Expression in *Escherichia coli* of the *Bacillus circulans* WL-12 Structural Gene for β-1,3-Glucanase A

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***Bacillus circulans* WL-12, a yeast and fungal cell wall lytic bacterium, secretes a variety of polysaccharide degrading enzymes into the culture medium. When β-1,3-glucanase was induced with pachyman, a β-1,3-glucose polymer obtained from the tree fungus *Poria cocus* Wolf, six distinct active molecules of the enzyme with different molecular weights were detected in the culture supernatant of this bacterium. Molecular cloning of one of the β-1,3-glucanase genes into *E. coli* was achieved by transforming *E. coli* HB101 cells with recombinant plasmids composed of chromosomal DNA fragments prepared from *B. circulans* WL-12 and the plasmid vector pUC 19. A recombinant plasmid containing 4.4 kb of inserted DNA in the *Pst*I site of pUC 19, designated as pNT003, conferred the ability to degrade pachyman on *E. coli* cells. The presence of pNT003 was harmful for *E. coli* cells and caused cell lysis, especially at higher temperatures of cultivation. β-1,3-Glucanase activity detected in *E. coli* was mainly recovered in the periplasmic fraction when cell lysis did not occur. SDS-PAGE analysis revealed that the periplasmic fraction contained four active molecules of β-1,3-glucanase which corresponded to four of the six active molecules produced by *B. circulans* WL-12.**

Bacillus circulans WL-12 has been isolated from soil as a yeast cell wall lytic bacterium. Earlier studies have shown that the bacterium is able to lyse the cell walls derived from a variety of yeast and fungal species such as *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Hansenula ciferrii*, *Candida parapsilosis*, *Pyricularia oryzae*, *Aspergillus oryzae*, and *Neurospora crassa*.[1,2] The cell walls of yeasts and fungi have complex structures and their components and compositions vary among species.[3] These facts indicate that the enzyme system of the bacterium involved in the cell wall lysis must be highly complex and versatile. In fact, a number of polysaccharide-hydrolyzing enzymes have been shown to be secreted by this bacterium. The production of β-1,3-glucanase, β-1,6-glucanase, xylanase, and chitinase have been reported.[4～6] Amylase and α-1,3-glucanase have also shown to be produced by the bacterium (unpublish data). Among these enzymes, β-1,3-glucanase has been suggested to be multiple and the ratio of these β-1,3-glucanases to be varied depending on the inducer substrates.[1]

Analysis of gene structures and organizations will provide important information necessary for understanding this complex enzyme system. In this paper, we report the cloning and expression of one of the β-1,3-glucanase genes of *B. circulans* WL-12 in *E. coli*.

Materials and Methods

Bacterial strains and media. Bacillus circulans WL-12[1] was grown in Yeast-Nitrogen-Base (YNB) medium (Difco Laboratories Inc., Detroit, Michigan) for enzyme

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production and in L-broth for extraction of DNA. *Escherichia coli* HB101 was used as a cloning host. For the isolation of an *E. coli* clone carrying the β-1,3-glucanase gene, L-broth agar plates containing 0.4% (w/v) of pachyman and 50 μg/ml of ampicillin were used.

Preparation of crude enzyme from culture fluid of B. circulans WL-12. Cells of *B. circulans* WL-12 were grown at 30°C for 72 hr in the YNB medium containing 0.4% of pachyman as described above. After centrifugation to remove cells and debris, the supernatant fluid was concentrated to about 1/5 of the volume in a rotary vacuum evaporator. The concentrated fluid was dialyzed against 0.02 M succinate buffer, pH 6.0, and centrifuged. Ammonium sulfate was added to achieve 80% saturation. The precipitate was dissolved in 0.02 M succinate buffer, pH 6.0, and dialyzed overnight against the same buffer.

Isoelectric focusing. Activity profiles of the crude enzymes were obtained with Ampholine Electrofocusing Equipment (LKB8100-10, LKB-Producer AB, Sweden) by the method described in the LKB instruction manual. A sucrose density gradient (5~50%) was established using an Ampholine Gradient Mixer (LKB8121). The concentration of carrier ampholyte was 1.3%. The sample was layered in the middle part of the column during the formation of the sucrose gradient, and isoelectric focusing was done at 700 V for 48 hr.

Construction of recombinant plasmid. Chromosomal DNA was prepared from *B. circulans* WL-12 by the method of Silhavy *et al.*[7] The chromosomal DNA was digested with *Pst*I, and 2- to 10-kb DNA fragments were collected by agarose gel electrophoresis and ligated to dephosphorylated-*Pst*I cut pUC19. *E. coli* HB101 was transformed with the ligated DNA and selected on L-broth agar plates containing 50 μg/ml of ampicillin. Ampicillin-resistant transformants were transferred onto L-broth agar plates containing 0.4% (w/v) of pachyman and 50 μg/ml of ampicillin. The plates were incubated at 37°C for 24 to 36 hr. Production of β-1,3-glucanase was judged by the formation of a visible clearing zone around the colony.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis in 10% slabs was done as described by Ames[8] using the buffer system of Laemmli.[9] After the electrophoresis was completed, renaturation of enzymes in the gel was done by the method of Blank *et al.*[10] with minor modifications as follows. SDS was removed from the gel by washing twice for 30 min in 0.1 M succinate buffer, pH 6.0, containing 25% isopropanol at room temperature on a slowly rotating platform shaker. Then, the gel was washed twice for 30 min in the same buffer without isopropanol. The gel was transferred onto a glass plate and excess liquid on the gel surface was removed. Detection of β-1,3-glucanase activity in the polyacrylamide gel was done by the method of Beguin which was originally designed for the detection of cellulase activity.[11] The gel was placed on a 2% agarose gel sheet 1.2 mm thick containing 0.1% pachyman and 0.1 M succinate buffer, pH 6.0, prepared on a glass plate. The polyacrylamide gel and the agarose gel sheet on the glass plate was gently wrapped and incubated for 1 to 3 hr at 37°C. The polyacrylamide gel was torn off from the agarose gel sheet and proteins remaining in the gel were stained with Coomassie brilliant blue R-250. The agarose gel sheet was dipped into 0.1% Congo red and stained for 30 min with gentle shaking. The Congo red was poured off and the gel sheet was washed with 1 M sodium chloride until excess stain was removed.

Subcellular fractionation of E. coli. E. coli HB101 carrying a recombinant plasmid was grown until O.D. (600 nm) = 0.7 in 5 ml of L-broth containing 50 μg/ml ampicillin at 33°C as a preculture. All of the preculture was inoculated into 200 ml of L-broth containing ampicillin and incubated. At a certain incubation time, cells were collected by centrifugation, and the cytoplasmic and periplasmic fractions were prepared by the method of Tsukagoshi *et al.*[12] The method is based on the treatment with lysozyme-EDTA to form spheroplasts as described by Birdsell *et al.*[13]

For the analysis of β-1,3-glucanase by SDS-PAGE, *E. coli* cells were grown at 30°C for 24 hr and the periplasmic fraction was prepared by the cold osmotic shock procedure of Neu *et al.*[14]

Deletion analysis of recombinant plasmid. Sequential deletions were introduced into the inserted DNA region of the recombinant plasmid using a Deletion Kit purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) by the method described in the instruction manual. This procedure is based on the methods of Yanisch-Perron *et al.*[15] and Henikoff.[16]

Enzyme assay. The assay for β-1,3-glucanase was done as described previously using laminarin as a substrate.[2] β-Galactosidase activity was measured by the method of Pardee *et al.*[17] β-Lactamase was assayed by the method of Sargent.[18]

Chemicals. Laminarin was prepared from *Laminaria japonica* by the method of Black *et al.*[19] with some modifications. Restriction enzymes and modification enzymes for genetic manipulation were purchased from Toyobo Co., Ltd. (Osaka, Japan).

Results

β-1,3-Glucanase produced by B. circulans WL-12

The production of multiple β-1,3-glucanases by *B. circulans* WL-12 have been suggested

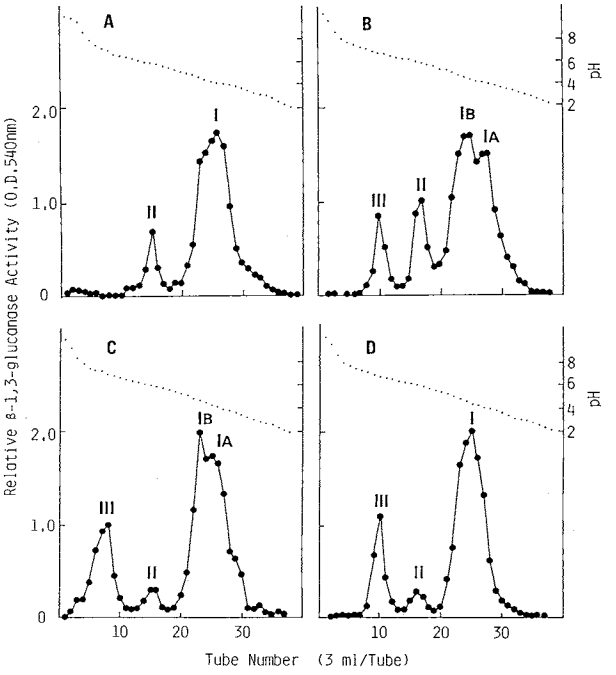

Fig. 1. Changes in the Activity Profile of β-1,3-Glucanase Obtained by Isoelectric Focusing during Cultivation.

Cells of *B. circulans* WL-12 were grown in YNB medium containing 0.4% pachyman. Samples of culture were withdrawn at 2 (A), 3 (B), 4 (C), and 5 (D) days of cultivation. Crude enzymes were prepared and analyzed by isoelectric focusing. ●, relative β-1,3-glucanase activity; · · ·, pH.

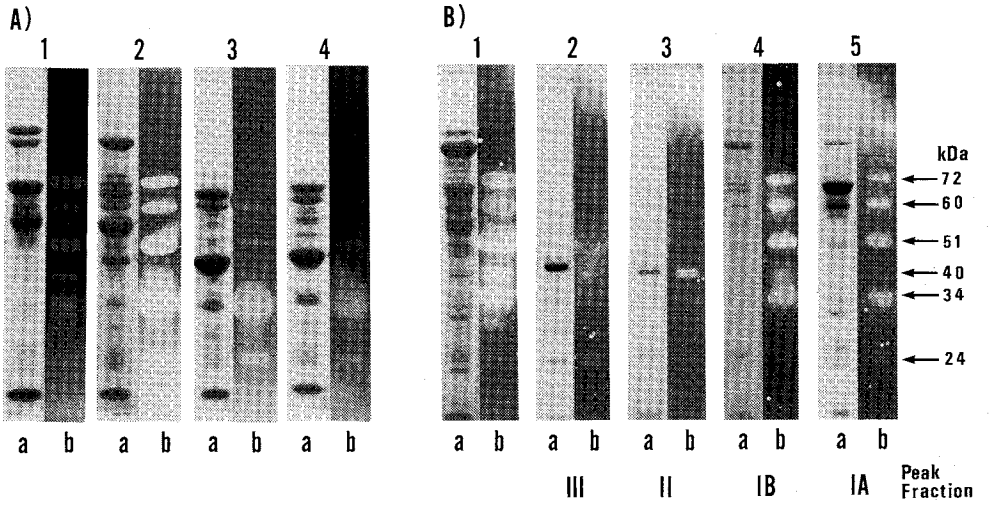

Fig. 2. A) SDS-Polyacrylamide Gel Electrophoresis of Crude Enzymes Prepared from 2 (lane 1), 3 (lane 2), 4 (lane 3), and 5 (lane 4) Day Culture Supernatants of *B. circulans* WL-12.

a, protein staining of polyacrylamide gel with Coomassie brilliant blue R-250; b, β-1,3-glucanase activity detected in agar replica of the polyacrylamide gel.

B) SDS-Polyacrylamide Gel Electrophoresis of Peak Fractions Obtained by Isoelectric Focusing Electrophoresis of Crude Enzyme Prepared from 2 Day Culture Supernatant (Fig. 1B).

Lane 1, total crude enzyme; lane 2, peak III of Fig. 1B; lane 3, peak II, lane 4, peak 1B; lane 5, peak 1A. a, protein staining; b, β-1,3-glucanase activity.

earlier.[1] To test this possibility with an inducer of known chemical structure, pachyman-induced crude enzymes were analyzed by electrofocusing and SDS-PAGE. Crude enzyme prepared from a 2-day-culture supernatant gave two major peaks of β-1,3-glucanase activity at p*I*s around 4 and 6 (Peak I and Peak II in Fig. 1) in isoelectric focusing. At three days of culture, a new peak (Peak III) at p*I* around 7 appeared and Peak I divided into two sub-peaks, Peak IA and Peak IB. Peak II almost disappeared at 4 days of cultivation. SDS-PAGE analysis of crude enzymes is shown in Fig. 2A. Glucanase activity in an polyacrylamide gel was detected by the degradation of pachyman in an agar replica. At least six distinct bands with β-1,3-glucanase activity (72, 60, 51, 40, 34, and 24 kDa) were detected. The intensity of the higher M_r bands decreased during the cultivation and almost disappeared at four days and significant increase of activity in lower M_r bands was observed suggesting that some proteolytic degradation occurred on secreted β-1,3-glucanase. Peak fractions of p*I*=4.0 (Peak IA), 4.4 (IB), 5.9 (II) and 6.7 (III) were collected and analyzed by SDS-PAGE (Fig. 2B). The peak III fraction at p*I*=6.7 gave a major band with a size of approx. 24 kDa and peak II at p*I*=5.9 gave a major band with a size of approx. 40 kDa, although significant cross contamination was observed. On the other hand, at least four glucanase bands were detected on the agar replica in the peak fractions IA and IB at p*I*=4.0 and 4.4. The active bands observed in the fraction IA by SDS-PAGE were almost identical to the fraction IB, except that the fraction IB contained an extra band of 40 kDa. The reason for the separation of the two fractions is unknown. The results described above clearly indicate that *B. circulans* WL-12 is able to produce at least six active molecules of β-1,3-glucanase, one at p*I* around 7, one at p*I* around 6, and four at p*I* around 4.

Cloning of the β-1,3-glucanase gene

*Pst*I-digested chromosomal DNA fragments, 2 to 10 Kb in size, were prepared from *B. circulans* WL-12. The fragments were ligated to *Pst*I-cleaved and alkaline phosphatase treated pUC19, and were used to transform *E. coli* HB101 cells. Ampicillin resistant-transformants (1200) were obtained by a single transformation experiment. All of the transformants were transferred onto L-broth agar plates containing 0.4% pachyman and 50 μg/ml ampicillin. Only one of the transformants had a clearing zone around the clony during two days of incubation at 37°C. The recombinant plasmid, designated pNT003, detected in this transformant was 7.1 kb in size, which contained a 4.4-kb fragment derived from *B. circulans* WL-12 chromosomal DNA. Retransformed *E. coli* HB101 with the isolated pNT003 all acquired the ability to degrade pachyman. HB101 cells without pNT003 never showed clearing zones on L-broth pachyman plates even after treatment with chloroform vapor, indicating that the ability to degrade pachyman was conferred by pNT003.

A restriction map of the recombinant plasmid pNT003 is shown in Fig. 3. The plasmid contains a 4.4-kb fragment derived from *B. circulans* WL-12 chromosomal DNA. A plasmid containing the 4.4-kb fragment in reverse orientation also conferred the ability to degrade pachyman. Thus the cloned β-1,3-glucanase gene is expressed by its own

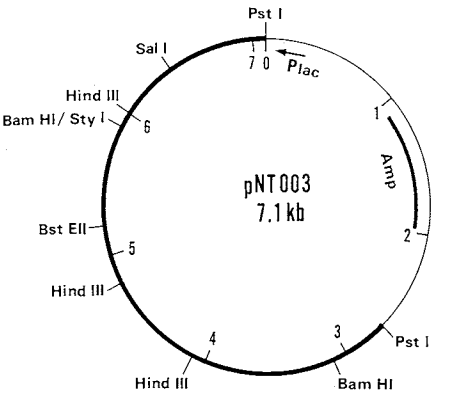

Fig. 3. Physical Map of Recombinant Plasmid pNT003, Obtained from Restriction Endonuclease Digestion and Agarose Gel Electrophoresis of Plasmid DNA.

The numbers indicate the size of the DNA in kb. Amp, β-lactamase gene; Plac, *lac* promoter.

promoter.

Expression of the β-1,3-glucanase gene in E. coli

The β-1,3-glucanase production by *E. coli* HB101 carrying pNT003 during cultivation at 33°C is shown in Fig. 4. Bacterial growth continued until O.D. = 1.6 and then the optical density decreased during further incubation. At higher temperatures, the cessation of growth and the decrease in optical density was observed earlier and at a lower O.D. of the bacterial growth. Under the same conditions, HB101 cells without the plasmid reached an O.D. over 2.0 and did not show any decrease in optical density during incubation. The production of β-1,3-glucanase activity associating with the cells was accompanied by the cell growth. Extracellular activity of β-1,3-glucanase was detected after the cessation of bacterial growth, indicating that the glucanase activity associating with the cell was released into the medium by the lysis of the cells.

β-1,3-Glucanase activity produced in *E. coli*

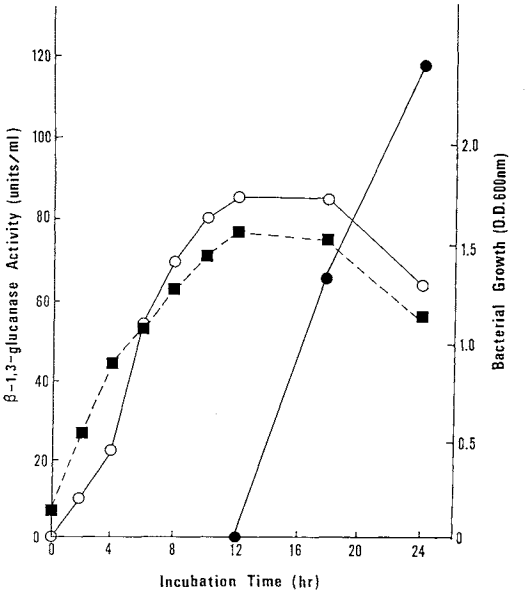

Fig. 4. Production of β-1,3-Glucanase by *E. coli* HB101 Harboring pNT003 during Cultivation.

■, bacterial growth measured by absorbance at 600 nm; ○, β-1,3-glucanase activity associating with the cells; ●, β-1,3-glucanase activity of the culture supernatant.

was subcellularly localized at 6 and 24 hr of cultivation (Table I). About 75% of the activity was detected in the periplasmic fraction, less in the cytoplasmic fraction, and no activity in the culture supernatant at 6 hr of cultivation. The β-galactosidase activity measured as a cytoplasmic marker enzyme was not detected in the culture supernatant. Thus cell lysis did not occur at the time of cultivation. On the other hand, the greater part of the β-1,3-glucanase activity was detected in the culture supernatant at 24 hr of cultivation. The activity associated with the cells was distributed in the periplasmic and cytoplasmic fractions with a similar ratio as at 6 hr cultivation. In addition, 68% percent of the β-galactosidase activity was detected in the culture supernatant at 24 hr of cultivation indicating the lysis of the cells. Thus, the overproduction of β-1,3-glucanase retards the growth rate and causes lysis of the cells at a late stage of bacterial growth.

β-1,3-Glucanase produced in E. coli

Periplasmic proteins were isolated from the *E. coli* HB101 cells carrying the recombinant plasmid pNT003 by a cold osmotic shock procedure[14] and analyzed by SDS-polyacrylamide gel electrophoresis. β-1,3-Glucanase activity in polyacrylamide gel was detected as the pachyman-degrading band formed in the agar replica. Surprisingly, four distinct bands with β-1,3-glucanase activity were observed when periplasmic proteins were analyzed (Fig. 5A). The apparent sizes of these bands were 72, 60, 51, and 34 kDa, respectively. They exactly corresponded to four of

Table I. Localization of the β-1,3-Glucanase Produced by *E. coli* HB101 Harboring pNT003

Subcellular fraction	Cultivation time	
	6 hr	24 hr
Extracellular	ND[a]	71.8 (%)
Periplasmic	74.9 (%)	22.6
Cytoplasmic	25.1	5.6

[a] Not detected.

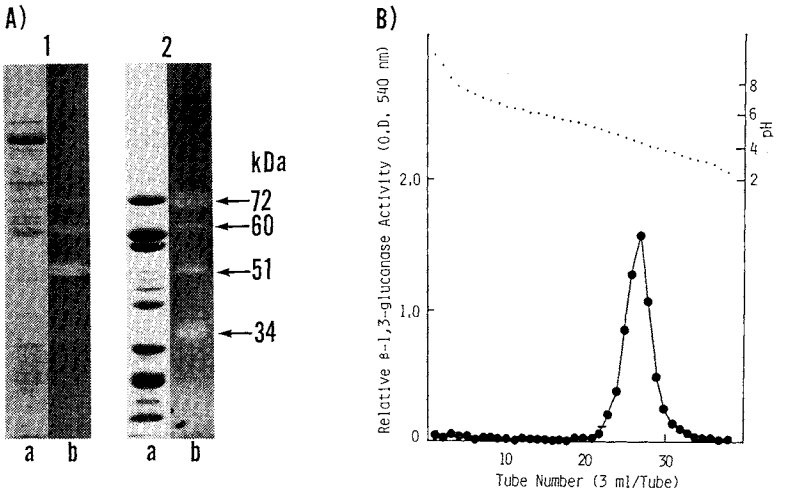

Fig. 5. Activity Profiles of β-1,3-glucanase Produced by *E. coli* HB101 Harboring pNT003.

Periplasmic fraction was prepared from *E. coli* HB101 (pNT003) grown at 30°C for 24 hr as described in the text. (A) SDS-polyacrylamide gel electrophoresis of the *B. circulans* WL-12 crude enzyme (lane 1) and the periplasmic protein of *E. coli* harboring pNT003 (lane 2). a, protein staining with Coomassie brilliant blue R-250; b, β-1,3-glucanase activity detected on the agar replica of the polyacrylamide gel. (B) Isoelectric focusing of the periplasmic protein. ··, pH; ●, relative β-1,3-glucanase activity.

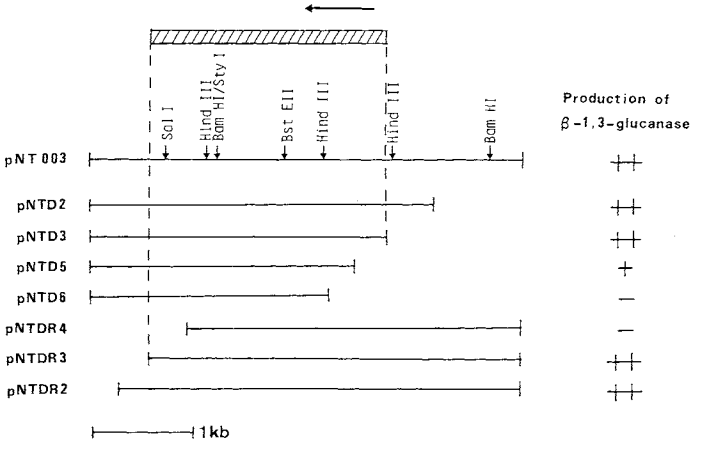

Fig. 6. Deletion Analysis of the β-1,3-Glucanase Gene.

Sequential deletions were introduced into *B. circulans* WL-12 DNA on the pNT003 as described in **Materials and Methods**. Deleted plasmids were introduced into *E. coli* HB101 by transformation. Production of the β-1,3-glucanase was judged by the formation of a clearing zone of pachyman around the transformant colonies. ++, size of clearing zone was indistinguishable from that of original pNT003; +, clearing zone was significantly smaller than that of pNT003; −, clearing zone was not observed. Hatched bar indicates the region of DNA required for the same level of β-1,3-glucanase production as the original pNT003. Horizontal arrow indicates the direction of transcription of the β-1,3-glucanase gene.

the six bands detected in *B. circulans* WL-12 crude enzyme. These four bands were major components of peak IA (p*I*=4.0) and IB (p*I*= 4.4) fractions of the crude enzyme in isoelectric focusing. The periplasmic protein fraction prepared from *E. coli* was also analyzed by isoelectric focusing (Fig. 5B) and it showed a single peak at p*I*=4.25. Thus, the gene we

have cloned appears to code for β-1,3-glucanase detected in the pI=4 fraction of isoelectric focusing analysis.

Deletion analysis of pNT003

To find the precise location of the β-1,3-glucanase gene within the 4.4-kb inserted fragment in pNT003, sequential deletions were introduced into the inserted fragment. *E. coli* HB101 cells were transformed with the sequentially deleted pNT003 derivatives. Production of β-1,3-glucanase was detected using L-broth pachyman plates as described above. Deletion of a 1.4-kb region from right to left (distal to the *lac* promoter on pUC 19, pNTD3) and 0.7 kb from left to right (proximal to the *lac* promoter, pNTDR3) on Fig. 6 did not affect the size of the clearing zone on the pachyman plate. Deletion of a 1.7-kb region proximal to the *lac* promoter (pNTD5) reduced the size of the clearing zone on the pachyman plate. The presence of a promoter in the *Hin*dIII fragment containing this region was tested using the promoter search vector pKO-1[10] carrying a galactokinase gene without it's own promoter. The galactokinase gene is only expressed when a DNA fragment containing a promoter is inserted upstream from the gene. The *Hin*dIII fragment was able to express the galactokinase gene, indicating the presence of a promoter for the β-1,3-glucanase gene in this region (data not shown).

β-1,3-Glucanase produced by *E. coli* carrying the deleted plasmids, pNTD3, pNTDR3, and pNTD5, were analyzed by SDS-PAGE. Four active molecules of β-1,3-glucanase were produced by *E. coli* carrying one of the three plasmids as shown in Fig. 7. These bands were identical in mobilities to the bands produced by *E. coli* carrying the original recombinant plasmid, pNT003.

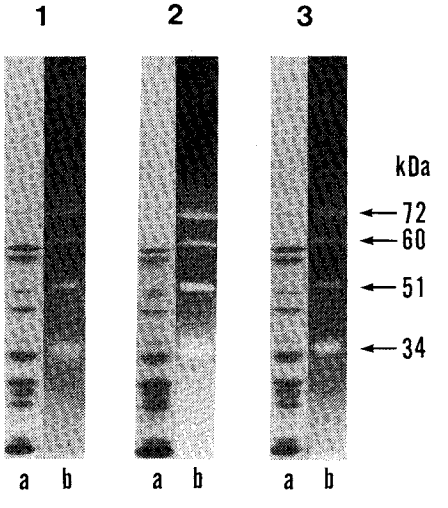

Fig. 7. SDS-Polyacrylamide Gel Electrophoresis of the Periplasmic Protein Prepared from *E. coli* HB101 Harboring the Deleted Plasmids pNTD3 (lane 1), pNTD5 (lane 2), and pNTDR3 (lane 3).

a, protein staining; b, β-1,3-glucanase activity detected on the agar replica of the polyacrylamide gel.

Discussion

The production of multiple β-1,3-glucanases by *B. circulans* WL-12 was suggested from native polyacrylamide gel electrophoretic analysis of crude enzyme induced by yeast or fungal cell walls. To make the experimental system simple, pachyman-induced crude enzyme and SDS-polyacrylamide gel electrophoresis were used in this study. SDS-PAGE analysis achieved clear separation of six active kinds of molecules of β-1,3-glucanases. Detection of distinct β-1,3-glucanases in denatured conditions is strong evidence for the multiplicity of this enzyme. The β-1,3-glucanases of approx. 40 kDa and 24 kDa on SDS-PAGE were the major constituents of the Peak II and Peak III fractions in isoelectric focusing, respectively. The peak fraction with pI around 4 contained four active kinds of molecules of β-1,3-glucanase on SDS-PAGE. Intensity of the higher M_r band decreased during cultivation, indicating that some proteolyic degradation of secreted β-1,3-glucanase occurred. Since crude enzyme prepared from culture supernatant at 4 days of culture still maintained the same level of β-1,3-glucanase activity in the Peak I fraction, degradation products must have β-1,3-glucanase activity. In fact, activity of lower M_r molecules increased significantly during cultivation. The concomitant appearance of Peak I and disap-

pearance of Peak II, and the M_r of the major active molecules of Peak I and Peak II may suggest that the β-1,3-glucanase of Peak III was generated from Peak II by proteolytic degradation or proteolytic processing.

The cloning strategy we used was based on the assumption that leakage of this enzyme into the medium might occur, thus enabling us to detect the β-1,3-glucanase clones. Our experimental data suggested that overproduction of β-1,3-glucanase retarded the growth rate and caused cell lysis. β-1,3-Glucanase produced in *E. coli* was successfully exported into the periplasmic space. No glucanase activity was detected in the culture supernatant early in the incubation, indicating that this protein itself does not penetrate the outer membrane at all. The visible clearing zone we detected around the colony of β-1,3-glucanase clone was presumably formed by the enzyme released from the periplasmic space by cell lysis, but not by the leakage of this enzyme through the outer membrane of *E. coli*. SDS-PAGE and isoelectric focusing analyses revealed that the products of β-1,3-glucanase gene we cloned corresponded to the four active molecules of the β-1,3-glucanase which appeared at p*I* around 4 in the isoelectric focusing. A DNA segment of 2.1 kb was shown to be sufficient for generating four active molecules of the enzyme in *E. coli*. These were distinct from the major β-1,3-glucanase of Peaks II and III in isoelectric focusing. Thus, it seems that *B. circulans* WL-12 has at least one or two additional β-1,3-glucanase gene(s) which is or are induced by pachyman.

Four active kinds of molecules of β-1,3-glucanase were generated from the 2.1-kb segment of inserted DNA in *E. coli*. The existence of four genes in this 2.1-kb DNA is unlikely, since it is only enough to direct synthesis of the 72-kDa β-1,3-glucanase. These active molecules may be generated by one of the following mechanisms. 1) proteolytic modification either inside or in the periplasm of the cell, 2) differences in size of transcripts, 3) overlap reading of mRNA. We can not exclude any of these possibilities at this moment. Recently Kawazu *et al.* reported that β-amylases with M_r 70, 56(or 58), and 42 kDa were produced in *E. coli* carrying 3.1-kb *Bacillus polymyxa* DNA on a vector plasmid.[21] One open reading frame of 2,808 nucleotides was found in the 3.1-kb DNA. From the experimental data using several protease inhibitors, they suggested that a large enzyme molecule was secreted and subsequently cleaved into several rather stable β-amylases with different sizes. A similar mechanism may be involved in the generation of multiple β-1,3-glucanases in our system. Experiments to elucidate the mechanism which is responsible for production of the multiple β-1,3-glucanases, including DNA sequencing of the 2.1-kb DNA segment, are now under way.

We focused on the β-1,3-glucanase in this study since this enzyme is crucial in hydrolyzing yeast and fungal cell walls. The results we obtained revealed that only one of the variety of polysaccharide-degrading enzymes consists of at least six distinct active molecules of the enzyme. The role of individual active molecules remains to be elucidated. Doi *et al.* described the production of two types of endo-β-1,3-glucanases by *Arthrobacter* sp. strain YCWD3, glucanases I and II, which act synergistically in the degradation of baker's yeast glucan.[22~25] Glucanase I rapidly solublizes yeast glucan and liberate laminaripentaose, but it can not attack short chain laminaridextrin. On the other hand, glucanase II can attack short-chain laminaridextrins and converts them to laminaribiose and glucose. The structural gene for glucanase I was cloned in *E. coli* by Doi and Doi.[26] The multiple nature of of β-1,3-glucanase has also been reported by various authors,[27~32]

Cooperative actions not only among various polysaccharide-degrading enzymes but among distinct β-1,3-glucanases must be required for the degradation and the utilization of yeast and fungal cell walls. *B. circulans* WL-12 may provide an excellent model system to study bacterial enzyme systems which are responsible for the utilization of complex substrates.

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