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Selection of Protease Hyperproducing Mutant Strain from *Serratia marcescens* ATCC 21074 and Enzymatic Properties of the Protease

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Serratia macescens ATCC 21074로부터 Protease 생산성이 높은 변이주의 선별 및 Protease의 효소학적 특성

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Abstract — A protease hyperproducer, ampicillin resistant mutant, *Serratia* sp. SMNT-1 was selected from *Serratia marcescens* ATCC 21074 by mutagenesis. The protease productivity of this strain was about 11 times as much as that of the parental strain. The enzyme showed maximal activity at pH 9.0 and 40℃, and was stable over the pH range from 6.0 to 10.0 at 4℃, whereas it was unstable at 37℃ in alkaline condition. the enzyme was inactivated by heating at 60℃ for 10 min. The enzyme was inactivated by EDTA and reactivated by Zn^{2+}, Co^{2+}, and Mn^{2+}, but the proteolytic activity of the enzyme was not affected by DFP. From the above results, the protease produced by *Serratia* sp. SMNT-1 was classified as a metalloprotese.

Proteases have been widely applied to the food and pharmaceutical industries. *Serratia marcescens*, a gram-negative bacterium belonging to the family Enterobacteriaceae, was known to produce a large amount of extracellular protease into the surrounding medium (1-3).

A protease produced by *Serratia* sp. has strong caseinolytic and fibrinolytic activities and the purified protease is used as an antiinflammatory agent. the enzyme was named tentatively as Serratiopeptidase from the reason of its origin.

There have been many reports on the regulation of protease formation by *Serratia* sp. and purification and its characteristics of the enzyme (4-9). However, only a few reports have dealt with the isolation of an extracellular nuclease deficient and antibiotic sensitive mutant of *Serratia marcescens* (10). We are not aware of any reports on the improvement of protease hyperproducing *Serratia* strain by using ampicllin-resistancy as guide marker.

Thus, in this report, we describe the selection of protease hyperproducing mutant from *Serratia marcescens* ATCC 21074 and the properties of the enzyme.

Key word: Protease, *Serratia* sp.
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Materials and Methods

Microorganism and cultivation

The strain of *Serratia marcescens* ATCC 21074 used in this study was obtained from the American Type Culture Collection. Mutant strains obtained from the parental strain after mutagenesis with NTG. The strain was maintained by transferring to fresh nutrient agar slant at intervals of 2 weeks. In experiment with shaking flask, a loopful of cells was inoculated into 100 m*l* of nutrient medium in a 1 *l* sagaguchi flask. Incubation was carried out at 30℃ with reciprocal shaking for 2 days. In experiment with a jar fermentor, cells was inoculated

into 100 m*l* of preculture medium in a 1 *l* sagaguchi flask and then incubated for 24 hr with shaking. A 200 m*l* aliquot of the preculture was inoculated into 15 *l* of main culture medium in 30 *l* jar fermentor (Marubishi MSJ. 30 *l*). The fermentation was operated for 2 days under following conditions; temperature was maintained at 30℃ and agitation and aeration were controlled to 300 rpm and 0.5 vvm, respectively. Bacterial growth in cultures was determined by measuring the optical density of 50 times diluted culture broth at 550 nm (1). The main culture medium was composed of 20g of casein, 30g of defatted soybean meal, 15g of glucose, 20g of sucrose, 5g of $(NH_4)_2HPO$, 1g of NaCl, 0.2g of KCl, 0.1g of $CaCl_2$, 0.1g of $MgSO_4 \cdot 7H_2O$, 2g of KH_2PO_4, 4g of K_2HPO_4 in 1 *l* H_2O and pH 7.0.

Assay of protease activity

Proteolytic activity was assayed by the nakamura method (12) with a slight modification. One m*l* of 0.6% milk casein in 50 mM Na-borate buffer (pH 9.0) was mixed with 1 m*l* of enzyme solution. After the mixture was incubated at 37℃ for 30 min, the reaction was terminated by the addition of 2 m*l* of 1% TCA solution and the mixture was allowed to stand at 37℃ for further 30 min. The insoluble part of the mxiture was filtered through Toyoroshi No. 2 filter paper and followed by adding 0.5 M Na_2CO_3 and 2 m*l* of 1/3 folin-ciocalteu phenol reagent to 1 m*l* of the filterate. The mixture was kept at 37℃ for 30 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 proteolytic activity was defined as the amount of the enzyme which gives the optical density at 660 nm equivalent to 1.0 μg of tyrosine in one minute under the above assay condition.

Mutagenesis and isolation of mutants

Several mutants capable of producing high amount of protease were obtained from *Serratia marcescens* ATCC 21074 as follows. Exponentially growing cells of *Serratia marcescens* ATCC 21074 on complete media (2% glucose, 1% peptone, 1% yeast extract, 0.5% NaCl, pH 7.0) at 30℃ were suspended in 50 mM tris-malate buffer (pH 6.0). To the above cell suspension, 500 μg/m*l* of NTG (N-methyl-N′-nitro-N-nitrosoguanidine) was added aseptically, and the mixture was incubated at 30℃ for 1 hr. After washing twice with the same buffer, the cells were suspended in the minimal medium (glucose (10 g/*l*), $(NH_4)_2SO_4$ (10 g/*l*), $MgSO_4 \cdot 7H_2O$ (50 mg/*l*), $FeSO_4 \cdot 7H_2O$ (10 mg/*l*), $MnSO_4 \cdot 7H_2O$ (10 mg/*l*), 0.05% NaCl, D-biotin (50 μg/*l*), vitamin B (200 μg/*l*), urea (3 g/*l*), agar (18 g/*l*) and then cultured in a shaker for 24 hr. After incubation, the culture broth was plated on to a minimal medium containing 50 μg/m*l* of ampicillin (Ap) (Ap medium) or 25 μg/m*l* of chloramphenicol (Cm) (Cm medium). After incubation of the plates at 37℃ for 2 days, the colonies which appeared were picked and tested for the protease productivity.

Purification of the enzyme

Unless otherwise noted, all purification procedures were conducted at 4℃ to avoid enzyme denaturation. The cultures were centrifuged at 15,000 ×g and ammonium sulfate was added slowly to the supernatant with gentle stirring to a final concentration of 70% saturation. After standing for 24 hr at 4℃, the precepitates were collected by centrifugation and dissolved in small amount of 10 mM Tris-HCl buffer (pH 7.0). the solution was subjected to dialysis through a cellophane membrane against the same buffer for 24 hr and the dialysate was apply to a column (2×25 cm, DE52, whatman, preswollen) of DEAE-cellulose.

The enzyme was eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer. The eluted enzyme fraction was concentrated with a membrane (MWCO 20,000, DDS Corp.). The concentrated enzyme solution was dialyzed against deionized water and then freeze dried.

Results and Discussion

Isolation of mutants

From approximately 3,200 colonies derived from *Serratia marcescens* ATCC 21074 by mutagenesis with NTG treatment, the mutant strain SM901 producing the largest amount of protease among them

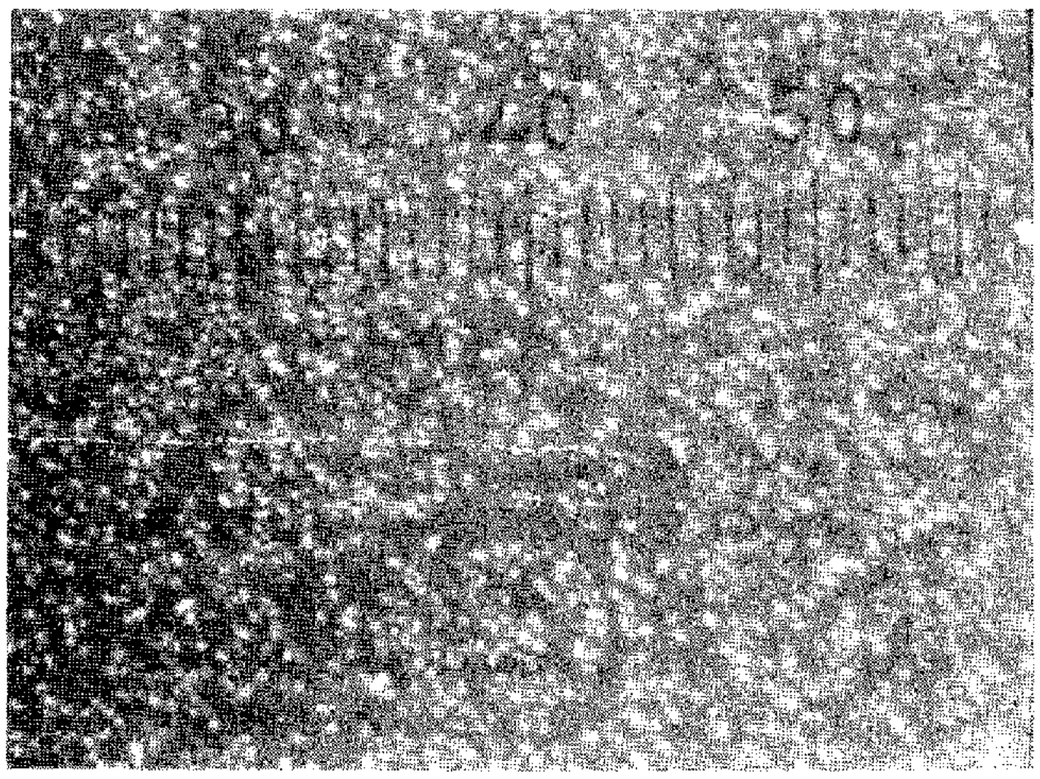

Fig. 1. Microphotograph of *Serratia* sp. SMNT-1.
*Microscopic obserbation showed that the *Serratia* sp. SMNT-1 grown on the nutrient agar medium for 1 day at 30℃.
*One scale indicates 1.0 μm.

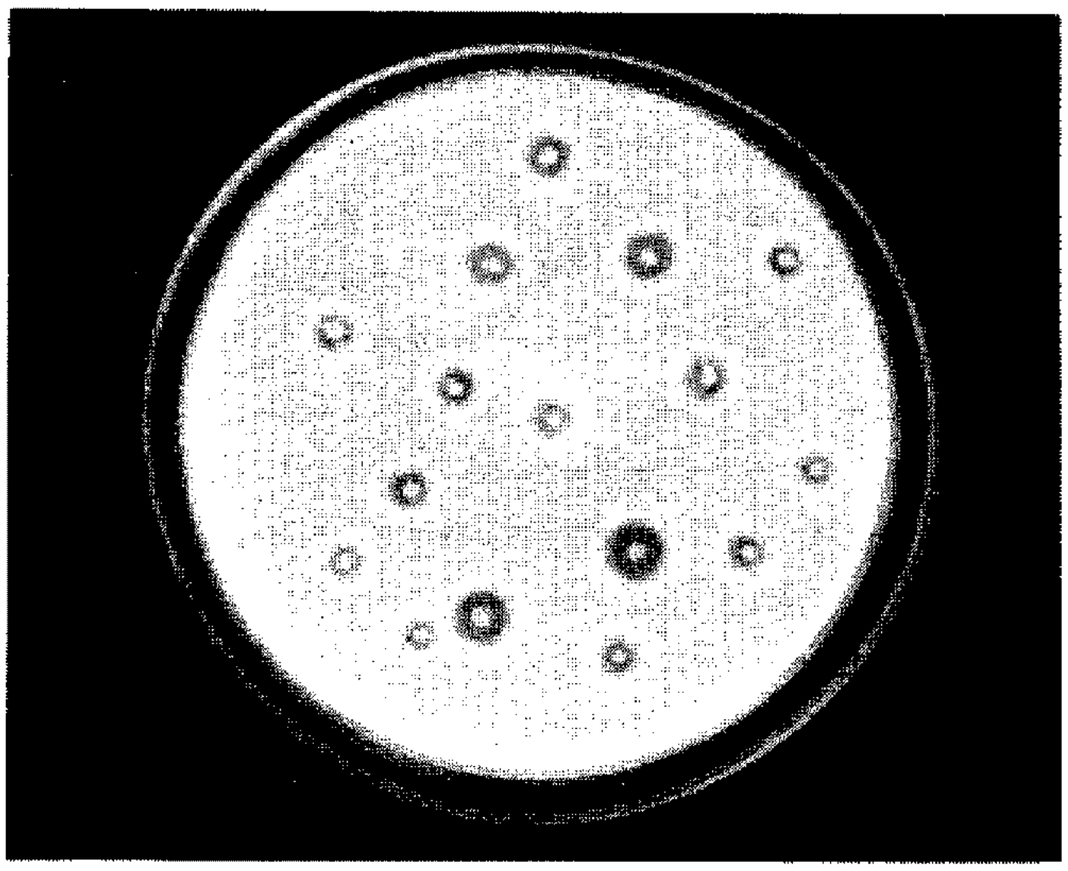

Fig. 2. Two-day old colonies, incubated at 30°C for 48 hr, surrounded by halo on nutrient agar medium containing 2.5% casein and 2% skim milk.

was primarily selected by comparison of the size of the clearing zone around the colonies (Fig. 2). The selected strain SM901 produced protease about 1.6 times (1,630 U/m*l*) more than parental strain. We performed test for antibiotics, ampicillin (25 μg/m*l*) and chloramphenicol (50 μg/m*l*). As the results of the test, parental strain ATCC 21074 was a ampicillin sensitive and chloramphenicol resistant strain, but the selected strain SM901 was a ampicillin and chloramphenicol resistant strain.

The selected strain SM901 was further treated with NTG, and resulting mutants, which were resistant to ampicillin, 7 colonies among them were selected and tested for the ability producing protease and the resistancy to antibiotics (25 μg/m*l* of ampicillin, 50 μg/m*l* of chloramphenicol). The highest protease producing strain SMN305 among them was selected and the selected strain produced protease about 2.9 times (4,800 U/m*l*) more than the strain SM901. As results of the test for antibiotics, although 5 mutant strains (SMN67, SMN2063, SMN2628, SMN4917, and SMN6905) were resistant to ampicillin and chlorampheicol, whereas SMN305 was resistant to ampicillin and sensitive to chloramphenicol. Strain SMN305 was further treated with NTG, and tested for the ability producing protease and antibiotics. The highest protease producing strain SMNT-1 among them was selected. As shown in Table 1, the enzyme productivity of the strain SMNT-1 was higher than any other mutant strain.

Table 1. Protease productivity and the relevant properties of mutant derived from *Serratia marcescens* ATCC 21074

Strain	Production of protease (U/m*l*)	Relevant properties
ATCC 21074	1,020	Ap^s, Cm^r
SM 901	1,630	Ap^r, Cm^r
SMN 67	2,282	Ap^r, Cm^r
SMN 305	4,800	Ap^r, Cm^s
SMN 2603	3,150	Ap^r, Cm^r
SMN 2628	2,420	Ap^r, Cm^r
SMN 4917	3,030	Ap^r, Cm^r
SMN 6905	2,650	Ap^r, Cm^r
SMNT-1	11,200	Ap^r, Cm^s
SMNT-38	9,800	Ap^r, Cm^s
SMNT-55	10,600	Ap^r, Cm^s

Abbreviation used for antibiotics and phenotypic markers; Ap^s: Ampicillin sensitive strain, Ap^r: Ampicillin resistant strain, Cm^s: Chloroamphenicol sensitive strain, Cm^r: Chloramphenicol resistant strain.

The selected strain SMNT-1 produced about 11 times (11,200 U/m*l*) higher than that of the parental strain ATCC 21074. *Serratia* sp. SMNT-1 was finally selected as the best strain and used in the following experiment. The final mutant strain SMNT-1 barely showed difference from the parental strain ATCC 21074 in apperance except the ampicillin resistant

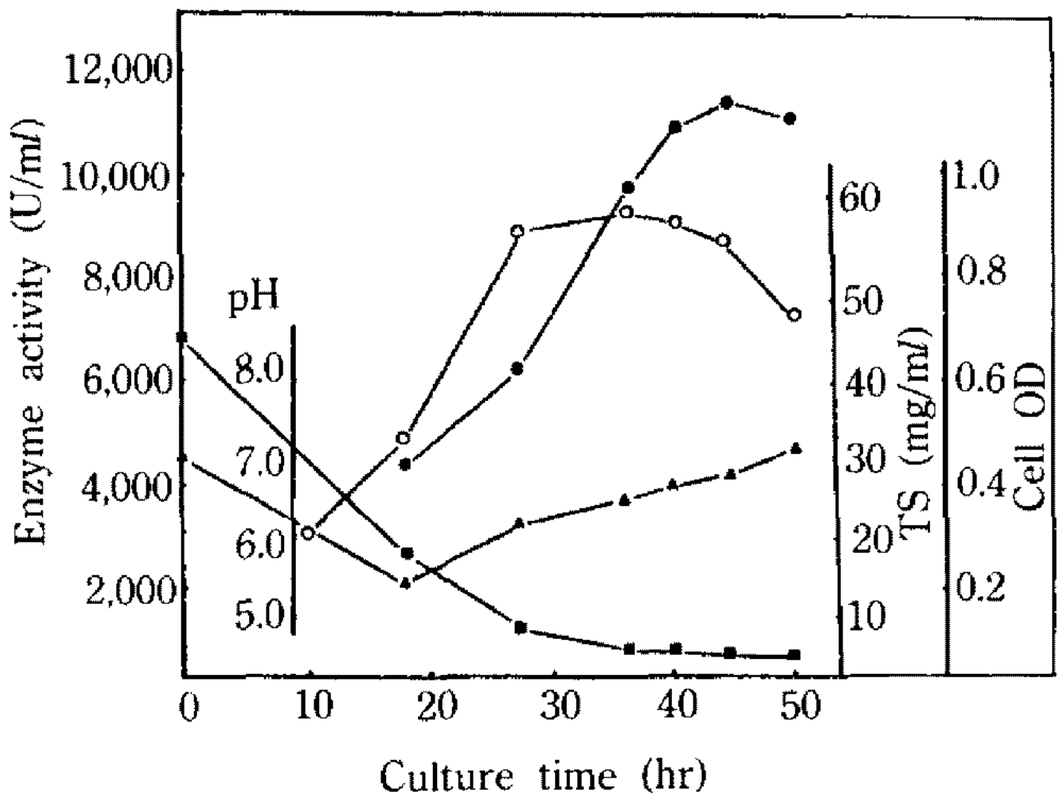

Fig. 3. Time course for the cultivation of *Serratia* sp. SMNT-11 in 30 *l* jar fermentor.
Serratia sp. SMNT-1 was cultivated in the main culture medium at 30℃. The pH, protease activity, cell growth and total sugar of culture broth during cultivation were measured.
●−●; Enzyme activity, ▲−▲; pH
■−■; Total sugar, □−□; Cell growth

and chloramphenicol sensitive strain.

The synthesis and secretion of the enzyme by bacteria are considered to be very complicated one affected by not only the transcription and translation efficiency of corresponding gene but also by many kinds of secretion mechanisms, and by other unknown mechanisms. Although we can not clearly explain the mechanism of mutation in present, the above results indicate that the ampicillin resistancy as a guide marker was effective in improvement of protease hyperproducing strain of *Serratia* sp.. And the results reported here were similar to those of Hitotsuganagi (13).

Production of protease by *Serratia* sp. SMNT-1

Serratia sp. SMNT-1 was cultivated in the main culture medium by using 30 *l* jar fermentor at 30℃. As shown in Fig. 3, the production of protease by strain SMNT-1 was found to begin at about 15 hr after incubation and was reached a maximal level at 45 hr of incubation. Prolonged fermentation led to a sharp decrease of proteolytic activity. Total sugar was almost completely consumed after 36 hr of cultivation. And pH profiles showed a decrease down to 5.4 until 19 hr and turned upward thereafter.

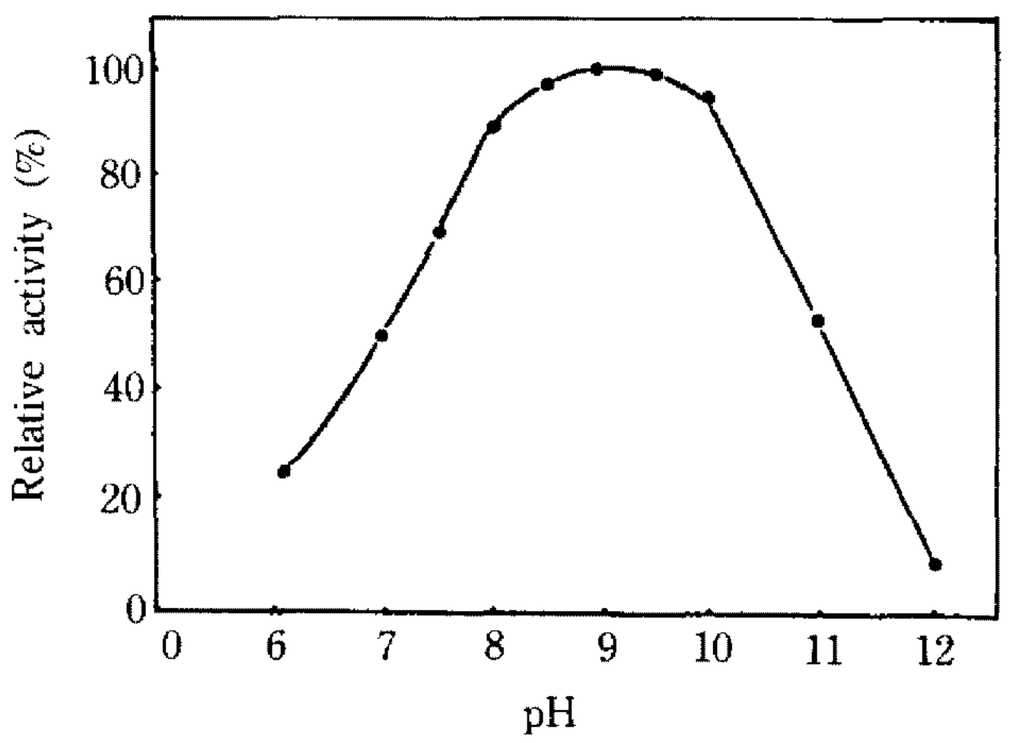

Fig. 4. Effect of pH on the enzyme activity.
Enzyme activity was measured by the standard assay method except that the reaction pH was varied.
pH 6~7 phospahte buffer, pH 8~9 Tris-HCl buffer
pH 9~11 glycine buffer.

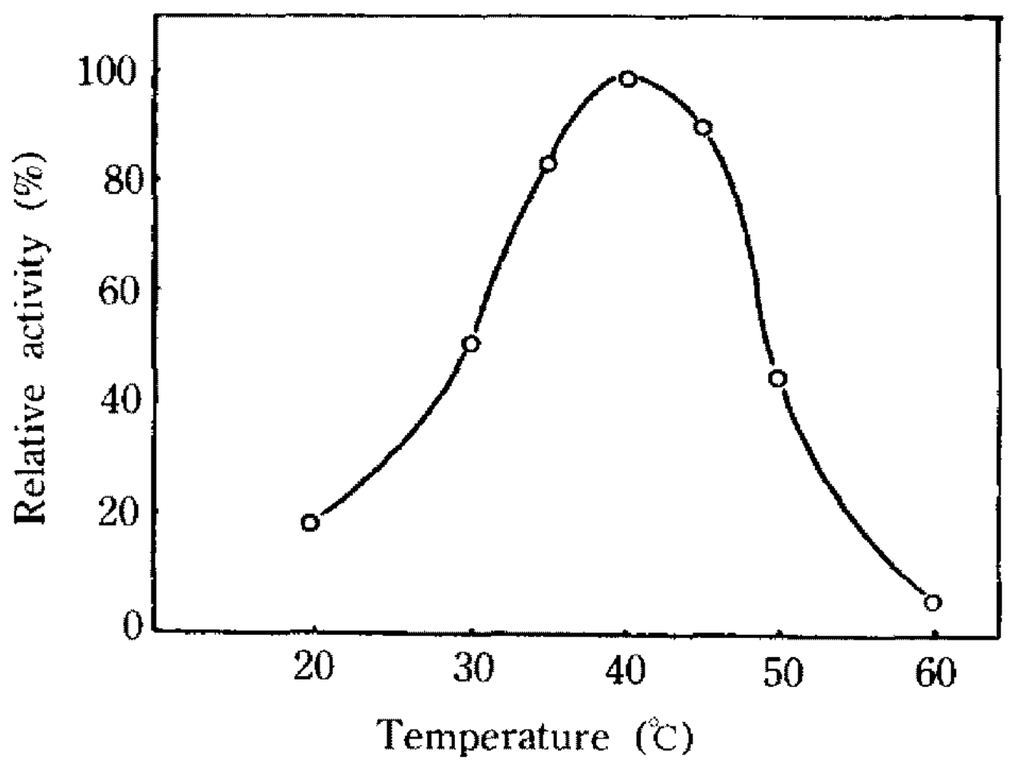

Fig. 5. Effect of Temperature on the enzyme activity.
Enzyme activity was measured by the standard assay method except that the reaction temperature was varied.

Enzymatic properties of the protease

Effect of pH on temperature on enzyme activity : As shown in Fig. 4 and 5, the enzyme showed maximal activity at around pH 9.0 and 40℃, and was stable under lower temperature over the pH range from 5 to 10, whereas it was unstable at 37℃ in alkaline coditions (Fig. 6). The enzyme was completely inactivated by heating at 60℃ for 20 minutes (Fig. 7). The pH and temperature for proteolytic activity of Serratial protease has been reported to be about pH 9.0 and 45℃ when casein was used as substrate (3, 6, 8).

These reports are in good agreement with our

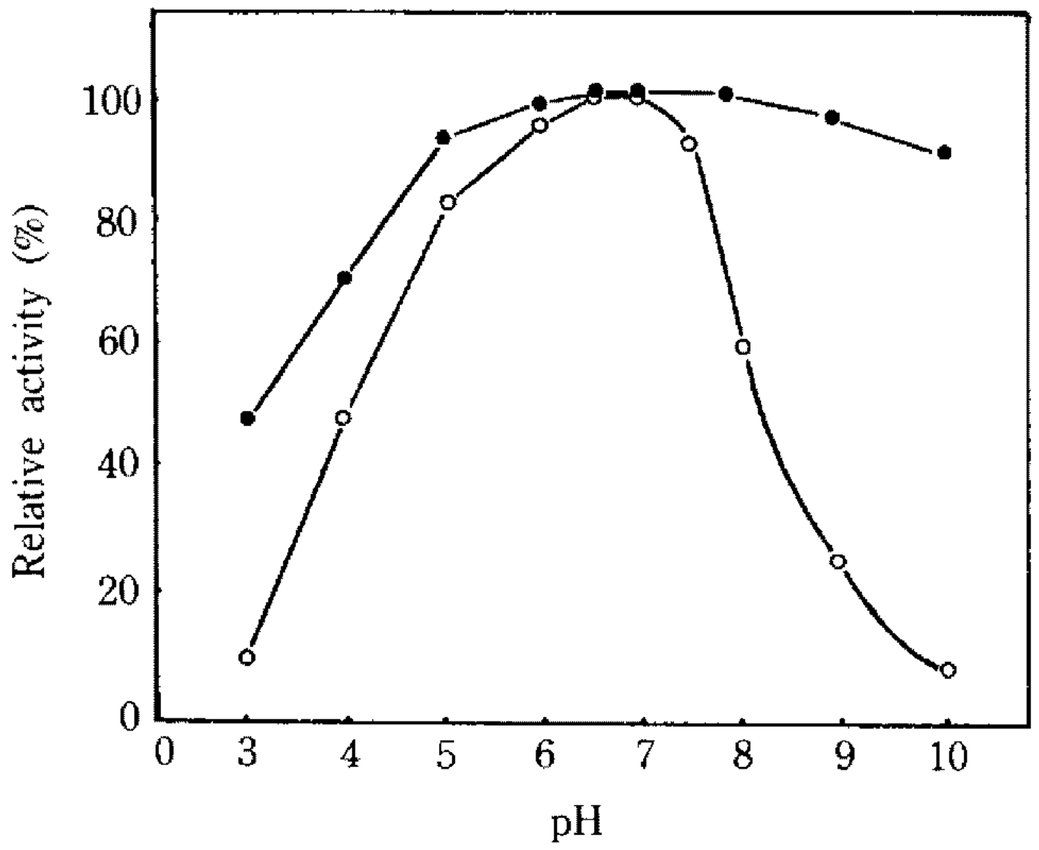

Fig. 6. Effect of pH on enzyme stability.
A 0.1% enzyme solution, adjusted to given pH, was incubated at 37℃ for 1 hr and at 4℃ for 24 hr, respectively. After incubation, the residual activity was measured by the standard assay method.
○−○; at 37℃ for 1 hr, ●−●; at 4℃ for 24 hr.

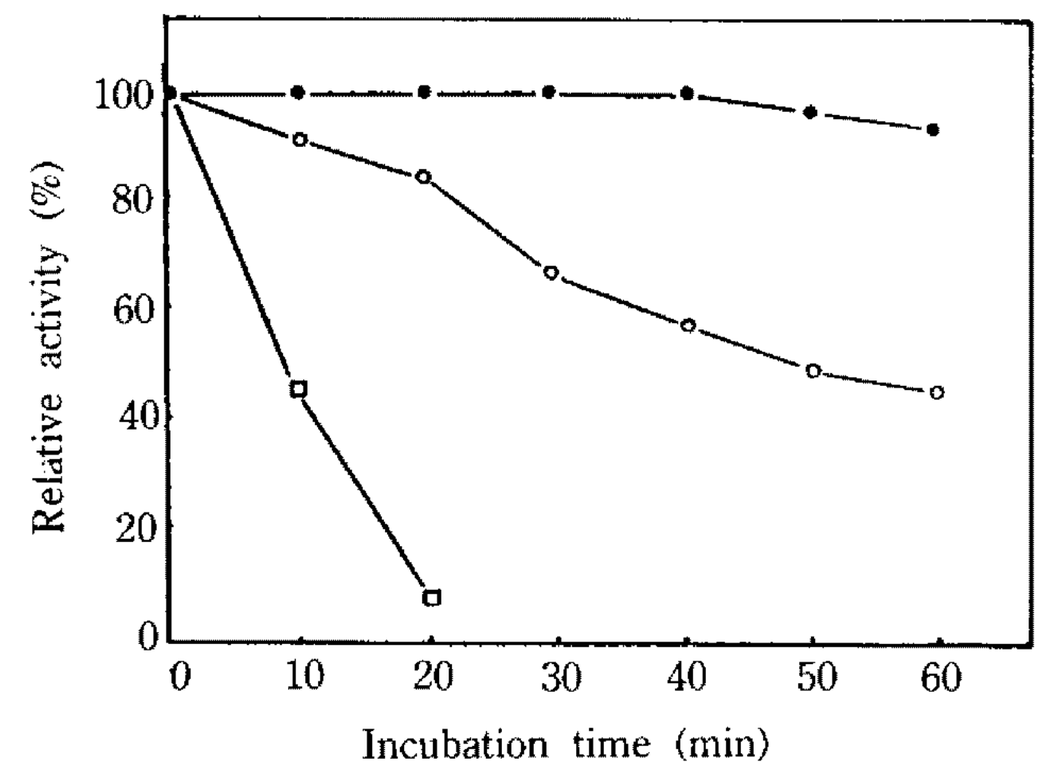

Fig. 7. Effect of temperature on enzyme stability.
A 0.1% enzyme solution in 0.1 M phosphate buffer, pH 6.5, was incubated at a given temperature. At intervals, the residual activity was assayed in the course of incubation.
●−● at 40℃, ○−○ at 50℃, □−□ at 60℃.

results decribed above.

Effect of various reagents on enzyme activity : The enzyme was preincubated with various reagents at 4℃ and then the remaining activity was measured. The results were summerized in Table 2.

Proteases are generally classified in four major groups (14). These groups are serine, metal, thiol and acid protease. Proteases and esterases which

Table 2. Effect of various reagents on enzyme activity

Reagent	Concentration	Relative activity (%)
No addition		100
EDTA	5 mM	0.42
pCMB (a)	1 mM	97
DFP (b)	1 mM	98
$MgCl_2$	2 mM	100
$CaCl_2$	2 mM	101
$MnCl_2$	2 mM	97
$CoCl_2$	2 mM	99
$NiCl_2$	2 mM	81
$ZnCl_2$	2 mM	100
$HgCl_2$	2 mM	20
$CdCl_2$	2 mM	63

*EDTA; etylenediaminetetraacetic acid
DFP; diisopropylfluorophosphate
pCMB; p-chloromercuribenzonate
*After the enzyme (1 mg/m*l*) was incubated in 0.01 M acetate buffer of a given reagent at pH 5.0, except (a), (b) at pH 7.0, at 4℃ for 1 hr, the residual activity was measured.

serine at their active centers, are inhibited by phosphorylation with diisopropylfluorophosphate (DFP). p-Chloromercuribenzoate (pCMB) inhibits thiol proteases which have an -SH group of cysteine at their active centers. Metalloproteases such as thermolysin require metal ions for activity or are activiated by metal ions.

The chelating agents, EDTA and o-phenanthroline, inhibited the proteolytic activity, while DFP and pCMB has no inactivating effect. Of various metal ions, Ni^{2+}, Cd^{2+} and Hg^{2+} inactivated the enzyme activity, whereas Co^{2+}, Mg^{2+} and Ca^{2+} did not inhibited. In order to investigate on the reactivation by the addition of various metal ions, we tested for the reactivation by adding of metal ions. As results of the test, Zn^{2+}, Co^{2+} and Mn^{2+} were effective for the reactivation as shown in Table 3.

The results obtained from the experiment using various reagents showed that the protease of *Serratia* sp. SMNT-1 is not a serine protease since it is not inhibited by DFP (15).

The enzye has an optimum pH for activity from 6.0 to 9.0, hence, it is not an acid protease but it can be grouped under neutral or semialkaline

Table 3. Reactivation of inactive enzyme with various metal ions

Added metal ions	Relative activity (%)
Native enzyme	100
Inactive enzyme	0.40
$MgCl_2$	23.2
$CaCl_2$	32.6
$MnCl_2$	68.4
$CoCl_2$	75.1
$NiCl_2$	9.5
$ZnCl_2$	92.7
$CdCl_2$	6.9
$HgCl_2$	2.5

protease. By virture of its inactivation by metal ion chelators and reactivation by Zn^{2+}, Co^{2+} and Mn^{2+}, the protease of *Serratia* sp. SMNT-1 can be classified as a metalloprotease. Miyata *et al.* (11) reported that Zn^{2+} was essential for proteolytic activity. On other hand, Aiyappa and Harris (5) reported that Fe was essential for proteolytic activity. Our results showed that Co^{2+}, Zn^{2+}, Mn^{2+} could effect reactivation of the EDTA-inactivated protease. On the point of this view, the properties of the protease from *Serratia* sp. SMNT-1 seems to be very similar to those reported by Miyata *et al.* (11).

Form the above results, the final selected strain SMNT-1 could be expected to be a microbial source of industrial production of serratial protease. Further studies including the detailed characteristics of serratial protease and optimal cultural conditions for large scale production of this enzyme are now in progress.

요 약

Serratia marcescens ATCC 21074로부터 돌연변이 유도에 의해 protease 생산능이 높은 ampicillin 耐性의 변이주 *Serratia* sp. SMNT-1을 분리하였다. 이 균주는 原 균주에 비하여 約 11배 정도 높은 protease 생산성을 나타내었다.

이 효소는 pH 9.0, 40℃에서 최대 활성을 나타내었으며, 저온하에서는 pH 6.0～10.0 범위에서 안정하였으나, 37℃, 알카리 조건하에서 불안정하였다. 이 효소는 60℃에서 10분간 열처리시 실활되었다.

이 효소는 EDTA에 의해 不活性化되었으며, Zn^{2+}, Co^{2+}, Mn^{2+}에 의해 再活性化되었다. 그러나 이 효소의 활성은 DFP에 의해 저해되지 않았다. 이상의 결과로부터 *Serratia* sp. SMNT-1에 의해 생산되는 protease는 metalloprotease로 확인되었다.

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