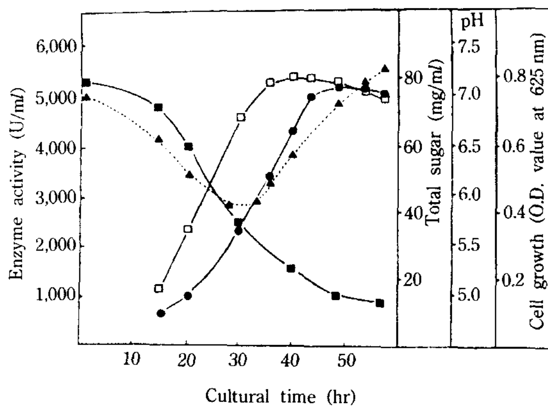

Fig. 3. Time course for the cultivation of *Bacillus* sp. KN103N by 30 *l* Jar Fermenter.
**Bacillus* sp. KN103N was cultivated in submerged culture medium at 37℃ using 30 *l* Fermenter. The pHs, protease activities, cell growth, and total sugar of culture broths during cultivation were measured.
●—●; Enzymee activity, ▲—▲; pH
■—■; Total sugar, □—□; Cell growth

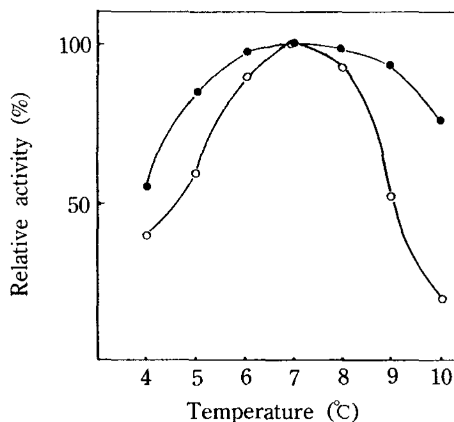

Fig. 4. Effect of pH on activity and stability of the enzyme.
*The following buffer systems were used: citric acid-sodium phosphate buffer (pH 4.0~6.5), Tris-HCl buffer (7.0~9.0), Glycine-NaOH buffer (10.0)
*Optimum pH; The pH was adjusted with the above described buffer systems and other conditions were the same as those of the standard assay method.
*pH stability; The reaction mixtures were incubated at 4℃ for 24 hours and the remaining activities were measured by standard assay condition.
○—○; Optimum pH, ●—●; pH stability

Properties of the extracellular protease from *Bacillus* sp. KN103N

As shown in Fig. 4, the optimum pH for enzyme

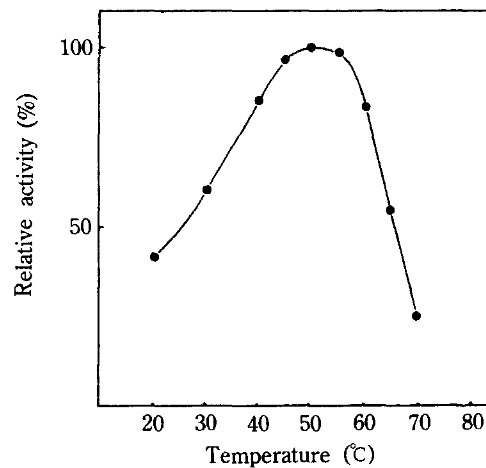

Fig. 5. Effect of temperature on the enzyme activity.
*The reaction was carried out under standard assay condition except that reaction temperature was varied.

reaction was between 6.5 and 7.5. The enzyme was stable between pH 6.0 and 8.0, but stability decreased sharply outside this range.

As shown in Fig. 5, the optimum temperature of the enzyme activity was 50℃. The enzyme thermostability was tested as follows; the enzyme was kept at each temperature for 30 min and remaining activity was measured at 37℃.

The extracellular protease from strain KN103N was stable below 40℃ (Fig. 6). The effect of reagents on the protease activity were tested (Table 3). Among the reagent tested, a chelating agent (EDTA) inhibited the enzyme activity, but DFP and PMSF did not inhibited the enzyme activity.

Keay, L. (4) reported that the neutral (metallo-) proteases have pH optima around 7.0 and are sensitive to metal chelating agents such as EDTA and o-Phenanthroline, but are not inhibited by DFP or thiol reagents.

On a point of this view, the properties of protease from *Bacillus* sp. KN103N studied by us agree with those reported by other investigations with respect to neutral protease, concluding that the protease from *Bacillus* sp. KN103N was a neutral protease.

From the above results, in the pratical view point, mutant strain KN103N has been proved to be a potent neutral protease hyperproducer. If the cultural conditions are optimized, this organism could be expected to be a microbial source for producing

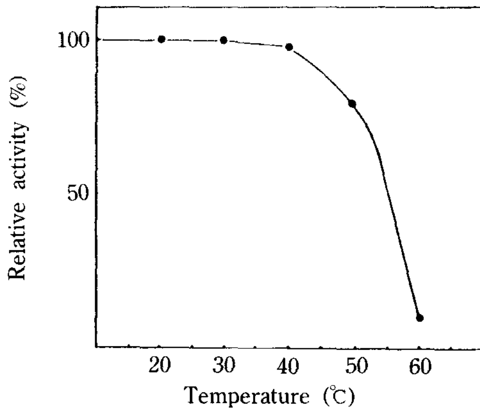

Fig. 6. Effect of temperature on the stability of enzyme.
*Enzyme solution in 50 mM Tris-HCl buffer (pH 7.0) was heated at various temperature for 30 min.
After heating, the remaining activities of quickly cooled enzyme were measured under the standard assay condition.

Table 4. Protease productivity of the selected mutant strains

Selected strain	Productivity (U/ml)
Strain KN	90
Strain KN5	765
Strain KN103	2,680
Strain KN205	980
Strain KN103N	5,090

neutral protease with aims at industrial application.

요 약

토양으로부터 protease 生産能이 높은 菌株(KN)를 분리하고 동정한 결과, 분리한 菌株는 *Bacillus* sp.로 확인되었다. 이 菌株로부터 여러 단계의 돌연변이 실험 및 protease 高 生産能의 菌株 선별실험을 통하여 protease 生産性이 가장 우수한 *Bacillus* sp. KN 103N 菌株를 선별하였다. 선별된 變異株의 protease 生産性은 原菌株인 *Bacillus* sp. KN보다 約 55배의 높은 生産性을 나타내었다.

이 酵素의 최적온도 및 pH는 각각 50℃와 7.0이었으며 pH 5.0~8.0, 온도 40℃ 이하의 범위에서 안정하였다. 이 酵素는 EDTA에 의해서 그 활성이 저해되었으나 DFP에 의해서는 저해되지 않았다. 上記의 결과로부터 *Bacillus* sp. KN103N 의해 生産되는 protease은 中性 protease인 것으로 확인되었다.

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