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Purification and Cellular Localization of Extracellular Nuclease of *Serratia marcescens* Expressed in *Escherichia coli*

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Nuclease was secreted to the environmental media from the *Escherichia coli* JM107 transformant harboring the extracellular nuclease gene of *Serratia marcescens* in the plasmid of pNUC4. Under the growth conditions, the amount of secreted enzyme was increased in parallel with bacterial growth. The enzyme was purified using chromatographic procedures of Matrex green gel and heparin agarose affinity gel, resulted in 50-fold purification with 15% recovery of the enzyme. The apparent molecular weight of the enzyme was estimated to be 29 KDa by sodium dodecylsulfate denaturing gel electrophoresis. Using the purified enzyme, polyclonal antibody was obtained from the rabbit. The specificity of the antibody was confirmed by immunoblotting and immunoprecipitation. For the investigation of cellular distribution of the enzyme, cells were fractionated into three fractions; cytoplasm, periplasm and extracellular fluid. While more than 80% of the enzymatic activity was detected in the extracellular fluid and periplasm, a little was found in the cytoplasm, indicating that the enzyme was likely to be immediately exported to the membrane for excretion after biosynthesis. These results were confirmed again by immunocytochemistry technique using the antibody.

KEY WORDS □ ***Serratia marcescens*, nuclease, secretion, localization**

In contrast to Gram positive bacteria, a most of enteric bacteria do not usually secrete proteins into the environmental media (22). Proteins synthesized in the cytoplasm can be exported into the membranes, although the detailed mechanism is not clear. N-terminal signal sequence found in the periplasmic protein is known to be involved in their exportation through the cytoplasmic membrane (3, 25). The proteins that are exported via signal sequence, therefore, have to be synthesized as larger precursor possessing signal peptide at N-terminus (8). However, exported proteins remain in the periplasmic membrane because the outer membrane functions as barrier prevents further excretion into the environmental medium (17). With exception, *Serratia marcescens* which is a Gram negative bacterium and belongs to the family of Enterobacteriaceae excretes a few extracellular proteins such as protease, chitinase, lipase, and nuclease (15). Considering these proteins are synthesized in the cytoplasm, it is important to know how these proteins are excreted to the environmental media without lethality.

Nucleases are produced from a wide variety of sources (20, 22). The enzymes hydrolyze polymerized native or denatured nucleic acid with the formation of a complex mixture of mono- to octanucleotides having a free 5′-phosphate terminal group. The properties of these enzymes are different in molecular weights, ligand binding sites, and substrate specificities. Most of them require the Mg^{2+}, Ca^{2+} or other metal cations for their catalytic activity, while they are inhibited by actinomycin D, ethidium bromide, and inhibitors from calf spleen (24).

Although it has been suggested that the extracellular nucleases catabolize the nucleic acid in the growth medium to use as nutrients, no definite physiological roles of the enzymes are firmly established. While the nuclease from *Staphylococci* had been studied extensively for the protein structure, substrate recognition sites and control mechanism (23, 24), little information has

been obtained for the extracellular nuclease secreted from *Serratia marcescens*, since the amount of the enzyme produced is too small to purify. To overcome this problem, Ball *et al.* (1) cloned the extracellular nuclease gene of *Serratia marcescens* into *E. coli* HB108. Using the recombinant *E. coli* JM 107 (pNUC4) containing the nuclease gene of *Serratia marcescens*, large quantities of the enzyme could be secreted into the medium.

In this paper, we purified the extracellular nuclease of *Serratia marcescens* from the over-expressed *E. coli*. Since the nuclease produced from *Serratia marcescens* was identical to the real exo-enzyme (1, 11) and the membrane composition of *E. coli* is smillar enough to that of *Serratia marcescens*, the distribution of the enzyme was investigated in overexpressed *E. coli* using the antibody.

MATERIALS AND METHODS

Materials

Calf thymus DNA, methyl green, IPTG and ampicillin were purchased from Sigma Chemicals. Matrex green gel was from Amicon Corp., and Heparin agarose was the product of Bethesda Research Lab. Nitrocellulose paper (BA-85) was obtained from Schleicher and Schull. Peroxidase-conjugated goat anti-rabbit Igs and its substrates were from Hyclone Lab.

Bacterial strains and media

E. coli JM 107(pNUC4) transformant which was kindly donated from Dr. Benedict of Texas A&M was used to purify the extracellular nuclease of *Serratia marcescens*. pNUC4 has 1.4 Kb insert of *Serratia marcescens* gene to the *Sma*I-site of pUC 18 and contains both promoters of nuclease gene itself and lac promoter. *E. coli* JM 107(pNUC4) was grown in 2 *l* of LB-broth containing 50 μg/m*l* of ampicillin and 100 μM IPTG at 37℃ with vigorous shaking. Extracellular nuclease of *Serratia marcescens* produced by *E. coli* JM 107 (pNUC4) was determined by plating on DNase test agar containing methyl green as an indicator (20).

Assay of nuclease

Nuclease activity was determined by measuring the conversion of DNA to its fragments which are soluble in 2% perchloric acid as described in Nestle and Roberts (16). Reaction mixture (500 μ*l*) containing 500 μg of DNA, 1 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 8.2), and a source of enzyme was incubated at 37℃ for 20 min. Reaction was terminated by the addition of 500 μ*l* of ice cold 4% perchloric acid. Absorbance of acid-soluble material obtained from centrifugation was measured at 260 nm. A unit of nuclease activity was assumed as the amount of enzyme causing an increase of 1.0 in absorbance at 260 nm in 20 min at 37℃.

Purification of the extracellular nuclease of *Serratia marcescens* from *E. coli* JM 107 (pNUC4)

All procedures were carried out at 4℃ unless otherwise specified.

Step 1. Ammonium sulfate fractionation: 20 *l* of *E. coli* JM 107(pNUC4) was grown at 37℃ in LB broth for 20 hrs in the presence of ampicillin (50 μg/m*l*) and 100 μM IPTG. It was centrifuged at 8,000×*g* for 30 min and obtained the culture supernatant. Ammonium sulfate fractionation was carried out by slowly adding the solid ammonium sulfate to the supernatant fluids. The brown precipitate obtained from 0 to 80% of ammonium sulfate fractionation was dissolved in 200 m*l* of 50 mM Tris-HCl buffer (pH 8.2) and dialyzed twice against 4 *l* of 10 mM Tris-HCl buffer (pH 8.2).

Step 2. Matrex green gel chromatography: The dialyzed solution from step 1 was applied to a Matrex green gel column (5×18 cm) equilibrated with 20 mM Tris- HCl buffer (pH 8.2). The green gel was washed with equilibrium buffer until it was free of unbound proteins. The protein was eluted at a flow rate of 4 m*l*/min with a 1.8 *l* linear gradient of equilibrium buffer containing 0 to 1 M KCl. The active fraction was pooled and concentrated by ultrafiltration.

Step 3. Heparin agarose affinity gel chromatography: The concentrated active fraction from step 2 was dialyzed, and applied to a heparin agarose affinity column (2.5×23 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.2). After washing the column with the same buffer, the protein was eluted with a linear gradient of KCl (50 to 500 mM) in the equilibrium buffer (Fig. 3). The active fraction was pooled, washed and concentrated by ultrafiltration. Purified enzyme was divided into aliquots and stored at −70℃ until used.

Preparation of polyclonal antibody

Purified nuclease (20 μg) emulsified with 1 m*l* of Freund's complete adjuvant was intramuscularly injected to rabbit three times with a time interval of two weeks. After one month from the third immunization, 30 μg nuclease was finally boosted without adjuvant. And then the serum was collected from the immunized rabbit ten days after the final boost as described previously (12). Prior to immunize, the preimmuneserum was collected and used as a control serum. The antiserum was purified by a protein A Sepharose CL-4B column. The column was equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 0.15 M NaCl at a rate of 1.5 m*l*/min. The antibody was eluted by 0.1 M glycine-HCl buffer (pH 3.0) and immediately buffered by adding appropriate volume of 1 M Tris-HCl buffer (pH 8.0) to neutralize.

Immunoblotting and immunoprecipitation

Proteins were separated by SDS-PAGE, and then transferred to nitrocellulose membrane electrophoretically. The nuclease was detected using peroxidase conjugated goat antirabbit IgG as described (10). For immunoprecipitation, 10 μg of antibody was incubated with appropriate amounts of enzyme sources for 12 hrs at 4℃. After incubation, 20 μl of Protein A (heat inactivated *Staphylococcus aureus* cells) was added and further incubated for 6 hrs. On centrifugation at 10,000×g for 10 min, the nuclease activities in the precipitates and supernatants were measured. As a control, the preserum was used as same way instead of antiserum.

Immunocytochemistry

E. coli cultured in LB broth was fixed for 3 hrs in a mixture of 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.7) containing 0.15 M $CaCl_2$ and 0.45 M sucrose. Fixation was continued overnight in the same buffer lacking glutaraldehyde and adjusted to pH 10.4. Rinsed cells were dehydrated in a graded ethanol series (up to 95%) and embedded in Lowicryl K4M (Polyscience). Ultrathin sections mounted on Formvar-coated gold grids were treated for 30 min with Tris buffered saline (20 mM Tris-HCl, pH 7.5 containing 0.5 M KCl), followed by 3% gelatin, and then for 2 hrs in nuclease antiserum at a 1:10,000 dilution in Tris buffered saline. Following a wash in 0.3% gelatin, the sections were incubated in a 1:20 dilution of protein A colloidal gold conjugate (12 nm) (Polyscience) for 60 min. The grids were finally washed with Tris buffered saline and then with distilled water, and examined using transmission electron microscope (Hitachi H-600). To reduce the nonspecific binding between the sections of *E. coli* and antiserum prepared in rabbits, the ultrathin sections were treated with preimmune serum followed by incubation on protein A gold.

Cellular fractionation

Cellular fractionation was done according to the method of Cornelis *et al.* (7). Collected by centrifugation, the cells were washed twice in half volume of the culture with 10 mM Tris-HCl buffer (pH 8.0) and then resuspended in the same volume of 25% sucrose. The supernatant was shaken for 10 min at room temperature in the presence of 1 mM EDTA. After centrifugation at 10,000×g for 10 min, the cells were quickly and vigorously resuspended in the same volume of ice cold water. The suspension was centrifuged at 12,000×g for 10 min and the supernatant cautiously removed. The cells were resuspended in the same volume of 10 mM Tris-HCl buffer(pH 7.5), and was sonicated (4 times at 7,000 cycles in a Branson sonicator). The combined fraction of culture supernatants, washed solution was designated as extracellular fluids, and the supernatant from the EDTA treatment was used as cytoplasmic fraction. The periplasmic fraction was the supernatant resulted from the cold water treatment.

Other methods

Protein concentrations were measured by Bradford's method (6) and the polyacrylamide gel electrophoresis was performed by the method of Laemmli (13) in 12% slab gels containing sodium dodecylsulfate.

RESULSTS

Growth of *E. coli* JM 107(pNUC4) and the production of extracellular nuclease of *Serratia marcescens*

Production of extracellular nuclease of *Serratia marcescens* from *E. coli* JM 107(pNUC4) was examined using DNase test agar containing methyl green. Yellow clear zone around colony was formed due to the nuclease excreted from the cell (data not shown). The enzyme produced in growth medium was monitored during the cell growth as shown in Fig. 1. As *E. coli* cells were grown, the extracellular nuclease activity was increased proportionally indicating that the nuclease synthesized in the cytoplam of *E. coli* JM 107(pNUC4) was secreted into the environmental medium as observed in *Serratia marcescens* (4, 11). Considering that the overexpressed cell contains only nuclease gene, no accessory protein is involved for the secretion. Therefore, the extracellular nuclease of *Serratia marcescens* might be transported to the medium by the information contained in nuclease protein itself. The maximal nuclease activity was attained from the culture supernatants grown for 20 hrs.

Purification of nuclease

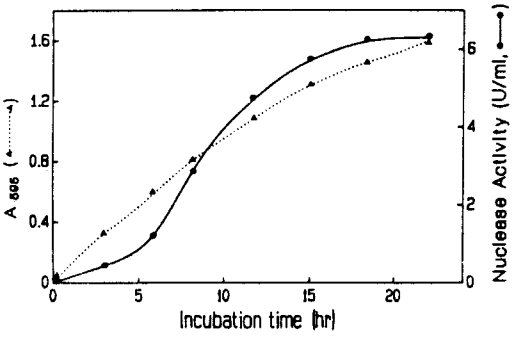

Fig. 1. *Secretion of extracellular nuclease from overexpressed E. coli during the growth.* Cells were cultivated at 37℃ with vigorous shaking. The aliquots taken out at the indicated time were used to measure the cell growth (A_{595}) and enzymatic activity.

Table 1. *Purification of nuclease of Serratia marcescens from overexpressed E. coli.*

Step	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Recovery (%)	Purification (fold)
Crude extract	1,350	160,560	119	100	1
Ammonium sulfate (0~80%)	720	100,096	139	62.3	1.2
Matrex green	7.5	25,680	3,424	26	28.8
Heparin agarose	4.2	24,566	5,611	15	47.2

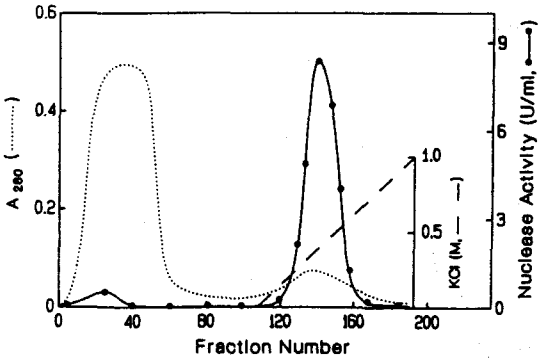

Fig. 2. *Matrex green gel column chromatography of extracellular nuclease.*
The nuclease from ammonium sulfate fraction (0~80%) step was loaded onto a gel column. The column was washed with 10mM Tris-HCl buffer (pH 8.2) containing 0.2 mM $MgCl_2$ to remove weakly bound protein, followed eluted by linear gradient of KCl (0~1 M). Protein peak (A_{280}) and enzymatic activity were monitored.

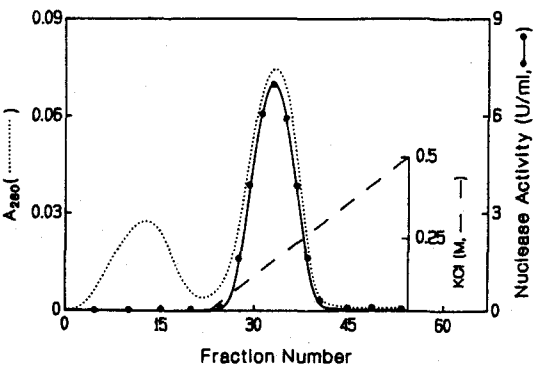

Fig. 3. *Heparin-agarose gel column chromatography of extracellular nuclease.*
The nuclease from Matrex green gel column chromatography was loaded onto a column. The column was washed with 10 mM Tris-HCl buffer (pH 8.2) containing 0.2 mM $MgCl_2$, and then the nuclease was eluted by linear gradient of KCl (0~0.5 M). Protein (A_{280}) and enzymatic activity was monitored.

The extracellular nuclease produced from *E. coli* JM 107(pNUC4) was homogeneously purified by column chromatography of Matrex green gel and heparin agarose affinity gel. As summarized in Table 1, 4.2 mg of pure nuclease was obtained from 1.35 g of soluble proteins of culture supernatants of *E. coli* JM 107(pNUC4). As shown in Fig. 2, column chromatography using Matrex green gel was useful to remove the huge amount of proteins. In unbound fraction, the nuclease activity was not detected. Although most impurities were removed by Matrex green gel, some minor impurities were still remained in the active fraction. However, minor impurities were removed by the heparin agarose affinity gel as shown in Fig. 3. Only one symmetrical protein peak was eluted from the heparin agarose gel which was coincided with the profile of nuclease activity.

Criteria of enzyme purity

Purity of nuclease was analyzed using 12% SDS-PAGE. The protein pattern of each purification step was shown in Fig. 4(A). A great number of protein bands were removed by the green gel chromatography and a single protein band was appeared from heparin agarose affinity gel chromatography. The molecular weight of the purified nuclease was estimated as a 29 KDa on SDS-PAGE, which is coincided with expected molecular weight calculated from DNA sequence. Polyclonal antibody prepared against the purified nuclease was reacted specifically even with the crude enzyme source. Although many protein bands were present as shown by Coomassie blue staining, only the nuclease protein band (29 KDa) was reacted with polyclonal antibody as shown in Fig. 4(B). The specificity of polyclonal antibody to nuclease was also confirmed by immunoprecipitation. The crude enzyme obtained from ammonium sulfate precipitation was incubated for 12 hrs with antibody. Antigen-antibody complex was precipitated by addition of protein A. When control serum was used instead of antibody, there

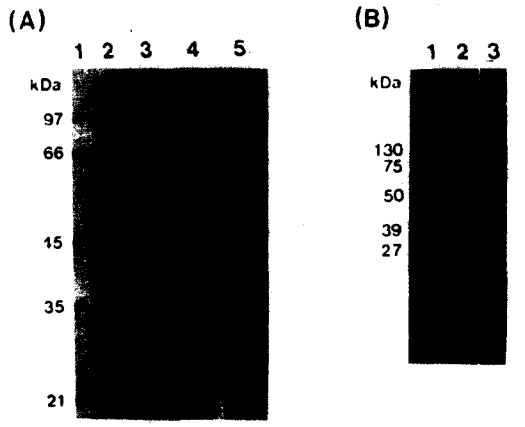

Fig. 4. *Analysis of nuclease by SDS polyacrylamide gel electrophoresis and immunoblot.*
(A) SDS polyacrylamide gel electrophoresis showing the progress of purification of extracellular nuclease. Protein bands were visualized by staining with Coomassie blue. Lane 1, standard marker; 2, crude extract; 3, ammonium sulfate fraction (0~80%) fraction; 4, pooled fraction of Matrex green gel chromatography; 5, purified nuclease from heparin-agarose chromatography. (B) Immunoblot of extracellular nuclease by antibody prepared against purified nuclease. 5 μg of protein of ammonium sulfate (0~80%) fraction was separated by 12% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Lane 1, prestained standard; 2, antiserum treated; 3, preimmune serum treated.

Table 2. *Nuclease activity recovered by immunoprecipitation.*

	Precipitate	Supernatant
	Total Activity (Unit)	
Nuclease only	N.D.[a]	2.072
Nuclease + antibody	2.530	N.D.
Nuclease + preimmune serum	N.D.	2.726

[a] Not Detected.

was little activity changes in the supernatants as shown in Table 2. However, most of the enzyme activity was recovered from the precipitates, showing the nuclese was specifically bound to the antibody. These results showed that the polyclonal antibody prepared specifically recognized the nuclease.

Distribution of nuclease in *E. coli* cell

To investigate the distribution of nuclease, cells were fractionated into the three fractions; extracellular space, periplasm and the cytoplasm. The enzymatic activities of alkaline phosphatase and β-galactosidase were assayed as marker enzymes for each fraction. As shown in Table 3, alkaline phosphatase was detected in the periplasmic fraction, while β-galactosidase was present in the cytoplasmic fraction. This result indicates that each fraction was separated with little cross-contamination. When the nuclease activity was measured using the fractions as enzyme sources, most enzymatic activity was found in the extracellular fluids (29%) and periplasmic fractions (54%). Only a little activity was recovered from the cytoplasmic fraction (16.5 %). Each protein fraction isolated from *E. coli* cellular fractions of cytoplasm, periplasm, and extracelluar fluids was immunoblotted by polyclonal antibody prepared against purified extracellular nuclease. The antibody was cross-reacted to all the nuclease separated from each fraction. In order to know whether the nucleases obtained from the cytoplasm, periplasm and extracellular fluids were active on its own nucleic acids or not, subcellular fractions were incubated with the nucleic acids extracted from the *E. coli* itself. All of them hydrolyzed its own *E. coli* DNA and RNA completely to mono-, di- and oligonucleotides, but the hydrolysis rate of DNA was faster than RNA (data not shown).

Localization of nuclease detected by immunoblot and immunocytochemistry

Immunolabelling experiments with the antiserum performed with cells prepared by the Lowicryl technique, exhibited that the nuclease was localized at the cell periphery. In the cytoplasm or the nuclear region, however, no significant amount of nuclease was present. Although nuclease activity was detected in cytoplasmic fraction, the enzyme was mainly retained in

Table 3. *Distribution of S. marcescens nuclease in overexpressed E. coli.*

Fraction	Nuclease activity	Alkaline phosphatase	β-Galactosidase
Extracellular fluids	30	2	8
Periplasm	54	88	2
Cytoplasm	16	N.D.[a]	90

Enzymatic activites were presented as a relative activity assuming the total activity being 100.
[a] Not Detected.

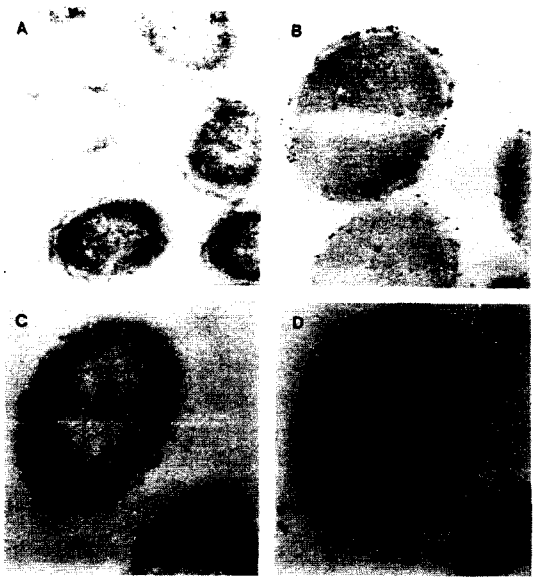

Fig. 5. *Localization of nuclease in overexpressed E. coli.*
Control cell (A, ×20,000) which has no plasmid harboring extracellular nuclease gene of Serratia marcescens was compared with overexpressed cells cultivated for 3 hrs (B, ×40,000), 8 hrs (C, ×40,000), and 15 hrs (D, ×60,000).

periphery. From the result shown in Fig. 5, the nuclease was likely to be synthesized maximally from the cell grown for 6 hrs (Fig. 5C) and then slowly decreased (Fig. 5D). Considering the fact that nuclease excreted to the environmental medium during growth, it seems likely that the synthesized enzyme was accumulated first in periplasm. This suggests that the nuclease synthesized in cytoplasm rapidly exported to the membrane, and then slowly secreted to the environmental medium. The nuclease like many other extracellularly secreted proteins also can be secreted to environmental medium in *E. coli* (27). It was supported the suggestions that at least some of the information required for secretion of the protein resides within the sequence was contained in *Serratia* nuclease itself. Therefore, the other gene product is not necessary for the secretion of *Serratia* nuclease in *E. coli* cell.

DISCUSSION

Many extracellular proteins produced by bacteria possessed enzymatic activity which might be toxic to the cell. However, they are synthesized in the cytoplasm without lethality. The self-protection of bacterium from those enzymes was explained by the presence of the inhibitors. For instance, colicins (18) and extracellular protease from *Erwinia caratovorocin* (21) were known to be co-expressed with specific inhibitors. In *Serratia marcescens*, extracellular protease (14) is secreted with its specific inhibitor, while nuclease, also potentially lethal protein, does not have known specific inhibitor. If active nuclease was present in the cytoplasm, it might cause DNA damage and make deleterious effects on the growth of bacteria. However, it was still unsolved how the chromosomal DNA or RNA were protected from the hydrolytic action of the substrate nonspecific nuclease. The transformed *E. coli* which over-expresses nuclease rapidly secreted the nuclease to the extracellular medium even though *E. coli* is normally not an exoprotein producer as shown in the result of Shin *et al.* (19). One possible explanation is that nuclease in the cytoplasm has not the proper folding due to the disulfide bonds. Disulfide bondings play an important role in the folding and stability of many exported preoteins including extracellular proteins. Nuclease of *Serratia marcescens* has four cysteine residues which form two disulfide bonds, on between C9 and C13 and a second C201 and C243 (5). This hypothesis is quite resonable that nuclease only becomes active after secretion to the periplasm. Recently, Ball *et al.* (2) found the disulfide bond formation is essential for the enzymatic activity by site directed mutagenesis. Although disulfide bond in nuclease may play an important role in preventing the lethal action of nuclease, it is not enough to explain the real mechanism in the bacterial cytoplasm. The mutants obtained from site directed mutagenesis has slight residual activity. In addition, β-mercaptoethanol which disrupt disulfide bonds cause only partial inactivation even at the high concentration (9). In this paper, we propose that the rapid exportation of the enzyme after synthesis to the membrane may help the prevention mechanism. For the study of secretion mechanism and localization, we purified enzyme and prepared antibody. It was possible to study the cellular localization of this enzyme using antibody. The results of this study indicate that most of the *Serratia* nucleases produced from *E. coli* were tansported to the membrane immediately after biosynthesis in ribosome. This was confirmed by the studies on distribution of nuclease activity in subcellular fractions in addition to electron microscopic analysis. Immunocytochemical studies demonstrated that large amounts of nucleases were transported into the outer membrane during the cell growth, as evidenced by the deposition of gold particles over the inner and outer membranes. It seems reasonable to assume that the typical N-terminal signalsequences of the nuclease and its processing are involved in

secretion through the inner cytoplasmic membrane (26), while the specific excretion of the nuclease through the outer membrane is directed by the nuclease gene itself. Therefore, this study suggests that the rapid secretion and disulfide bond forming of the nuclease may play a crucial role in protecting the cell from the lethal activity of the enzyme.

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초 록: 대장균에 발현된 *Serratia marcescens*의 Nuclease의 정제와 세포내 분포

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*Serratia marcescens*가 세포외로 분비하는 nuclease의 유전자가 발현된 *Escherichia coli* JM107을 배양하여 다량의 효소를 정제하였다. Matrex green gel과 heparin agarose gel column chromatography법으로 약 50배 정제한 효소는 분자량이 29 KDa였으며, 전기영동 상에서 단일 띠를 보였다. 이 단백질을 이용하여 polyclonal antibody를 만들고, 면역조직화학법으로 세포내의 분포를 조사하였다. Nuclease는 주로 세포막에 존재하였고, 이를 토대로 효소가 세포질에서 합성된 후 세포막으로 빠르게 이동함을 알 수 있었다. 이 결과는 세포의 막분획에서 효소의 활성의 대부분이 회수되며, 면역블럿 방법으로 효소의 대부분이 세포막에서 검출된다는 결과와 일치하였다.