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Cloning and Expression of *Serratia marcescens* Protease Gene in *Escherichia coli*

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A 5.8 kb chromosomal DNA fragment of *Serratia marcescens* ATCC 27117 including an extracellular serine protease gene was cloned in *Escherichia coli*. The cloned gene(pSMP18) caused specific excretion of the protease into the extracellular medium through the outer membrane of *E. coli* host cells. The protease purified from *E. coli* harboring pSMP18 was inactivated not by 100 mM EDTA but by 10 mM phenyl methyl sulfonyl flouride (PMSF). The molecular weight of the purified serine protease was about 66,000 in the SDS-PAGE and the isoelectric point was approximately 5.7 in IEF·Gel electrophoresis. The optimal pH and temperature for reaction of the purified serine protease were 9.5 and 45°C, respectively.

Serratia is known as a good model system for the study of the excretion of protein in gram-negative bacteria because it excretes several degradative enzymes such as nuclease (1), proteases (3, 14, 23), chitinase (9) and phospholipase (6) into the extracellular culture medium. Among the enzymes of *Serratia*, there have been various studies on the proteases. One of the serine protease genes has been cloned and the nucleotide sequence has been determined (23). This cloned protease gene was comprised of prepro structure and also excreted into the extracellular medium through the outer membrane of *E. coli*. The metallo protease gene from the *Serratia* sp. E-15 was cloned and the nucleotide sequence was determined (14). Unlike serine protease, metallo protease was produced as a precursor form but was not excreted into the culture medium when expressed in *E. coli*. These findings suggested that the prepro-form of the *Serratia* protease is very essential and plays an important role in excretion of the protease into the extracellular medium in *E. coli*.

Although there are many reports concerning the excretion of proteins (18, 19, 21, 22), the mechanism of the excretion of proteins from gram-negative bacteria has been not extensively studied. Hence, we cloned minor serine protease gene from the *Serratia marcescens* ATCC 27117, analyzed its expression in *E. coli* and characterized the protease for the studies of its excretion in gram-negative bacteria.

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MATERIALS AND METHODS

Bacterial Strains, Media and Enzymes

S. marcescens ATCC 27117 was used as a DNA donor. *E. coli* JM109[*recA1 supE44 endA1 hadR17 gyrA 96 relA1 thi* Δ(*lac-proAB*) *F'*(*traD36 proAB*$^+$ *laclq lacZ* Δ*M15*)] and *E. coli* HB101[*supE44 hsdS20* (*rB*$^-$*mB*$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] were used as hosts. *S. marcescens* ATCC 27117 and *E. coli* were grown in LB broth at 30℃ and 37℃, respectively. *E. coli* transformants were plated on LB medium containing 50 μg of ampicillin per m*l* and 1% skim milk. Halo-forming colonies were detected after 24 hours of cultivation at 37℃. Restriction enzymes, T_4 DNA ligase, and calf intestinal alkaline phosphatase (CIP) were purchased from Boehringer Mannheim and used according to the manufacturer's instructions.

DNA Manipulations

Chromosomal DNA of *S. marcescens* was purified by the modified Marmur method (13). Preparation of a large amount of plasmid was performed by the method of Godson and Vapnek (7). For small-scale plasmid isola-

tion, the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke(1981) were employed with modifications (2, 8). For cloning, chromosomal DNA was completely digested with *Pst*I. The fragments were ligated to the linear pUC18 DNA cleaved with *Pst*I, and dephosphorylated with CIP. The ligated DNA was transformed into *E. coli* JM109 by the method of Cohen *et al.* (4).

Southern Blot Analysis

Chromosomal DNA from *S. marcescens* and recombinant DNA pSMP18 digested completely with *Pst*I were electrophoresed on 0.8% agarose gel and then transferred to a nylon membrane by Trans VAC (Hoefer Scientific Instruments, San Francisco). The 1.2 kb fragments of pSMP18 digested with *Kpn*I and *Bst*EII were labeled with Digoxigenin (Boehringer Mannheim Biochemica) and used as a hybridization probe. The detection of the labeled probes was done according to manufacturer's instructions using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (NBT).

Assay of Enzyme Activity

Protease activity was measured by a colorimetric procedure using azocasein. A mixture of 250 μl of prewarmed 2% azocasein in distilled water and 150 μl of enzyme solution was incubated for 20 minutes at 37℃. The reaction was terminated by adding 1.2 ml of 10% trichloroacetic acid. After the reaction mixture was allowed to stand for 20 minutes at room temperature, it was centrifuged at 8,000 g for 5 minutes, and then 1.2 ml of the supernatant fluid was transferred to a test tube containing 1.4 ml of 1 N NaOH. The absorbance of this solution was measured at 440 nm. One unit of the protease activity was defined as the amount of protease causing an increase in absorbance of 0.1 during the 20 minutes incubation period. β-galactosidase activity was measured by the method of Pardee *et al.* (17).

Localization of Enzyme Activity

Fractionation of extracellular, periplasmic, and cytoplasmic enzymes in *E. coli* cells was performed by the method of Cornelis *et al.* (5). *E. coli* cells were harvested at the early-stationary phase and washed twice with 10 mM Tris hydrochloride buffer (pH 7.5) containing 25% sucrose. The washed cells were suspended in the same buffer containing 25% sucrose and 1 mM EDTA, and the suspension was shaken for 10 minutes at room temperature. After centrifugation at 7,000 g for 10minutes, the cells were quickly and vigorously suspended in ice-cold water. The suspension was further shaken for 10 minutes. at 4℃ and then centrifuged at 9,000 g for 10 minutes. The precipitated cells were resuspended in 10 mM Tris hydrochloride buffer (pH 7.5) and disrupted by sonication. The extracellular enzyme fraction activity was the sum of the activities in the culture supernatant, the two washes, and the supernatant of the EDTA treatment. The periplasmic fraction activity was the activity found in the supernatant after treatment with cold water. The cytoplasmic fraction activity was the activity in the supernatant after sonication.

Purification of Cloned *Serratia* Protease

E. coli HB101(pSMP18) was cultured in LB media at 37℃ for 24 hours. After centrifugation the protein fractionation was acheived by doing ammonium sulfate precipitation (50 to 70%) of the culture supernatant. Ion exchange chromatography was done with DEAE Sepharose CL-6B using a 0 to 400 mM NaCl gradient in 10 mM potassium phosphate buffer (pH 7.2). Gel filtration was performed with Sepharose 6B using 10 mM potassium phosphate buffer (pH 7.2) as an elution buffer.

Enzymatic Properties of the Cloned Protease

SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (11). The isoelectric point of the purified enzyme was determined by IEF gel electrophoresis using a Phast system (Phamacia Co.). After the native gel electrophoresis was done, overlaying the 0.8% agarose gel containing 1% skim milk onto the native gel and staining gel with Coomassie Brilliant Blue produced the activity staining. To measure the effect of inhibitors, the enzyme was preincubated with the inhibitors at 37℃ for 5 minutes, then assay was performed.

RESULTS AND DISCUSSION

Cloning of the Serine Protease Gene

*Pst*I fragments of chromosomal DNA from *S. marcescens* ATCC 27117 were inserted into the *Pst*I site of pUC18, and the ligated DNA was transformed into *E. coli* JM109. Transformants were detected on X-gal agar plate and the white colonies were selected. Among 3,000 transformants, one clone formed a distinct clear zone around the colony on the LB agar plate containing 1% skim milk. The plasmid DNA isolated from this clone was transformed into *E. coli* JM109 again. All the resulting transformants also formed a halo on skim milk (Fig. 1). The recombinant plasmid DNA was named pSMP18. Restriction mapping of pSMP18 was performed by digestion with various restriction enzymes and analysis of the resulting fragments by agarose gel electrophoresis. As shown in Fig. 2., pSMP18 harbored a 5.8 kb insert including sites of *Eco*RI, *Sal*I, *Hin*dIII, *Kpn*I, *Bst*EII and *Stu*I. There were no *Sac*I, *Sma*I, *Bam*HI, *Xba*I, *Xho*I, *Bgl*II or *Pst*I sites. This is in contrast to the serine protease gene cloned from *S. marcescens* IFO3046 by Yanagida *et al.*. That gene harbored *Bam*HI and *Pst*I sites (23).

Southern Hybridization

To identify the foreign gene fragment originating from

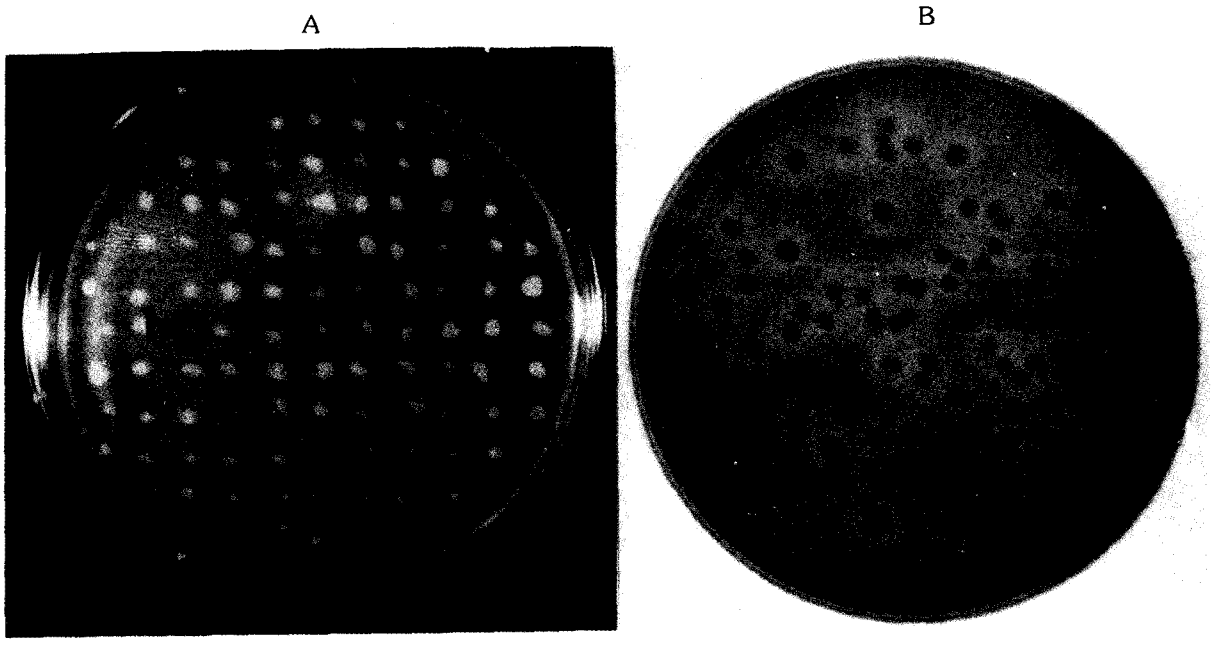

Fig. 1. Screening of *E. coli* JM109 harboring recombinant protease gene onto LB agar plates containing 1% skim milk.
A: a; *Serratia marcescens*, b; Protease positive clone, B: Retransformation of the recombinant plasmid pSMP18.

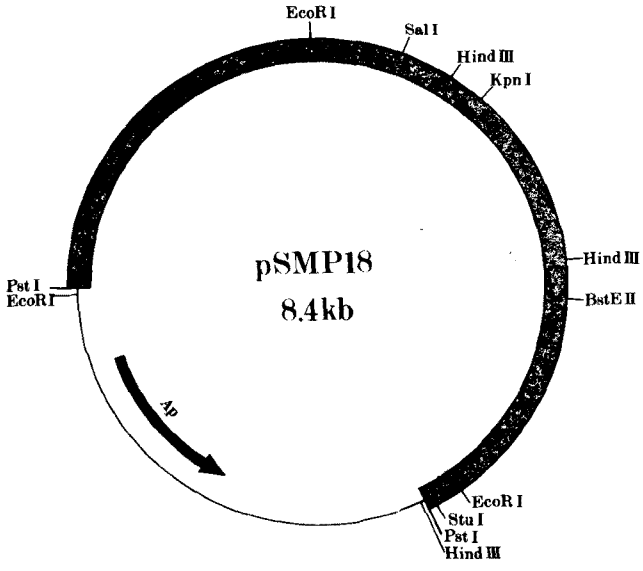

Fig. 2. Restriction map of recombinant plasmid pSMP 18 carrying the protease gene.
The thick line represents the 5.7 kb chromosomal DNA of S. marcescens, and the thin line represents pUC18.

the chromosomal DNA of *S. marcescens* ATCC 27117, a hybridization experiment was performed. Fig. 3 shows the result of Southern hybridization. 1.2 kb of labeled DNA fragments hybridized with *Pst*I-digested *S. marcescens* chromosomal DNA fragments corresponded to the size of the *Pst*I-digested fragments of the insert. This result indicates that the cloned protease gene originated from *S. marcescens* ATCC 27117.

Localization of Enzyme Activities

To analyze the effect of recombinant DNA pSMP18 introduced into *E. coli* HB101, the distribution of protease activity was assayed. As shown in Table 1, when *E. coli* HB101 harboring pSMP18 was cultured in LB broth aerobically at 37℃, almost all the protease activity was found in the extracellular fraction of the *E. coli* cells, while most of the β-galactosidase activity was localized in the cytoplasm. Thus, most of the expressed protease in *E. coli* was excreted through the membrane into the culture medium and not released by cell lysis. Generally, because gram-negative bacteria have an inner and an outer membranes, the excretion of protein is known to be more difficult than in gram-positive bacteria. For this reason the excretion mechanism has not been extensively studied. But there were several efforts at excretion through the gram-negative bacteria's two membranes such as in the case of α-hemolysin from some strains of *E. coli* by *hlyA*, *hlyB*, *hlyC* and *hlyD*, and *E. coli* cells harboring the recombinant plasmid containing penicillinase gene excreted by *kil* gene (10, 15, 16, 20). Because *Serratia* excretes with a different mechanism from these, it is suggested that *Serratia* protease gene cloned

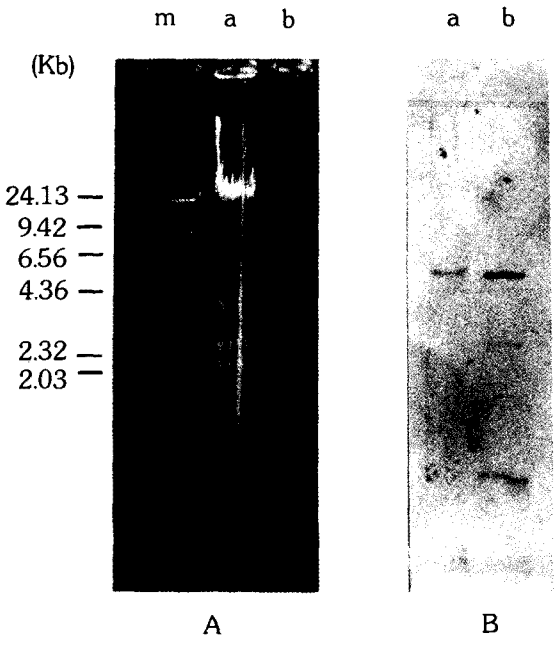

Fig. 3. Southern blot analysis.
The 1.2 kb DNA fragments derived from pSMP18 were used as the probe. The host chromosomal DNA was digested with *Pst*I and separated by agarose gel electrophoresis (A). The DNA was transfered and hybridized with the probe (B): m; size marker (λ/*Hin*dIII), a; *Pst*I-digested host chromosmal DNA, b; *Pst*I-digested pSMP18.

Table 1. Localization of protease and β-galactosidase in *E. coli* HB101 harboring pSMP18.

Enzymes	Plasmids carried	Extracellular activity (U/ml)	Periplasmic activity (U/ml)	Cytoplasmic activity (U/ml)
Protease	pSMP18	4.3014	0.1340	*
	pUC18	0.2077	0.1005	*
β-Galactosidase	pSMP18	31.69	34.16	2570.61
	puc18	21.55	9.11	2084.82

*Not determined

in this work may be good for studies about excretion in gram-negative bacteria.

Enzymatic Properties of the Cloned Protease

The molecular weight of the enzyme purified from the *E. coli* HB101 (pSMP18) was estimated to be about 66,000, and the isoelectric point was approximately 5.7. The purified enzyme was completely inhibited by 10 mM of phenyl methyl sulfonyl fluoride (PMSF), but not by 100 mM EDTA (Table 2). When the culture supernatant of *S. marcescens* was electrophoresed the band having the same mobility as purified protease was hardly visible, whereas the major metallo protease was clearly visible (Fig. 4). This result indicates that the cloned protease is a serine protease, a small amount of which is produced and excreted extracellularly by *S. marcescens* with a large amount of the extracellular metallo protease reported by Yanagida *et al.* in 1986. However, when the *S. marcescens* was cultured in the presence of 1 mM EDTA, a small amount of metallo protease was detected in the culture medium (Fig. 5). The optimal pH (Fig. 6) and temperature (Fig. 7) for activity of the purified serine protease were 9.5 and 45℃, respectively. These are similar to the optimal pH and temperature of the cloned serine proteases of Lee (12) and Yanagida *et al.* (23).

Table 2. Effect of inhibitors on protease activity.

Inhibitors	EDTA					PMSF				
Conc. (mM)	100	50	10	5	1	10	5	1	0.5	0.1
Relative activity (%)	110	107	104	108	105	2	16	80	80	96

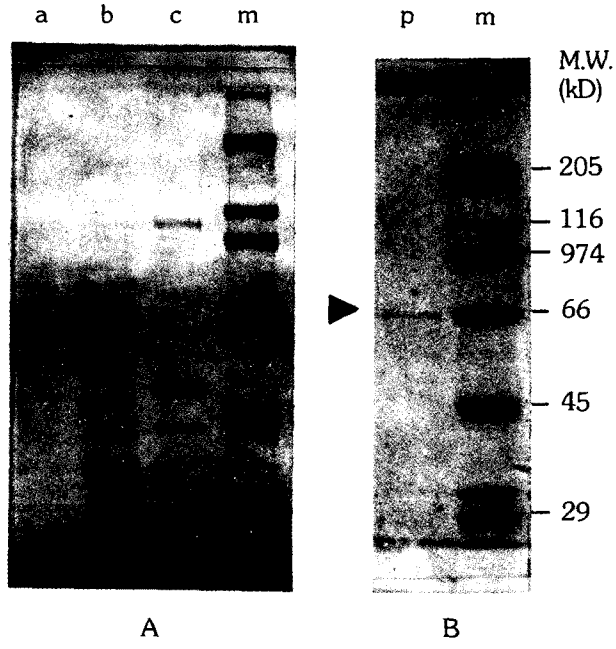

Fig. 4. SDS-PAGE analysis of *E. coli* JM109 and *S. marcescens* culture supernatant (A) and purified protease from *E. coli* HB101 harboring pSMP18 (B).
a; *E. coli* JM109(pUC18), b; *E. coli* JM109(pSMP18), c; *S. marcescens* ATCC 27117, p; purified protease, m; M.W. marker

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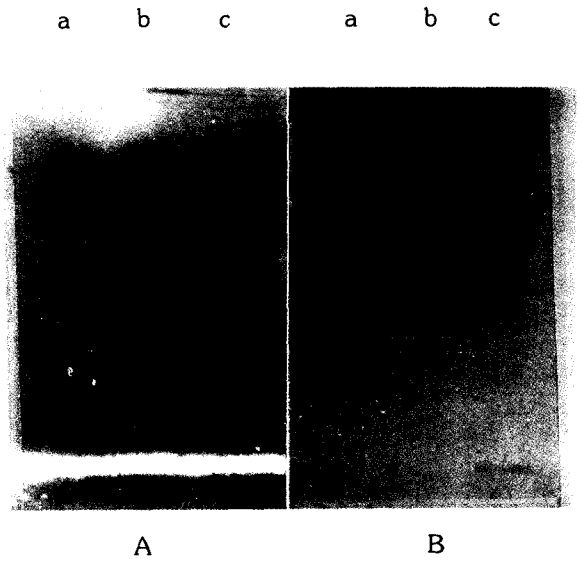

Fig. 5. Activity staining (A) and native gel electrophoresis (B).
a; purified protease from *E. coli* HB101(pSMP18), b; cultured medium of *S. marcescens* (−EDTA), c; cultured medium of *S. marcescens* (+EDTA).

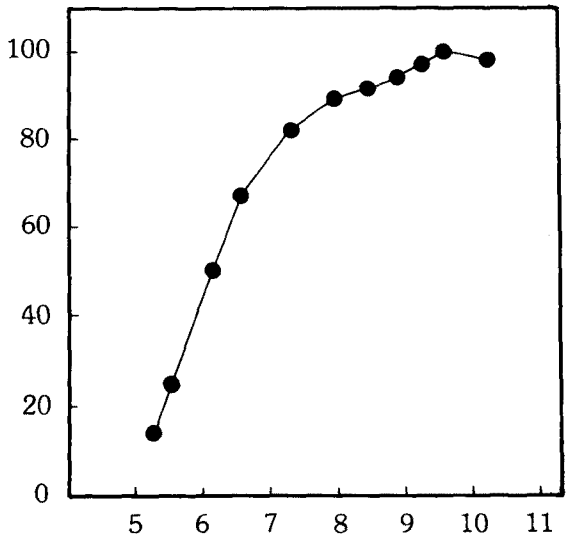

Fig. 6. Optimal pH for reaction of purified protease.

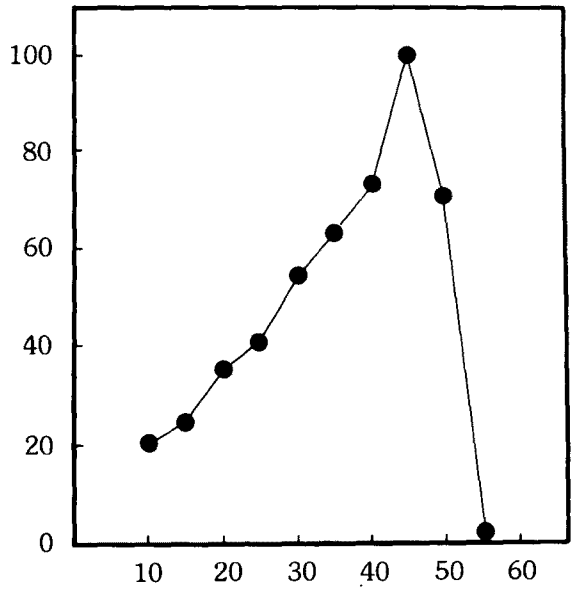

Fig. 7. Optimal temperature for reaction of purified protease.

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