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Expression and Secretion of *Serratia marcescens* 58 KD Chitinase in *Escherichia coli*

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대장균에서 *Serratia marcescens* 58 KD 키티나아제의 발현과 분비

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Abstract — We subcloned a 58 KD chitinase gene of *Serratia marcescens* into *Escherichia coli* and investigated the expression and secretion of the chitinase. Chitinase was produced in *E. coli* by using its own promoter but the levels of enzyme were very low, less than 5 mU/m*l*. However, by the combined action of the chitinase and lac promoters, the chitinase activity increased up to about 80 mU/m*l*. The most of the chitinase produced in *E. coli* was localized in periplasm and the small amounts were observed in cytosol and culture medium. Intracellular chitinase activities increased in proportion to the growth of *E. coli* up to the early stationary phase but rapidly decreased thereafter, which was assumed to be degradation of the chitinase by *E. coli* proteolytic enzymes.

Chitinase hydrolyzes chitin, a β-1,4-polymer of N-acetyl glucosamine (NAG) and is widely found in a variety of organisms such as bacteria (1-6), fungi (7, 8), plants (9-12), insects (13, 14), crustaceans (15), and some vertebrates (16, 17). The biological roles of the chitinase are somewhat different for the organisms they belong: in fungi, crustaceans, and insects, chitinases play an important role in the modification of their structural constituent; in higher plants, chitinases are involved in the host-defense mechanism for the fungal pathogens by hydrolyzing the fungal cell wall containing chitin as a structural element; in bacteria, chitinases are one of the hydrolytic enzyme they produce for utilizing the chitin as carbon and energy sources.

A gram negative bacterium *Serratia marcescens* has been well-known for producing extracellular chitinases. Their chitinases are considered as composed of three kinds of enzyme, an endochitinase, a chitobiase, and a factor required for the hydrolysis of crystalline chitin (1). *S. marcescens* has been believed to produce at least five chitinase isozymes consisting of 58, 52, 48, 36, and 21 KD in their molecular weight (18-20). Several investigators have reported that these chitinases are partly responsible for the antifungal activity of *S. marcescens*, presumably by hydrolyzing fungal cell wall (19, 21-23). Recently, Chi A and Chi B genes, encoding 58 KD and 52 KD chitinases are cloned and sequenced (18, 19, 21, 24). These chitinase genes have been applied to construct transgenic plants and breed the root-colonizing bacteria for repelling the plant-pathogenic fungi (18, 19, 21-23, 25-27). However, still these five chitinase enzymes are not investigated in detail with respect to their enzymatic characterizations and antifungal avtivities of each purified isozyme, and to their interrelationships for hydrolyzing insoluble chitin and fungal cell wall. To solve these problems, previously we cloned 58 KD chitinase gene from *S. marcescens* ATCC 27117 and examined its antifungal activity (21). At present paper,

Key words: Subcloning, *Serratia marcescens* 58 KD chitinase, localization and production in *E. coli*.
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we described the subcloning and expression of 58 KD chitinase gene in *E. coli*.

Materials and Methods

Chemicals and enzymes

Crab shell chitin (Sigma C-4666) was purchased from Sigma Chemical Co. (St. Louis. Mo., USA), restriction enzymes, T_4 DNA ligase, and calf intestinal phosphatase (CIP) from Promega Biotech (Madison, Wi., USA), and standard marker proteins, SDS-PAGE reagents, and Econo-PacQ cartridge from Bio-Rad (Richmond, Calif., USA). Other chemicals and reagents were purchased from Sigma Chemical Co. and were of reagent grade.

Bacterial strains, plasmids, and media

E. coli JM109 was used as a host microorganism for transformation and plasmid pUC19 as a cloning vehicle for all cloning experiments. Plasmids pCHI 89, pCHI43, pCHI30, and pCHI26 were constructed in this study and had an 8.9 Kb, 4.3 Kb, 3.0 Kb, and 2.6 Kb insert DNAs in pUC19, respectively. *E. coli* was routinely grown in Luria Bertani (LB) medium at 37℃. In order to screen *E. coli* transformants showing chitinolytic activity, the transformants were tooth-picked on LB agar plate supplemented with ampicillin (50 μg/m*l*), isopropyl thiogalactoside (IPTG, 0.1 mM), and swollen chitin (1%, w/v), and incubated for two weeks at 37℃. The swollen chitin was prepared from crab shell chitin by the method of Monreal and Reese (1).

Subcloning and DNA manipulation

Unless stated otherwise, all recombinant DNA techniques were performed according to the laboratory manual of Sambrook *et al.* (28). By using the plasmid pCHI89 constructed in our previous paper, subcloning was carried out and the resulting subcloned plasmids are shown in Fig. 1. Large scale plasmid DNA was extracted by cleared lyzate method and purified by Econo-PacQ cartridge. DNA fragments after the treatment of restriction enzyme were eluted with Biotrap 1000 (Schleicher & Schuell, Germany) or Gene-Clean kit (BIO 101, Calif., USA) from 0.7% agarose gel. Transformation of *E. coli* JM109 was carried out by the method of Cohen *et al.* (29).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and activity staining of chitinase

SDS-PAGE was conducted according to the method of Laemmli (30) with SE 600 electrophoresis system (Hoeffer Scientific Ins., Calif., USA). After SDS-PAGE, the activity staining of chitinase was con-

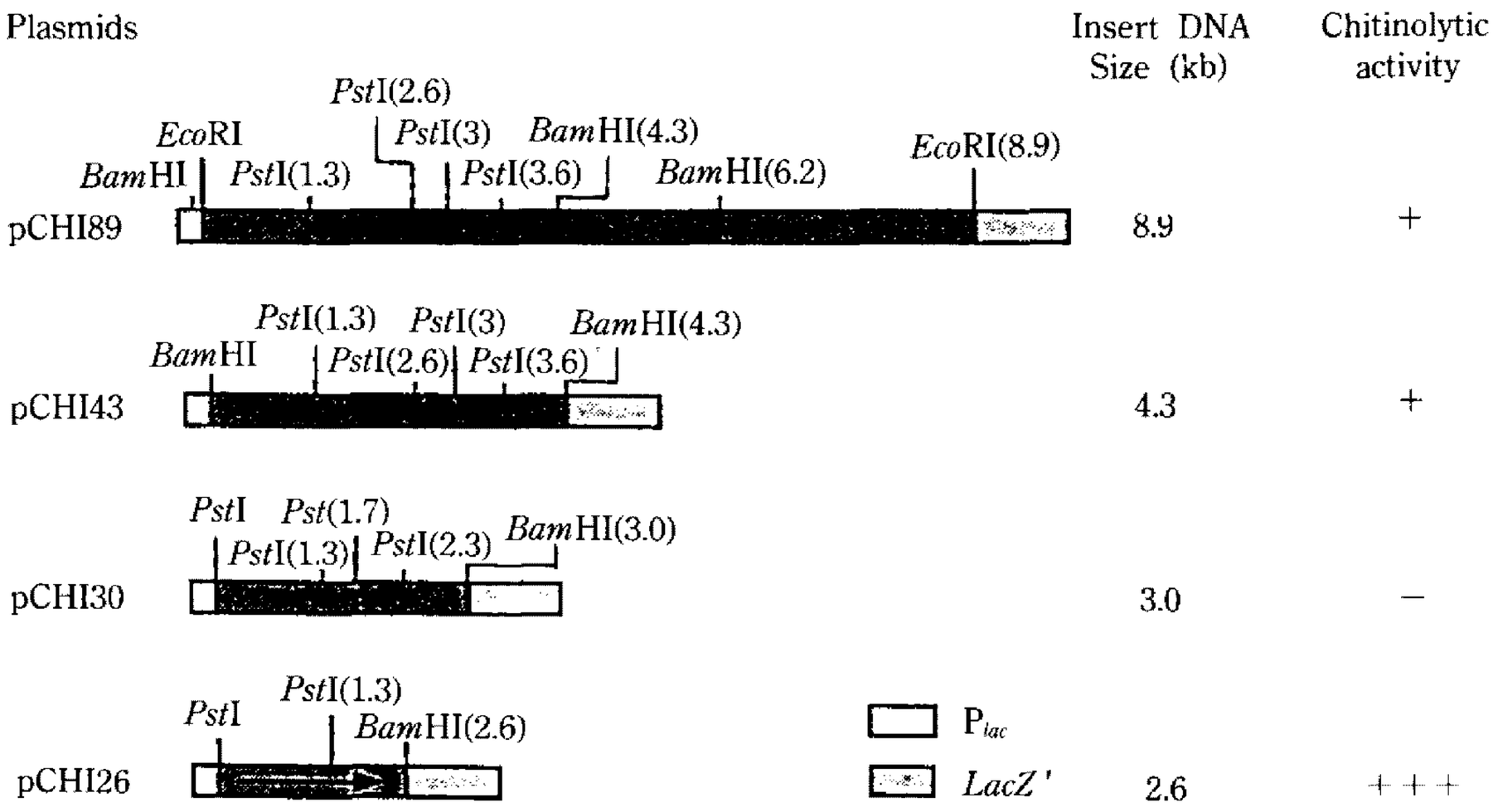

Fig. 1. Relationship between subcloned fragments and enzyme activities.

ducted by the sligtly modified method of Trudel and Asselin (31) described in our previous paper.

Cellular fractionations

The recombinant *E. coli* was grown in 100 m*l* LB media with ampicillin (50 μg/m*l*) and IPTG (0.1 mM) and the culture supernatant was collected by centrifugation at 5000 rpm (Beckman, rotor JA-10) for 10 min at 4℃. Proteins in culture supernatant were precipitated by saturation with 80% ammonium sulfate and collected by centrifugation at 15,000 rpm (Beckman, rotor JA-20) for 30 min at 4 ℃. The protein precipitate was dissolved and dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and used for extracellular enzyme fraction. For the preparation of total intracellular enzyme, if necessary, the washed cell suspension was disrupted by sonication (Vibra-Cell, Sonics & Materials Inc., Conn., USA) for 1.5 min and the supernatant was collected after centrifugation at 10,000 rpm for 10 min at 4℃. Periplasmic enzyme fractions were prepared from the harvested cells according to the method of Cornelis *et al.* (32) by osmoti%c shock. After extraction of periplasmic enzyme, the remaining precipitated cells were resuspended in 5 m*l* of 10 mM Tris-HCl buffer (pH 7.5), sonicated, and centrifuged at 10,000 rpm for 20 min. The supernatant was collected as cytosolic enzyme fraction. From 100 m*l* of culture broth, final 5 m*l* of extracellular, periplasmic, cytosolic fractions were prepared (20-fold concentration).

Chitinase assay

Chitinase activities were determined according to the our previous paper (21). One unit(U) of enzyme was defined as the amount of enzyme that produced one micromole of NAG from swollen chitin per hr under the experimental conditions. One milli unit (mU) was also defined as 1/1000 U and used for convenience.

Results and Discussion

Subcloning of 58 KD chitinase gene

Previousely, it was confirmed that plasmid pCHI 89 encoded 58 KD chitinase of *S. marcescens* ATCC 27117. *E. coli* containing the plasmid pCHI89 produced very low level of chitinase, only detectable on swollen chitin agar plate after 7 days-incubation. However, chitinase activities produced by this *E. coli* strain were not detectable by the colorimetric method of Somogyi-Nelson (33). For the further characterizations of 58 KD chitinase gene and its product, the subcloning of 58 KD chitinase gene from pCHI89 was carried out and resulting recombinant plasmids are shown in Fig.1 with restriction enzyme sites. The plasmid pCHI43 containing 4.3 Kb-*Bam*HI DNA fragment of pCHI89 gave similar chitinolytic activities on swollen chitin agar plate with pCHI89. But pCHI30, deleted 1.3 Kb *Bam*HI-*Pst*I DNA fragment downstream the lac promoter of pCHI43, gave no chitinolytic activity, which suggested that the 1.3 Kb *Bam*HI-*Pst*I DNA fragment is prerequisite for the chitinolytic activity. A 2.6 Kb DNA fragment including this essential 1.3 Kb DNA was prepared by double-digestion of pCHI43 with BamHI and PstI, and used to construct pCHI26. Chromosomal DNA fragment of pCHI26 was located in pUC19 with opposite-orientation as compared with those of pCHI89 and pCHI43. The pCHI26 showed larger chitinolytic activities than pCHI89 and pCHI43. Clear halo was observed on the swollen chitin agar plate of *E. coli* JM109(pCHI26) after 24 hr incubation. This result suggested that open reading frame (ORF) of chitinase in pCHI89 and pCHI43 has the opposite-orientation to the lac promoter, producing low levels of chitinase by its own promoter, while ORF in pCHI26 has the same orientation to the lac promoter, producing high levels of enzyme by the combined action of both lac and chitinase promoters.

Chitinase activities of subcloned fragments and their products analysis

To compare the chitinase activities of the subcloned fragments, 5 m*l* enzymes of intracellular and extracellular fractions were prepared from 100ml culture of *E. coli* (20-fold concentration) and their chitinase activities are shown in Table 1. As described previousely, *E. coli* containing pCHI89 gave very low level of chitinase activities, below the detectable range of Somogyi-Nelson method (less than

Table 1. Chitinase activities of *E. coli* strains harboring various recombinant plasmids

Plasmid	Chitinase activity (mU/ml)	
	Intracellular	Extracellular
pUC19	N.A.[a]	N.A.
pCHI89	<5[b]	<5[b]
pCHI43	<5[b]	<5[b]
pCHI26	80	<5[b]

Enzyme fractions were prepared by the method described in Materials and Methods. [a]No chitinase activity. Cells gave no chitinase activity not only on swollen chitin agar plate but also in culture broth. [b]Cells gave chitinase activity on swollen chitin agar plate but produced very low levels of enzyme in culture broth, not detectable with reproducibility by Somogyi-Nelson method.

10 μg reducing sugar per ml per 2 hr). Intracellular and extracellular chitinase activities of pCHI43 were also very low level, less than 5 mU/ml. But pCHI26 produced about 80 mU/ml of chitinase intracellularly. Extracellular chitinase activities produced by pCHI26 were less than 10% of intracellular enzymes. This indicated that most of the chitinase produced by *E. coli* are accumulated intracellularly.

From our DNA sequencing data of 58 KD chitinase gene, it was revealed that ORF of chitinase gene in pCHI89 and pCHI43 was located in opposite-orientation to the lac promoter (data not shown). Therefore, it seemed that pCHI89 and pCHI43 expressed the chitinase gene by using their own promoters and consequently produced low levels of enzyme. While ORF of pCHI26 was located in the same orientation with lac promoter. This plasmid produced higher chitinase activities, more than 20 times of those of pCHI89 and pCHI43.

When the gene products of subcloned fragments were analyzed by SDS-PAGE and activity staining methods, all subcloned fragments produced 58 KD chitinase (Fig. 2). One unexpected result was that pCHI26 produced a minor 20 KD chitinase as well as a major 58 KD chitinase. This minor chitinase activity has been also found frequently in the expression of pCHI89 and pCHI43 but never been found in the cells harboring a cloning vector pUC19. It seemed that the 20 KD chitinase band was derived from 58 KD chitinase by proteolytic digestion of *E. coli*. The similar observations were also found in chitinase A1 of *Bacillus circulans* WL-12: chitinase A2 was originated from chitinase A1 by proteolytic removal of a C-terminal portion of chitinase A1 (34-36). However, the possibilities are still remained that the minor band on SDS-PAGE is another chitinase-like enzyme such as *E. coli* lysozyme. Purification, characterization, and relationship between 58 KD and 20 KD chitinases are thought to be important task to be done in the near future.

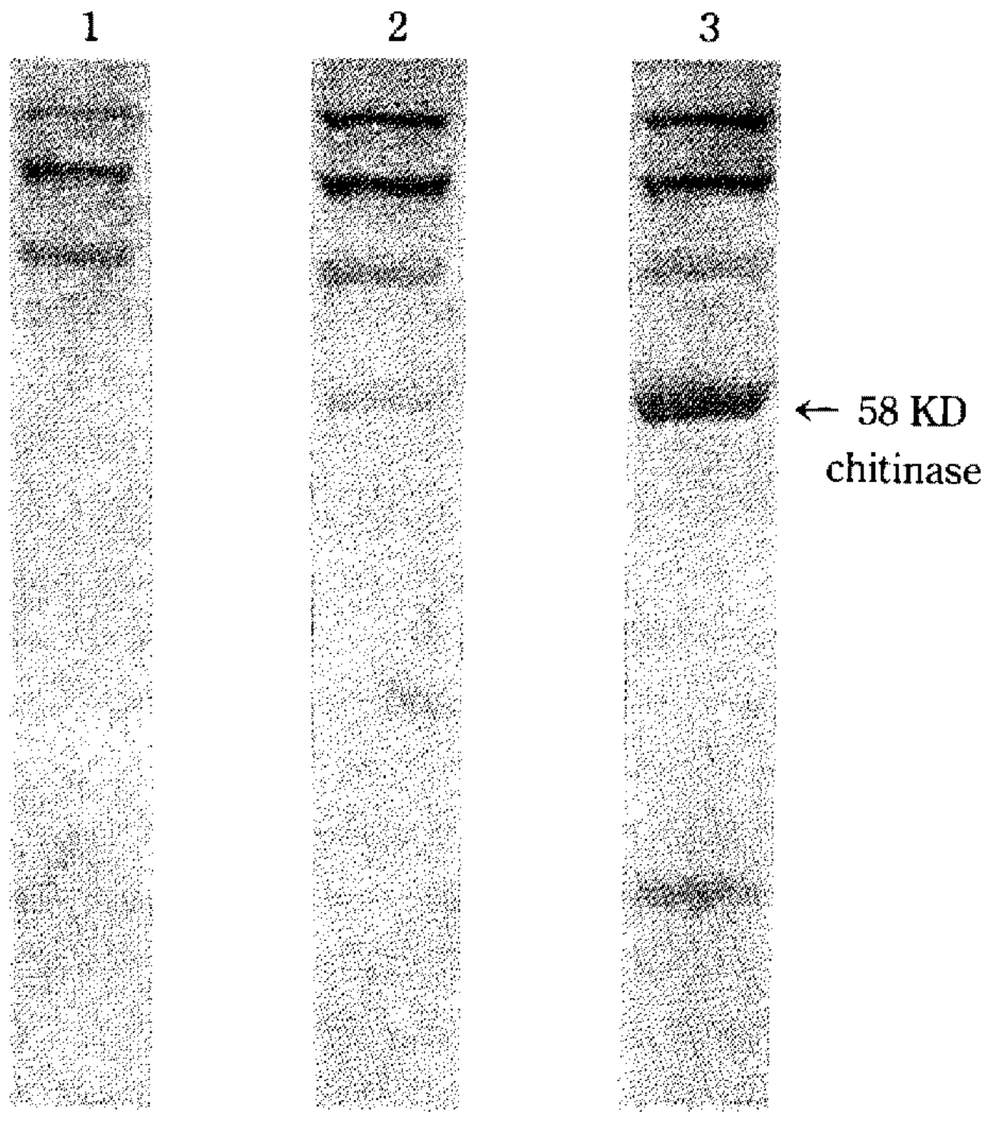

Fig. 2. SDS-polyacrylamide gel electrophoresis of proteins produced by *E. coli* strains harboring subcloned recombinant plasmids.
After SDS-PAGE, bands were visualized by activity staining of chitinase. Lanes 1, pUC19; 2, pCHI43; and 3, pCHI26.

Effect of IPTG on chitinase expression

Plasmid pCHI26 contains two promoters of lac and chitinase promoters at the upstream of 58 KD chitinase ORF. To investigate the effect of lac promoter on the expression of chitinase gene, final 0.1 mM IPTG was added to the culture broth as an inducer and chitinase activities were determined (Table 2). In the presence of IPTG, about three times of enzyme activities, 71 mU/ml, were produced in comparison with 26 mU/ml of chitinase produced in the absence of IPTG. When the chitinase

Table 2. Effect of IPTG on the expression of 58 KD chitinase in *E. coli* JM109(pCHI26)

Inducer	Culture time (hr)	Cell Growth (O.D. at 600 nm)	Chitinase activity (mU/ml)
−IPTG	6	1.44	26
	18	1.66	29
+IPTG	6	1.41	71
	18	1.79	47

100 ml of LB broth containing ampicillin (50 μg/ml) and IPTG (0.1 mM) in 500 ml Erlemeyer flask was inoculated with 2 ml of overnight culture of *E. coli* JM109(pCHI 26) and cultivated in a rotary shaker at 37℃, 200 rpm. The intracellular enzyme was prepared and measured by the method described in Materials and Methods.

ORF was located in the opposite-orientation to lac promoter as pCHI43, chitinase was expressed at low-level, less than 5 mU/ml (Table 1) by using its own promoter. This result suggested that lac promoter contributes to the increase of expression level of chitinase gene. The pCHI26 showed higher chitinase activities even in the absence of IPTG when compared with pCHI43. This result seemed to be owing to the partial induction of chitinase by lac promoter in the absence of IPTG. Because pUC plasmids are such high-copy-number plasmids, lac promoter would not be completely repressed by the repressor proteins, even when overexpressed from the lac Iq allele present in *E. coli* JM109. The levels of chitinase activities produced in the presence of IPTG decreased for the extended cultivation of *E. coli* (Table 2), indicating that chitinase proteins may be degraded by the proteolytic enzymes of *E. coli*.

Localization of 58 KD chitinase in *E. coli*

E. coli harboring pCHI26 was cultivated to $O.D._{600}$ =1.0 and cellular fractionations were carried out. As shown in Table 3, 7.3, 87.8, and 4.9% of total chitinase activities were found in extracellular, periplasmic, and cytoplasmic fractions, respectively. Most of the chitinases produced in *E. coli* are excreted into periplasmic space through the inner membrane and remained in this place without further excretion into culture medium. This result was not

Table 3. Localization of 58 KD chitinase in *E. coli* JM 109(pCHI26)

Location	Chitinase activity (mU/ml)
Cytoplasm	5.0(4.9)[a]
Periplasm	90.0(87.8)
Growth medium	7.5(7.3)

[a] Parenthesis represents percent of total activity.

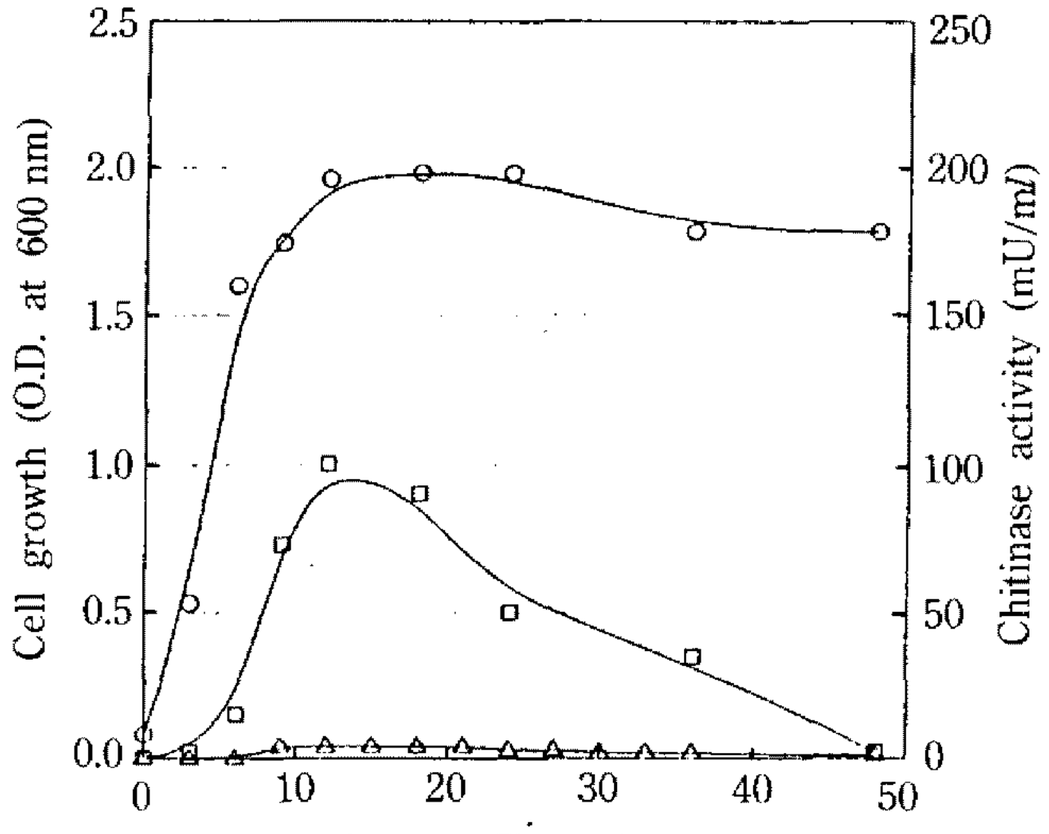

Fig. 3. Production of 58 KD chitinase from *E. coli* JM 109 (pCHI26) according to culture time.
500 ml of LB broth containing ampicillin (50 μg/μl) and IPTG (0.1 mM) in 3 l Erlenmeyer flask was inoculated with 10 ml of overnight culture of *E. coli* JM109(pCHI 26) and cultivated at 37℃, 200 rpm in a rotary shaker. The enzyme fractions were prepared by the method described in Materials and Methods. Symbols: ○, cell growth; □, intracellular chitinase activity; △, extracellular chitinase activity.

coincided with our previous paper (21) reporting that nearly the same intensities of chitinase bands were observed in both intracellular and extracellular fractions when visualized by the activity staining after SDS-PAGE. We thought that this controversy might be due to the non-linearity of band intensity with the chitinase activity at the very low concentration of enzyme. Considering the fact that *S. marcescens* excretes the chitinases exclusively into culture medium, *E. coli* seems to be deficient a secretion machinery for the excretion of chitinase into culture medium.

Production of 58 KD chitinase in *E. coli*

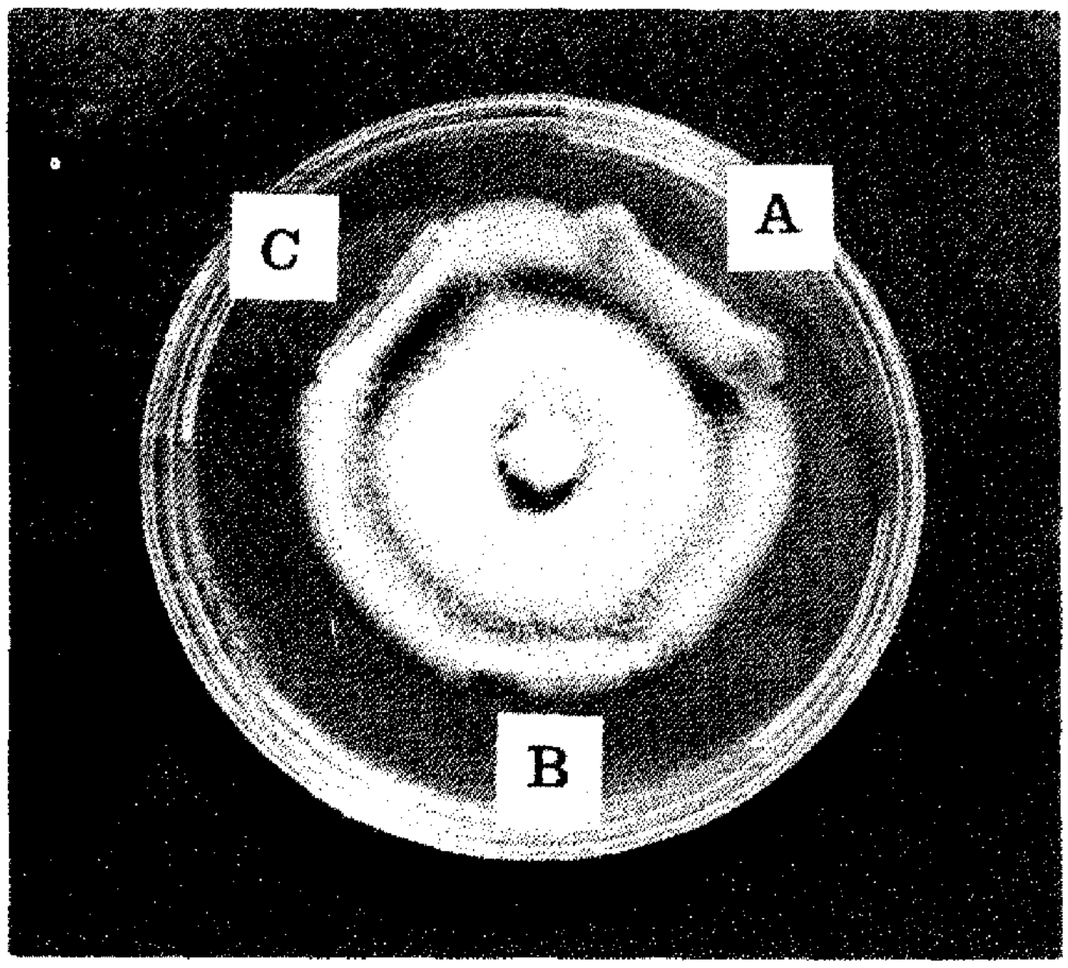

Fig. 4. Growth inhibition of a plant pathogenic fungus, *Fusarium oxysporum* by *E. coli* JM109(pCHI26) and *S. marcescens* ATCC 27117.
A: *S. marcescens*, B: *E. coli* JM109(pUC19), and C: *E. coli* JM109(pCHI26).

Fig. 3 shows the chitinase production of *E. coli* according to the culture time. As the cell grow, intracellular chitinase activities increase up to 88 mU/m*l*. However, during the stationary and early death phases, the intracellular chitinase activities were rapidly decreased. But the extracellular chitinase activities remained nearly at a constant level during these phases. Decreases in the intracellular chitinase activities seemed to be occurred by the proteolytic digestion of chitinases accumulated in periplasmic space of *E. coli*.

Antifungal activity of *E. coli* JM109(pCHI26)

Our previous paper(21) reported that *E. coli* JM 109(pCHI89) shows the growth inhibition of *Fusarium oxysporum*. But the inhibition of fungal growth was very low. When *E. coli* JM109(pCHI26) constructed in this paper was tested for its antifungal activity, it also showed a low growth inhibition of *Fusarium oxysporum*, as shown in Fig. 4. Increases in chitinase activity by the aid of lac promoter did not enhance the fungal growth inhibition of *E. coli* appreciably. Inhibitory effect of *S. marcescens* on fungal growth was very similar to that of *E. coli* JM 109(pCHI26). From this result, we thought that the chitinases of *S. marcescens* are not effective for hydrolyzing the fungal cell wall. Hydrolytic patterns of 58 KD chitinase and chitinase complex of *S. marcescens* revealed that they only produced N-acetylglucosamine and chitobiose from swollen chitin by exochitinase activities(data not shown). Exochitinase activity of *S. marcescens* seemed to be less effective for hydrolyzing the fungal cell wall than endochitinases produced by plants as their defense mechanism.

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요 약

Serratia marcescens ATCC 27117에서 부터 클로닝한 58 KD 키티나아제 유전자를 subcloning하여 2.6 Kb DNA 삽입단편을 가진 플라스미드 pCHI26을 제조하고 대장균에서의 발현과 분비를 살펴보았다. 키티나아제 유전자는 대장균에서 자신의 promoter를 이용하여 매우 낮은 수준(<5 mU/m*l*)으로 발현되었으며 lac promoter를 이용하는 경우 키티나아제 발현이 증가되어 약 80 mU/m*l*가 되었다. 발현된 키티나아제는 거의 전적으로 대장균의 periplasm에 위치(약 87.8%)하고 있었다. 배양시간에 따라서 세포내 키티나아제 활성을 측정해본 결과 초기정지기까지는 균체 성장과 비례해서 세포내 효소활성이 증가되었으나 정지기부터 세포내 효소활성이 급격히 줄어드는 양상을 보였다. 그러나 이 기간 동안 세포외 효소활성의 변화는 거의 없었다. 이러한 결과로 보아 periplasm에 위치한 키티나아제가 대장균의 단백분해효소에 의해서 분해되는 것으로 추정되었다.

References

1. Monreal, J. and E.T. Reese. 1969. The chitinase of *Serratia marcescens*. *Can. J. Microbiol.* **15**: 689-696.
2. Joshi, S., M. Kozlowski, G. Selvaraj, V.N. Iyer, and R.W. Davies. 1988. Cloning of the genes of the chitin utilization regulon of *Serratia liquefaciens*. *J. Bacteriol.* **170**: 2984-2988.

3. Soto-Gil, R.W. and J.W. Zyskind. 1984. Cloning of *Vibrio harvaeyi* a chitinase genes in *Escherichia coli*. Pp. 209-223. *In* J.P. Zakikas (ed.), *Chitin, chitosan, and related enzymes*. Academic Press, Inc., New Yor.
4. Wortman, A.T., C.C. Somerville, and R.R. Colwell. 1986. Chitinase determinants of *Vibrio vulnificus*: gene cloning and applications of a chitinase probe. *Appl. Environ. Microbiol.* **52**: 142-145.
5. Robbins, P.W., C. Albright, and B. Benfield. 1988. Cloning and expression of a *Streptomyces plicatus* chitinase(chitinase-63) in *Escherichi coli*. *J. Bacteriol.* **263**: 443-447.
6. Watanabe, T., K. Suzuki, W. Oyanagi, K. Ohnishi, and H. Tanaka. 1990. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* **172**: 4017-4022.
7. Pedraza-Reyes, M.and E.Lopez-Romero. 1989. Purification and some properties of two forms of chitinase from mycelial cells of *Mucor rouxii*. *J. General Microbiol.* **145**: 211-218.
8. Correa, J.U., N. Elango, I. Polacheek, and E. Cabib. 1982. Endochitinase, a mannan-associated enzyme from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **257**: 1392-1397.
9. Boller, T. 1985. Induction of hydrolases as a defense reaction against pathogens. Pp. 247-262. *In* J.L. Key and T. Kosuge(ed.), *Cellular and Molecular Biology of Plant Stress*. Alan R. Liss. Inc., New York.
10. Samac, D.A., C.M. Hironaka, P.E. Yallaly, and D.M. Shah. 1990. Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*. *Plant Physiol.* **93**: 907-914.
11. Keefe, D., U. Hinz, and F. Meins Jr. 1990. The effect of ethylene on the cell-type specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. *Planta* **182**: 43-51.
12. Joosten, M.H.A.J. and P.J.G.M. De Wit. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum*(syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. *Plant Physiol.* **89**: 945-951.
13. Hamamura, Y., and Y. Kanehara. 1940. Enzymatic studies on exuvial fluid of *Bombyx mori*. II. Chitinase. *J. Agric. Chem. Soc. Jpn.* **16**: 907-909.
14. Koga, D., J. Jilka, and K.J. Kramer. 1983. Insect endochitinases: glycoproteins from moulting fluid, integument and pupal hemolymph. *Insect Biochem.* **13**: 295-305.
15. Lunt, M.R., and P.W. Kent. 1960. Chitinase system from *Carcinus maenas*. *Biochim. Biophys. Acta* **44**: 371-373.
16. Danulat, E. 1986. Origin of chitinase activity in cod, *Gadus morhua*, p. 241-243. *In* R. Muzzarelli, C. Jeuniaux, and G. W. Gooday(ed.), *Chitin in Nature and Technology*. Plenum Press, New York.
17. Aronson, N.N. Jr., M. Backes, and M.J. Kuranda. 1989. Rat liver chitobiase: purification, properties, and role in the lysosomal degradation of asn-linked glycoproteins. *Arch. Biochem. Biophys.* **272**: 290-300.
18. Jones, J.D.G., K.L. Grady, T.V. Suslow, and J.R. Bedbrook. 1986. Isolation and charaterization of genes encoding two chitinase enzymes from *Serratia marcescens*. *The EMBO Journal* **5**: 467-473.
19. Fuchs, R.L., S.A. Mcpherson, and D.J. Drahos. 1986. Cloning of *Serratia marcescens* gene encoding chitinase. *Appl. Environ. Microbiol.* **51**: 504-509.
20. Hwang, J.R., S.W. Gal, K.A. Lee, Y.C. Shin, M.J. Cho, and S.Y. Lee. 1991. Identification of five chitinase isozymes from *Serratia marcescens*. *Korean Biochem. J.* **24**: 264-270.
21. Chang, K.I., K.S. Kim, M.J. Cho, S.Y. Lee, and Y.C. Shin. 1992. Molecular clonining of *Serratia marcescens* chitinase gene into *Escherichia coli*. *Kor. J. Appl. Microbiol. Biotechnol.* **20**: 129-135.
22. Ordentlich, A., Y. Elad, and I. Chet. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathol.* **78**: 84-87.
23. Roberts, W. and C.P. Selitrennikoff. 1988. Plant and bacterial chitinases differ in antifungal activity. *J. General Microbiol.* **134**: 169-176.
24. Mark, H.H. and P. Dunsnueir. 1988. Nucleotide sequence of the chitinas B gene of *Serratia marcescens* QMB 1466. *Nucleic Acids Res.* **17**: 5395.
25. Mitchell, R. and M. Alexander. 1962. Microbial processes associated with the use of chitin for biological control. *Soil Sci. Soc. Am. Proc.* **26**: 556-558.
26. Shapira, R., A. Ordentlich, I. Chet, and A. Oppenheim. 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathol.* **79**: 1246-1249.
27. Lund,P., R.Y. Lee, and P. Dunsmuir. 1989. Bacterial chitinase is modified and secreted in transgenic tobacco. *Plant Physiol.* **91**: 130-135.
28. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning, A laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York.
29. Cohen, S.N., A.C.Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bateria: genetic transformation of *Escherichia coli* by R

factor DNA. *Proc. Natl. Acad. Sci. USA.* **69**: 2110-2114.
30. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
31. Trudel, J. and A. Asselin. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* **178**: 362-366.
32. Cornelis, P., C. Digneff and K. Willemot, 1982. Cloning and expression of a *Bacillus coagulans* amylase gene in *Escherichia coli*. *Mol. Gen. Genet.* **186**: 507-511.
33. Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* **195**: 19-23.
34. Watanabe, T., W. Oyanagi, K. Suzuki, K. Ohnishi, and H. Tanaka. 1992. Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to the other prokaryotic chitinases ans class III plant chitinases. *J. Bacteriol.* **174**: 408-414.
35. Watanabe, T., K. Suzuki, W. Oyanagi, K. Ohnishi, and H. Tanaka. 1990. Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. *J. Biol. Chem.* **265**: 15659-15665.
36. Watanabe, T., W. Oyanagi, K. Suzuki, and H. Tanaka. 1990. Chitnase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* **172**: 4017-4022.

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