Joural of Microbiology and Biotechnology
Vol. 5, No. 1, 26-30, 1995

Internal Cleavage of *Bacillus subtilis* BSE616 Endo-β-1,4-glucanase expressed in *Escherichia coli*

KIM, HOON*, SUNGMIN F. KIM[1], DONG HO AHN[1], JIN HO LEE[1] AND MOO YOUNG PACK[1]

Department of Agricultural Chemistry, Sunchon National University, Sunchon, 540-742, Korea, and [1]Department of Biotechnology, KAIST, Taejon, 305-338, Korea

The cytoplasmic endo-β-1,4-glucanase (endoglucanase) was purified from cell extracts of *Escherichia coli* (pBS1) transformant carrying the *Bacillus subtilis* endo-β-1,4-glucanase gene after full growth, and its molecular weight was found to be 52 kilodaltons (kDa). The endo-β-1,4-glucanase isolated from the periplasmic space was smaller than 52-kDa cytoplasmic enzyme. The 52-kDa endoglucanase was found to be cleaved in the periplasm and finally converted to 34.5-kDa protein. Small amounts of both 52-kDa and 34.5-kDa proteins were secreted into the culture broth. The cleavage took place in the C-terminal portion of the enzyme. The N-terminal amino acid residues of both 52-kDa and 34.5-kDa enzymes were determined to be the same, Ala, the 30th residue of the primary translation product. Cleavage of the C-terminal portion showed to have no significant effect on the basic enzyme properties.

Bacillus species have several advantages for the production of useful proteins because of their apathogenecity and high secretion capacity (20). Among them, *Bacillus subtilis* has been extensively studied for the secretion of endogeneous and heterologous proteins. Several endoglucanase genes have recently been cloned and characterized from *Bacillus subtilis* strains DLG (18, 19), PAP115 (10, 12), IFO3034 (7, 14), and BSE616 (16, 17). These four endoglucanase genes show high degree of homology, over 93% with one another, and they all encode proteins composed of 499 amino acid residues. For *B. subtilis* DLG, intracellular endoglucanase is initially translated as a large (ca. 51.5 kDa) precursor, however, the mature exocellular enzyme is 35.2 kDa. *Escherichia coli* transformants carrying the *B. subtilis* DLG endoglucanase gene produce an intracellular form (51 kDa) and a cell-associated form of the enzyme (39 kDa) (18, 19). *B. subtilis* PAP115 and *E. coli* transformants produce active extracellular endoglucanases of 27~40 kDa and 30~49 kDa, respectively (10, 12).

Our previous works showed that the purified *B. subtilis* BSE616 endoglucanase overproduced in *B. megaterium* (pCK98) transformant had a molecualr weight of 33 kDa (5) and it was the final product of a progressive C-terminal cleavage of the secreted endoglucanase in *B. megaterium* transformant (6). Recently, we have reported that a proteolytic enzyme was responsible for the cleavage of the enzyme (1). We have also purified the protease from *B. megaterium* (1). Here, we present the localization and internal cleavage pattern of the endoglucanase produced in a *E. coli* transformant and the comparison of the cleaved products with those of *B. megaterium* and *B. subtilis* BSE616.

*Corresponding author
Key words: Internal cleavage, *Bacillus* endoglucanase, Recombinant *Escherichia coli*

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions.

The bacterial strains used in this study were *B. subtilis BSE616* and *E. coli* (pBS1) (16). *E. coli* (pBS1) strain carries *B. subtilis* BSE616 endo-β-1,4-glucanase gene in *E. coli* HB101. *E. coli* (pBS1) was grown at 37°C in LB medium containing 15 μg/ml of tetracycline. *B. subtilis* BSE616 was grown at 37°C with vigorous agitation in the modified GS medium (18) containing 0.5% glucose. *B. megaterium* (pCK98) transformant was used for the comparison of the cleavage patterns of the gene products.

Preparation and Purification of Endo-β-1,4-glucanase.

For the preparation of the periplasmic enzyme, *E. coli* (pBS1) cells were harvested by centrifugation after full growth and washed with cold 10 mM Tris/HCl buffer, pH 8.0. The cells were suspended in 10 mM Tris/HCl-20% sucrose buffer containing 1 mM EDTA, pH 8.0 (80 ml/g wet cell), and shaken for 10 min at room tem-

perature (15). The suspension was centrifuged and resuspended in ice-cold water (80 ml/g wet cell). After 10 min shake, the cells were collected by centrifugation at 9000 g for 10 min and was used for the preparation of the cytoplasmic enzyme. The supernatant was concentrated by ultrafiltration (PM-10, Amicon, USA) and used for preparation of the periplasmic enzyme.

The collected cells were resuspended in a minimal volume of 50 mM sodium citrate buffer (pH 5.5) and sonicated for 5 min with Branson Sonifier (Model 350) at 40% output. Cell debris was removed by centrifugation for 15 min at 9,000 g, and the supernatant was precipitated with 40~80% ammonium sulfate. The crude cytoplasmic enzyme preparation was dialyzed against a large volume of 50 mM Tris/HCl buffer, pH 7.5, applied onto a Sephadex G-75 column (2.4 × 74 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.5, eluted with the same buffer (20 ml/h). Fractions containing the major enzyme activity were chromatographed on a SP-Sephadex C-50 column (2.8 × 40 cm) with a linear gradient of NaCl (0~1.0 M) in 50 mM of sodium citrate buffer, pH 5.0. The enzyme was further purified by Bio-Gel HT hydroxylapatite chromatography. The column (2.0 × 10 cm) was equilibrated with 10 mM phosphate buffer, pH 7.0, and the enzyme was eluted at a rate of 18 ml/h with linearly increasing concentration of phosphate buffer (10~400 mM, pH 7.0). The periplasmic enzyme was purified by two-step chromatography, Sephadex G-75 and SP-Sephadex C-50, as described above. Fractions with endo-β-1,4-glucanase activity were pooled, dialyzed against 50 mM sodium citrate buffer (pH 5.5), and concentrated by ultrafiltration.

To prepare the total cell extract, *E. coli* (pBS1) cells were harvested, washed with 50 mM sodium citrate buffer, pH 5.5, and resuspended in a minimal volume of the sodium citrate buffer. The cell suspension was sonicated for 3 min and cell debris was removed by centrifugation.

Western Blotting.

The purified cytoplasmic endo-β-1,4-glucanase was used to produce antibody. The antisera of rabbits were prepared as described previously (6). *E. coli* (pBS1) was grown and harvested by centrifugation at various time intervals as indicated. The cells were fractionated into two portions, periplasmic and cytoplasimc, by osmotic-shock and sonication method as described above. Proteins in each culture supernatants were precipitated with acetone (final concentration 60%, v/v). The protein pellets were electrophoresed according to Laemmli (8) and used for Western blot. Western blotting was performed according to Harlow and Lane (4) using alkaline phosphatase-conjugated secondary antibody (Promega, USA).

Endo-β-1,4-glucanase Assay.

Enzyme activity was determined by measuring reducing sugars released from carboxymethyl-cellulose (CMC) by the dinitrosalicylic acid method (13). One unit of the enzyme activity was defined as the amount of enzyme that catalyzed liberation of 1 μmol glucose equivalent per min.

Protein Analysis.

The N-terminal sequences of 52 kDa and 34.5 kDa enzymes were determined by an automatic amino acid sequencer (Millipore). Molecular weights of the enzymes were determined by 12% polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecylsulfate (SDS) according to Laemmli (8). Protein concentrations were determined according to Lowry et al. (11).

RESULTS AND DISCUSSION

Enzyme Purification.

Most of the endoglucanase produced remained within the *E. coli* (pBS1) cells. The intracellular enzyme of the *E. coli* (pBS1) transformant was distributed almost equally in periplasmic and cytoplasmic compartments of the cell (16). Only about 5% of the total endoglucanase was secreted into the culture medium. Robson and Chambliss have also reported that only a negligible amount of the enzyme was found in the culture medium (18). However, about 25% of the activity of the cloned endoglucanase from *B. subtilis* IFO3034 was found in the extracellular fraction of *E. coli* (7), and even higher degree, about 70%, of secretion of *B. subtilis* PAP115 endoglucanase was reported in *E. coli* (pC6.3) (10). Lo et al. suggested that the differences are the result of an intrinsic property of either the endoglucanase protein or the host (10). However, the exact reason for the difference is not known at this time.

When the cytoplasmic extract was fractionated on a Sephadex G-75 column, two activity peaks, one major and one minor, were observed (Fig. 1). The fractions containing major endoglucanase activity (peak II) was purified by SP-Sephadex C-50 column chromatography. The enzyme was eluted with 0.30~0.45 M of NaCl on the column. The endoglucanase was further purified by Bio-Gel HT column chromatography. The enzyme was eluted with 70~90 mM of phosphate buffer. The purified cytoplasmic enzyme was found to be homogeneous by SDS-PAGE (Fig. 2, lane A).

The periplasmic enzyme could be purified by a two-step chromatography using Sephadex G-75 and SP-Sephadex C-50 as described above. The chromatographic elution pattern of the enzyme activity was similar to that of the cytoplasmic enzyme. However, the mag-

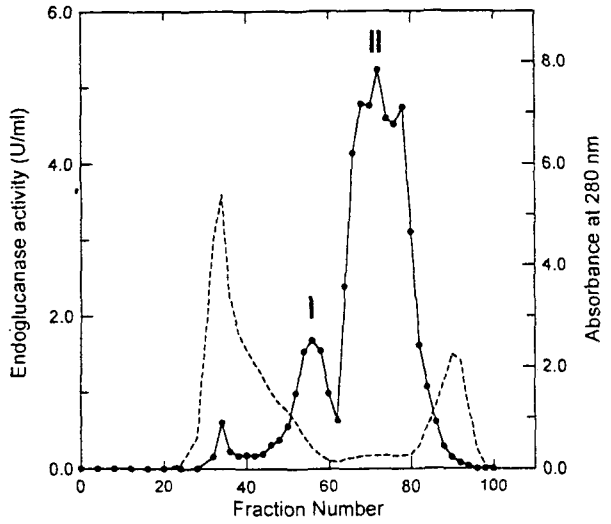

Fig. 1. Chromatogram of the cytoplasmic endoglucanase of *E. coli* (pBS1) by Sephadex G-75. ●—●, endoglucanase activity; ——, absorbance at 280 nm.

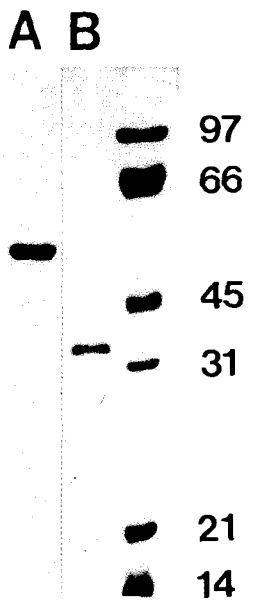

Fig. 2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the determination of molecular weights of the purified endoglucanases.
Lane A, the cytoplasmic endoglucanase (52 kDa); B, the cleaved endoglucanase from periplasmic space (34.5 kDa). The 52 kDa (lane A) and the cleaved 34.5 kDa (lane B) endoglucanases were purified from peak II of Sephadex G-75 chromatography using the cytoplasmic source and from peak I of Sephadex G-75 chromatography using the periplasmic source, respectively. Numbers in the right margin are molecular weights in kDa.

nitude of activity peak I portion was much higher, about 4 times, than that of the cytoplasmic sample (data not shown). By comparing the ratio of the amounts of the cytoplasmic and the periplasmic enzymes, it is thought that the peak I could be that of the cleaved endoglucanase which is shorter than the cytoplasmic protein. The activity peak I was found to be homogeneous by SP-Sephadex C-50 column chromatography (data not shown) and SDS-PAGE (Fig. 2, lane B).

Molecular Weights of the Purified Endoglucanases.

Molecular weight of the purified cytoplasmic enzyme was estimated to be 52 kDa by SDS-PAGE (Fig. 2). The purified enzyme was homogeneous enough to prepare polyclonal antibody. The molecular weight of the primary translation product expected from the nucleotide sequence of the B. subtilis BSE616 endoglucanase gene is about 55 kDa (17), indicating that the primary translation product would be cleaved at signal sequence region during the transport of the protein. The molecular weight of cleaved protein in the periplasm was found to be 34.5 kDa by SDS-PAGE (Fig. 2).

Immunoblotting.

The transport and secretion of the endoglucanase in *E. coli* (pBS1) was investigated by immunoblotting. During the cell growth from 6 to 21 h, only 52-kDa protein was found in the cytoplasm (Fig. 3A). The amount of 52-kDa protein in the cytoplasm was continuously increased until 15 h of growth and no further increase was observed. In the periplasm, only 52-kDa protein was detected at the initial stage of cell growth until 9 h, but after 12 h of growth, one additional protein band corresponding to 34.5 kDa was observed (Fig. 3B). In the culture supernatant, two protein bands which correspond to the periplasmic protein bands were detected (Fig. 3C). In the periplasm and supernatant, the amount of the larger protein was more significant, at least 5 times, than that of the smaller protein. It seems that the ratio of the quantity of the larger to the smaller protein was not changed indicating that no extra cleavage of protein occurred during or after the protein transport via outer membrane. Similar results were reported which indicate that the secreted endoglucanase was apparently processed to about 36-kDa and 43-kDa proteins in *Saccharomyces cerevisiae* transformant having complete signal sequence of *B. subtilis* BSE616 (3).

B. subtilis BSE616, the endoglucanase gene donor, produced only extracellular endoglucanases. In this case, three protein bands were observed, corresponding to 52, 43.5, and 36.5 kDa, respectively (Fig. 4). This result was similar to that of *B. megaterium* (pCK98) transformant experiment (6), but was different in that the rate of cleavage of *B. subtilis* BSE616 enzyme was slower than that of *B. megaterium* (pCK98). That is, the ratio of the quantity of the larger protein, 52 kDa, to the smaller proteins, 43.5 and 36.5 kDa, was much higher in *B. subtilis* BSE616 than in *B. megaterium* (pCK98) after 21 h of growth. Even then, the 52-kDa protein band was much more prominent than the bands of two smaller proteins.

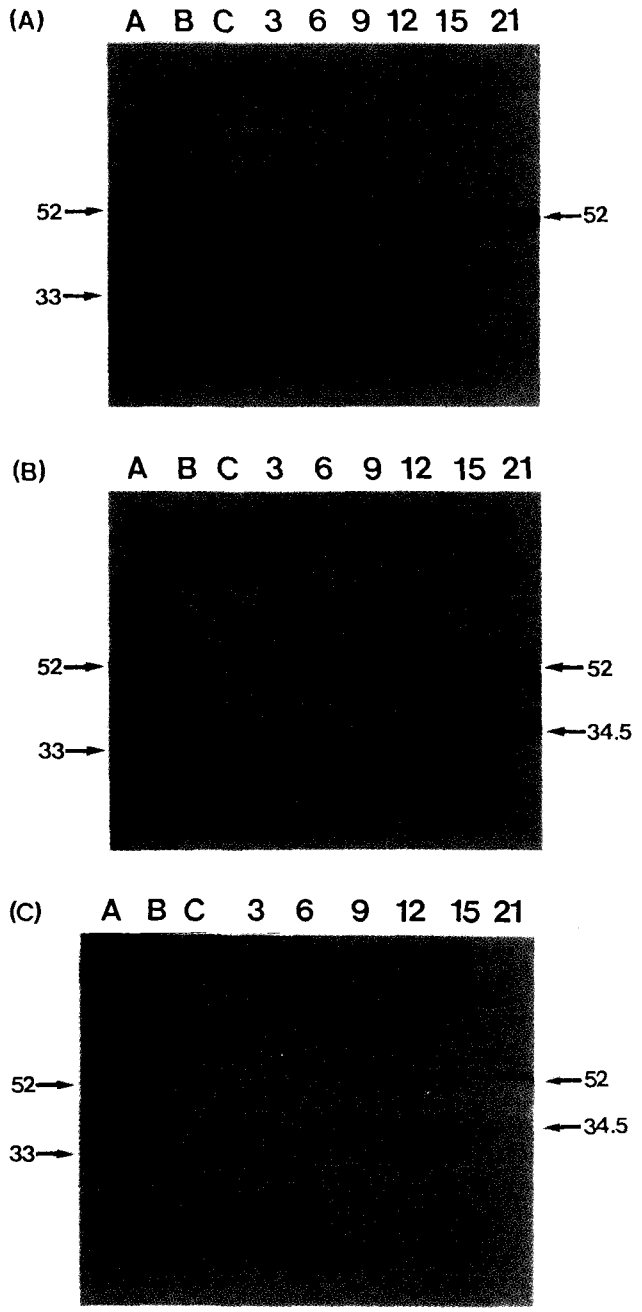

Fig. 3. Western blot analysis for the endoglucanase produced in *E. coli* (pBS1) and secreted into the culture medium with anti-52 kDa endoglucanase as a function of time.
At the indicated times (in hour), cells were withdrawn from the culture and fractionated as described in the text. (A), the cytoplasmic; (B), the periplasmic; (C), the extracellular sample. Lane A, *E. coli* HB101 extract as a control; B, the purified 52 kDa endoglucanase from *E. coli* (pBS1) as a size marker; C, the purified 33 kDa endoglucanase from *B. megaterium* (pCK98) as a size marker. Numbers above each figures indicate the sampling time of *E. coli* (pBS1).

With *B. megaterium* (pCK98) transformant, the smallest protein, 33 kDa, was the predominant product (6). At early log phase, 6 h, the *B. megaterium* (pCK98) endoglucanase was secreted as a 52-kDa protein, and then progressively cleaved into smaller proteins in the culture

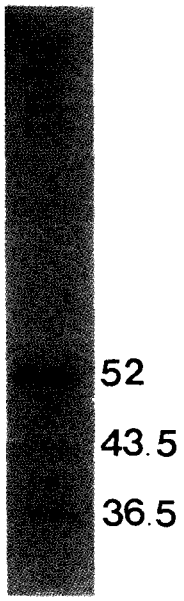

Fig. 4. Western blot analysis for the endoglucanase secreted from *B. subtilis* BSE616 after 21 h of growth.
Numbers in the right margin are molecular weights in kDa.

broth during further growth. At about 9 h, the smaller proteins (44, 42 and 41 kDa) were found in the culture broth, and these proteins were further processed to the final product, 33-kDa protein, in the culture broth after 21 h of growth.

N-terminal Sequence Analysis.

The amino acid sequence of the N-terminus of the 52-kDa protein of *E. coli* (pBS1) was determined to be Ala-Gly-Thr-(?)-Thr-(Pro)-Val-. The N-terminus amino acid sequence of the 34.5-kDa protein from *E. coli* (pBS1), Ala-Gly-Thr-Lys-Thr-Pro-Val-Ala-, was the same as that of 52-kDa protein. This result could have been predicted from the experiment with *B. megaterium* (pCK98) (6). With the deduced amino acid sequence (17), it could also be concluded that the signal peptide is composed of 29 amino acid residues and the N-terminal amino acid of both 52-and 34.5-kDa proteins is Ala-30 in *E. coli* (pBS1). The endoglucanase precursor (55 kDa) undergoes signal sequence processing (about 3 kDa) and the 52-kDa protein appears in the periplasmic space and in the culture medium. The difference in the size of proteins might be due to the significant C-terminal cleavage of the protein.

It is suggested that the primary translation product is transported through the membrane via cleavage of the signal sequence, and the secreted enzyme is further cleaved by progressive removal of its C-terminal portion by proteolytic cleavage.

Enzymatic Properties.

The cleavage of the C-terminal portion did not affect the enzyme properties since the enzymatic properties

Table 1. Properties of 52 kDa and 34.5 kDa endoglucanases (EG) purified from *E. coli* (pBS1) transformant

	52 kDa EG	34.5 kDa EG
Km for CMC[a]	0.14%	0.16%
Km for PNPC[b]	2.6 mM	2.8 mM
Optimum pH[c]	6.0	6.0
Optimum temperature[d]	60°C	60°C
Heat stability[e]	52%	48%
Inhibition by 50 mM cellobiose[f]	43%	50%

[a]The values were determined using 0.1~2.0% of CMC as a substrate. [b]The values were determined using 1.0~10 mM PNPC as a substrate. Reactions were carried out at 40°C. After 30 min, 3 ml of 1 M Na_2CO_3 solution was added to the reaction mixtures and then the absorbance was measured at 400 nm. [c]Enzyme activity at pH 5.5 was 95% of the maximum activity at pH 6.0. [d]Enzyme activity was measured in a standard assay condition. [e]Remaining enzyme activity was measured after preincubation of the enzyme without the substrate for 30 min at 60°C. [f]The values represent the degree of inhibition when 50 mM cellobiose was added to the reaction mixtures.

of 52-and 34.5-kDa proteins were very similar to each other when the affinity for substrates, CMC and p-nitrophenyl cellobioside (PNPC), optimum pH, optimum temperature, heat stability, and sensitivity to inhibition by cellobiose were investigated (Table 1). However, the C-terminal portion of the endoglucanase seems to have its own function when the enzyme acts on native substrates such as microcrystalline cellulose (manuscript in preparation).

Acknowledgment.

This research was supported by the Korea Science and Engineering Foundation grant (893-0301-007-2, 1989-1991) and in part by Non Directed Research Fund, Korea Research Foundation (1990~1992), for which authors are very grateful.

REFERENCES

1. Ahn, D.H., H. Kim, and M.Y. Pack. 1993. Cleavage of Bacillus *subtilis* endo-β-1,4-glucanase by *B. megaterium* protease. *Biotechnol. Lett.* **15**: 127-132.
2. Ferrari, E., A.S. Jarnagin, and B.F. Schmidt. 1993. Commercial production of extracellular enzymes, P. 917-937. *In* Sonenshein, A.L., J.A. Hoch and R. Losick (ed.), *Bacillus subtilis* and *other Gram-positive bacteria*: Biochemistry, physiology, and molecular genetics, American Society for Microbiology, Washington, D.C.
3. Han, Y.J., D.O. Kang, S.C. Lee, B.Y. Kim, H.H Suh, J.M. Kim, T.I. Mheen, and J.S Ahn. 1994. Secretion of *Bacillus* endoglucanase in *Saccharomyces cerevisiae* by its own signal sequence. *J. Microbiol. Biotechnol.* **4**: 24-29.
4. Harlow, E., and D. Lane. 1988. Immunoblotting, p. 471-510. *In Antibodies*: A Laboratory Manual. Cold Spring Harbor, New York.
5. Kim, H., and M.Y. Pack. 1988. Endo-β-1,4-glucanase encoded by *Bacillus subtilis* gene cloned in *Bacillus megaterium*. *Enzyme Microb. Technol.* **10**: 347-351.
6. Kim, H., S.F. Kim, and M.Y. Pack. 1991. C-terminal processing of *Bacillus subtilis* BSE616 endo-β-1,4-glucanase in *Bacillus megaterium*. *Biotechnol. Lett.* **13**: 799-804.
7. Koide, Y., A. Nakamura, T. Uozumi, and T. Beppu. 1986. Molecular cloning of a cellulase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. *Agric. Biol. Chem.* **50**: 233-237.
8. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
9. Lee, D.S., and M.Y. Pack. 1987. Use of Bacilli for overproduction of exocellular endo-β-1,4-glucanase encoded by cloned gene. *Enzyme Microb. Technol.* **9**: 594-597.
10. Lo, A.C., R.M. Mackay, V.L. Seligy, and G.E. Willick. 1988. *Bacillus subtilis* β-1,4-endoglucanase products from intact and truncated genes are secreted into the extracellular medium by *Escherichia coli*. *Appl. Environ. Microbiol.* **54**: 2287-2292.
11. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
12. Mackay, R.M., A. Lo, G. Willick, M. Zuker, S. Baird, M. Dove, F. Moranelli, and V. Seligy. 1986. Structure of a *Bacillus subtilis* endo-β-1,4-glucanase gene. *Nucleic Acids Res.* **14**: 9159-9170.
13. Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination reducing sugar. *Anal. Chem.* **31**: 426-428.
14. Nakamura, A., T. Uozumi, and T. Beppu. 1987. Nucleotide sequence of a cellulase gene of *Bacillus subtilis*. *Eur. J. Biochem.* **164**: 317-320.
15. Neu, H.C., and L.A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplast. *J. Biol. Chem.* **240**: 3685-3692.
16. Park, S.H., and M.Y. Pack. 1986. Cloning and expression of a *Bacillus subtilis* cellulase gene in *Escherichia coli*. *Enzyme Microb. Technol.* **8**: 725-728.
17. Park, S.H., H.K. Kim, and M.Y. Pack. 1991. Characterization and structure of the cellulase gene of *Bacillus subtilis* BSE616. *Agric. Biol. Chem.* **55**: 441-448.
18. Robson, L.M., and G.H. Chambliss. 1986. Cloning of the *Bacillus subtilis* DLG β-1,4-glucanase gene and its expression in *Escherichia coli* and *B. subtilis*. *J. Bacteriol.* **165**: 612-619.
19. Robson, L.M., and G.H. Chambliss. 1987. Endo-β-1,4-glucanase gene of *Bacillus subtilis* DLG. *J. Bacteriol.* **169**: 2017-2025.
20. Simonen, M., and I. Palva. 1993. Protein secretion in *Bacillus* species. *Mirobiol. Rev.* **57**: 109-137.

(Received September 29, 1994)