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Chitin Binding Protein (CBP21) in the Culture Supernatant of *Serratia marcescens* 2170

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A chitin binding protein (CBP21) 21 kDa in size, is a major protein in the culture supernatant when *Serratia marcescens* 2170 is grown in the presence of chitin. The gene (*cbp*) for CBP21 was found to be located in a region 1.5 kb downstream of the *chiB* gene encoding chitinase B. The *cbp* gene encodes a polypeptide of 197 amino acids with a calculated size of 21.6 kDa containing a putative signal sequence of 27 amino acids. Comparison of the amino acid sequence of the deduced polypeptide with that of other proteins showed that CBP21 is similar (45.3% amino acid identity) to CHB1 of *Streptomyces olivaceoviridis*. Purified CBP21 prepared from the periplasmic fraction of *Escherichia coli* carrying the cloned *cbp* gene showed its highest binding activity to squid chitin (β-chitin) followed by colloidal chitin and regenerated chitin. Binding of CBP21 to regenerated chitin was affected by pH, in particular, low pH reduced binding activity markedly.

The presence of similar chitin binding proteins in the distantly related microorganisms, *Streptomyces* and *Serratia*, suggests a wide distribution of this type of chitin binding protein in chitinolytic microorganisms.

Key words: chitin binding protein; *Serratia marcescens*; chitinase; nucleotide sequence

Chitin is the second most abundant carbohydrate polymer in nature next to cellulose. It consists of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) units and is present in the exoskeleton of arthropods, coelenterates, nematodes, protozoa, mollusks, insects, and crustaceans as well as in the cell walls of many fungi. Enzymes that degrade chitin are not only produced by organisms containing constituent chitin but also by bacteria, higher plants, and mammals. Bacterial chitinases play a vital role in maintaining the ecological balance by degrading and converting chitin into a biologically useful form.

Serratia marcescens is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. The *chiA* and *chiB* genes encoding chitinase A and B of three *S. marcescens* strains, QMB1466, BJL200, and 27117, have been cloned and sequenced.[1–7] The three-dimensional structure of chitinase A from strain QMB1466 has been recently solved by Perrakis *et al.*[8] Brurberg *et al.* recently speculated on a role for chitinase A and B of *S. marcescens* BJL200 in chitin breakdown based on chitinase characterization, sequence comparisons, and cellular localization.[9]

To initiate genetic analysis of chitin degradation and use by chitinolytic bacteria, we have chosen *S. marcescens* 2170 as a model organism since this bacterium is an active producer of chitinase and several techniques often used in bacterial genetic study are applicable. A limited number of proteins including four chitinases, A, B, C1, and C2, were detected in the culture supernatant when *S. marcescens* 2170 was grown in the presence of chitin. Sequence analysis showed that the amino acid sequences of chitinases A and B were very similar to those of *S. marcescens* strains.[10] Four chitinases in the culture supernatant were all adsorbed to a chitin affinity column and could be eluted with acetic acid solution. It is known that many microbial chitinases have chitin binding activity and, in some cases, participation of the chitin binding domain in the binding activity has been demonstrated experimentally.[11,12] In addition to four chitinases, we found a 21-kDa protein with chitin binding activity in the culture supernatant of *S. marcescens* 2170.[10] This protein (designated as CBP21) probably corresponds to a 21-kDa protein of *S. marcescens* QMB 1466 described by Fuchs *et al.* as a chitinolytic protein.[3] CBP21 bound to a chitin affinity column but did not show chitinase activity in our experiments.

In this paper we describe the nucleotide sequence of the gene encoding CBP21 and the biochemical characterization of CBP21. Amino acid sequence comparison of CBP21 with other proteins found significant similarity to the CHB1 of *Streptomyces olivaceoviridis* previously reported by Schnellmann *et al.*[13]

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Abbrevations: YEM medium, yeast-extract-supplemented minimal medium; LB medium, Luria-Bertani medium; IPTG, Isopropyl-β-D-thiogalactopyranoside: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CBDs, cellulose binding domains.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. *Serratia marcescens* 2170[14] was grown in yeast-extract-supplemented minimal (YEM) medium[15] containing various carbon sources at 30°C with shaking. *Escherichia coli* DH5α was used as a host organism, and pUC119 was used as a vector for cloning of the gene encoding CBP21 of *S. marcescens* 2170. *E. coli* DH5α cells harboring plasmids were grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin.

Production and purification of CBP21. CBP21 was purified both from culture supernatant of *S. marcescens* 2170 and the periplasmic fraction of *E. coli* DH5α cells harboring pSBP4 carrying the cloned *cbp* gene as follows. Proteins in the day 2 culture supernatant of *S. marcescens* 2170 cultivated in YEM medium containing 0.5% (w/v) colloidal chitin were collected by ammmonium sulfate precipitation (80% saturation). CBP21 and other proteins were separated by chitin affinity column chromatography as described elsewhere.[10] The fractions containing only CBP21 were collected, dialyzed, and lyophilized. Lyophilized CBP21 was dissolved in a small volume of 20 mM phosphate buffer and further purified by Sephadex G-75 gel filtration chromatography.

For purification of CBP21 produced by *E. coli*, DH5α cells harboring plasmid pSBP4 carrying the cloned *cbp* gene were grown in LB medium with 100 μg/ml ampicillin and 0.4 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) for 24 h. The CBP21 secreted into the periplasmic fraction was extracted by the cold osmotic shock method as described by Manoil and Beckwith.[16] The extracted periplasmic proteins containing CBP21 were collected by ammonium sulfate precipitation (80% saturation), dissolved in 20 mM phosphate buffer (pH 6.0), dialyzed against 2 mM sodium phospate buffer (pH 6.0), and lyophilized. Lyophilized proteins were dissolved in a small volume of 20 mM phosphate buffer (pH 6.0) containing 0.5 M NaCl and put onto a chitin affinity column (3.5 by 13 cm) previously equilibrated with the same buffer. Elution was done with 20 mM acetic acid and peak fractions containing CBP21 were collected, dialyzed against 2 mM sodium phospate buffer (pH 6.0), and lyophilized. Lyophilized CBP21 was dissolved in a small volume of 20 mM phosphate buffer and further purified by Sephadex G-75 gel filtration chromatography.

Purification of chitinase A. Chitinase A produced by *E. coli* carrying the cloned *chiA* gene was purified from the culture supernatant. *E. coli* DH5α cells harboring plasmid pNCA112[10] were grown on LB medium with 100 μg/ml ampicillin and 0.4 mM IPTG for 24 h. After the cells were removed by centrifugation, proteins in the culture supernatant were collected by ammonium sulfate precipitation (80% saturation) and the precipitate was dissolved in 20 mM phosphate buffer (pH 6.0), and dialyzed against 2 mM sodium phospate buffer (pH 6.0). Dialysate containing chitinase A was put onto a chitin affinity column previously equilibrated with 20 mM phosphate buffer (pH 6.0) containing 0.5 M NaCl. After washing with 3 column volumes of the same buffer and 3 column volumes of 20 mM sodium acetate buffer (pH 5.5), chitinase A was eluted with 20 mM acetic acid. Peak fractions containing purified chitinase A were collected, dialyzed against 2 mM sodium phosphate buffer and lyophilized.

Nucleotide sequencing and sequence analysis. Overlapping deletions were introduced into the DNA fragment of the plasmid carrying the *cbp* gene and a plasmid with the fragment in reverse orientation with a deletion kit purchased from Takara Shuzo Co., Ltd. (Osaka). Regions of the deletion derivatives of appropriate size were sequenced with an automated laser fluorescence sequencer (Model 4000L; LI-COR). Sequencing reactions were done by using the Thermosequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) according to the supplier's instructions with a double-stranded template. Nucleotide sequence data were analyzed using the Genetyx system (Software Kaihatsu Co., Tokyo). The amino acid sequence was compared with those available in the SWISS-PROT protein data bank and translated EMBL database by using the Lipman-Pearson algorithm.[17]

N-terminal amino acid sequence analysis. Purified protein obtained by chitin affinity chromatography was run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins in the polyacrylamide gel were electroblotted onto a polyvinylidene difluoride membrane as described.[18] The membrane was briefly stained with Coomassie brilliant blue R-250 for protein bands and the excised protein band was sequenced with an Applied Biosystem 473 gas phase sequencer (Foster City, USA).

Chitin binding assay. The binding assay mixture contained 4 mg of regenerated chitin (or another insoluble polysaccharide) and 1 M NaCl in 20 mM sodium phosphate buffer, pH 6.0 (total volume 500 μl). After adding various amounts of protein, the mixture was incubated for 1 h on ice with stirring every 15 min. The supernatant, containing unadsorbed protein and regenerated chitin, was separated by centrifugation and the protein in the supernatant was measured. When chitosan was used as a binding assay substrate, unadsorbed protein in the supernatant was precipitated with deoxycolate and trichloroacetic acid,[19] and collected by centrifugation. The protein was then measured after dissolving the precipitate in 2% SDS.

When the chitin binding assay mixture contained both CBP21 and chitinase A, the concentration of individual proteins in the supernatant was estimated by high performance liquid chromatography (HPLC) with a Diol-150 gel filtration colum in a liquid chromatograph LC-10A system (Shimadzu Corp., Kyoto).

Enzyme and protein assay. Chitinase activity was measured by a modification of Schales' procedure[20] with colloidal chitin as the assay substrate. One unit of chitinase activity was defined as the amount of enzyme

that produced 1 μmole of reducing sugar per min. Protein concentration was measured by the method of Lowry *et al.*[21] using bovine serum albumin as the standard.

SDS-PAGE. SDS-PAGE in 12.5% slabs was done as described,[22] using the buffer system of Laemmli.[23] When necessary, renaturation of enzymes in polyacrylamide gel and detection of chitinase activity were done as described previously.[24]

Chemicals. Glycol and colloidal chitin were prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo) as described by Yamada and Imoto,[25] and Jeuniaux,[26] respectively. Chitin EX (powdered prawn shell chitin) used in the chitin affinity column chromatography, and chitosan 7B (approximately 70% deacetylated), 8B (approximately 80% deacetylated), and 10B (more than 99% deacetylated) were purchased from Funakoshi Chemical Co. (Tokyo). Regenerated chitin for the chitin binding assay was prepared from chitosan 8B by the method of Molano *et al.*[27] Colloidal pachyman was prepared from the tree fungus *Poria cocus* Wolf as described previously.[28] The chitooligosaccharide mixture [$(GlcNAc)_3+(GlcNAc)_4$] was obtained from Pias Co. (Osaka). Cellulose powder was purchased from Toyo Roshi Kaisha, Ltd. (Tokyo). Restriction and modification enzymes were purchased from Toyobo Biochemicals (Osaka) and New England Biolabs (Beverly, MA).

Results

Production of CBP21 in medium containing various carbon sources

Chitin binding protein, 21 kDa in size (CBP21), was a major protein in the culture supernatant of *S. marcescens* 2170 grown in the presence of chitin. CBP21 did not have chitinase activity nor did it seem to be a chitin binding domain proteolytically released from the chitinases present in the culture supernatant.

Since the number of proteins observed in the culture supernatant of *S. marcescens* 2170 is limited and CBP21 was a major protein along with the chitinases, it is of interest whether this protein is produced concurrently with the chitinases. Therefore, we examined the production of chitinase activity and CBP21 by *S. marcescens* 2170 in medium containing various carbon sources. Protein concentration and chitinase activity detected in the culture supernatant during cultivation are shown in Fig. 1. Chitinase activity detected in the medium containing colloidal chitin reached 0.13 unit/ml at day 1 and gradually increased with further cultivation. In contrast, in the medium containing powdered chitin, chitinase activity, absent for the two days, increased steeply from day 3 to day 4 and reached 0.35 unit/ml at day 5, the highest activity observed in this series of experiments. Oligomers of *N*-acetylglucosamine also induced the production of chitinase. The activity reached 0.1 unit/ml on the first day of cultivation and remained at this level during further cultivation. Partially *N*-acetylated chitosan (approximately 30% acetylated) also induced production of chitinase but at a very low level. Essentially no chitinase activity was detected in the medium containing glycerol. Glucose and fully deacetylated chitosan did not induce chitinase production either (data not shown).

Figure 2 shows SDS-PAGE analysis of the proteins in the day 4 culture supernatant containing various carbon sources. A significant amount of CBP21 was detected in the medium containing colloidal chitin, powdered chitin, partially *N*-acetylated chitosan (30% acetylated), and $(GlcNAc)_{3-4}$. The level of chitinase activity varied depending on the various carbon sources but all of these

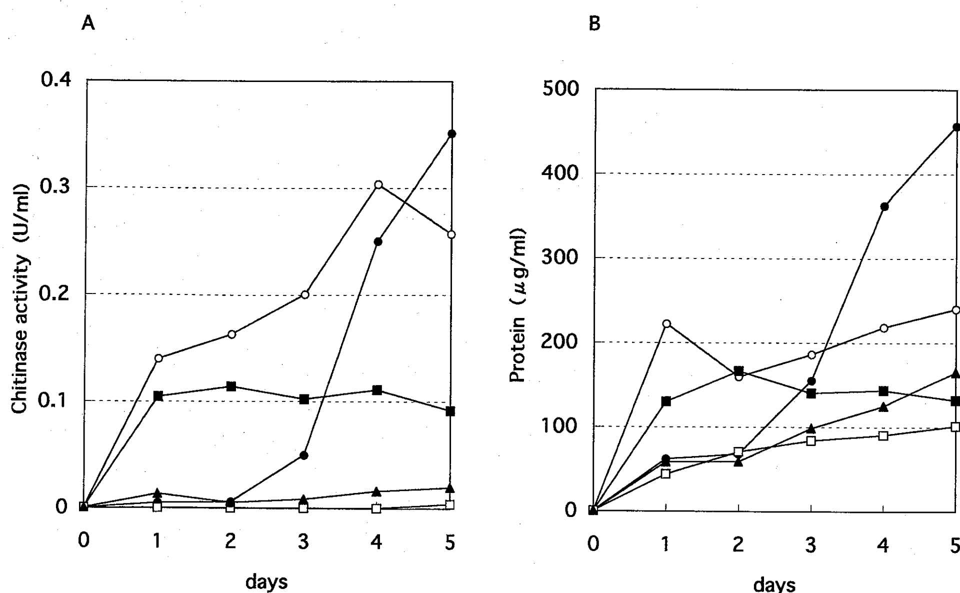

Fig. 1. Chitinase Activity (A) and Protein Concentration (B) Detected in the Culture Supernatant of *Serratia marcescens* 2170.

S. marcescens 2170 was grown in medium containing colloidal chitin (○), chitin powder (●), 30% *N*-acetylated chitosan (▲), $(GlcNAc)_{3-4}$ (■), and glycerol (□). Chitinase activity in the culture supernatant was measured by a modification of Schales' procedure using colloidal chitin as an assay substrate. Protein concentration was measured by the method of Lowry *et al.* using bovine serum albumin as the standard.

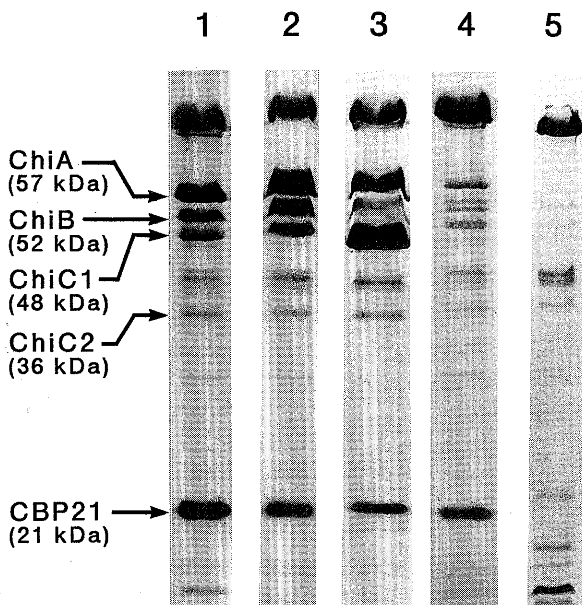

Fig. 2. SDS-PAGE Analysis of CBP21 in the Culture Supernatant of *S. marcescens* 2170 Grown in Medium Containing Various Carbon Sources

Proteins (50 μg in each lane) in the day 4 culture supernatant of *S. marcescens* 2170 grown in medium containing 0.5% of powdered chitin (lane 1), colloidal chitin (lane 2), $(GlcNAc)_{3\text{-}4}$ (lane 3), 30% acetylated chitosan (lane 4), and glycerol (lane 5) were analyzed by SDS-PAGE.

carbon sources supported the production of chitinase. Those media that supported greater production of chitinase activity tended to support greater production of CBP21. On the other hand, CBP21 was not clearly detected in the medium containing glucose, glycerol, or fully deacetylated chitosan. In these media, no or very low levels of chitinase activity were detected, although significant levels of protein concentration were detected as shown in Fig. 1B. Therefore, the production of the chitinases and CBP21 are regulated in parallel, in the sense that the substrate which induces production of chitinase also induces production of CBP21.

Nucleotide sequence of the gene (cbp) encoding CBP21

The N-terminal amino acid sequence of CBP21 purified from the culture supernatant of *S. marcescens* 2170 is HGYVESPASRAYQXKLQLNT.[10] By comparing with sequences in the translated EMBL database, it was found that the deduced amino acid sequence from the open reading frame located downstream of the *chiB* gene of *S. marcescens* 27117[4] contains a region identical to the N-terminal amino acid sequence of CBP21. Since the nucleotide sequence of the *chiB* gene of *S. marcescens* 2170 and 27117 found in EMBL database[4] are very similar, we decided to sequence the downstream region of the *chiB* gene of strain 2170 to see whether it contains the gene (*cbp*) encoding CBP21.

A charomid clone (pCOSB) carrying the *chiB* gene previously isolated contains 18.0 kb of inserted DNA. From this clone, a 6.4 kb *Sac* I fragment containing the *chiB* gene with an approximately 3.6 kb downstream region was subcloned using pUC119 as a vector and pSBP1 was obtained as shown in Fig. 3. Then, the 3-kb *Kpn*I-*Sac*I fragment was subcloned and its nucleotide sequence analyzed.

An open reading frame of 591 base pairs located approximately 1.5 kb downstream from the *chiB* gene was identified as shown in Fig. 4. The open reading frame encodes a polypeptide of 197 amino acids that contains the region (from His-28 to Lys-42) identical to the N-terminal amino acid sequence of CBP21. The calculated size of the deduced polypeptide was 21,586 Da. The N-terminal portion (Met-1 to Ala-27) of the predicted amino acid sequence had a typical signal sequence. The molecular mass of the mature form of the deduced protein (without the putative signal sequence) is 18.8 kDa, and differs only slightly from the apparent molecular mass (21 kDa) of CBP21 estimated by SDS-PAGE analysis. These observations strongly suggest that this is the gene (*cbp*) for CBP21.

The presumed ribosome binding site, AGGAG, was found to immediately precede the assigned ATG initiation codon. An 11-bp inverted repeat, which may serve as a transcription termination signal, was observed downstream of the translation termination codon. Several possible promoter sequences were predicted but the actual promoter for this gene could not be deduced.

CBP21 produced by E. coli harboring pSBP4

E. coli DH5α cells harboring pSBP4 carrying the putative *cbp* gene produced 21 kDa protein which was mainly recovered in the periplasmic fraction. The size of the protein was identical to that of CBP21 purified from culture supernatant of *S. marcescens* 2170 as shown in Fig. 5 (lane 5). The 21-kDa protein was purified by chitin affinity followed by gel filtration chromatography. The behavior of this protein in chitin affinity column chromatography was indistinguishable from that of CBP21 produced by *S. marcescens* 2170, that is it was adsorbed by the chitin column and eluted at a pH of around 5.5 (Fig. 6). The protein in the peak fraction (fraction 22 and 23) of chitin affinity column chromatography was pooled and further purified by gel filtration chromatography with Sephadex G-75. A total of 30.1 mg of purified CBP21 was obtained from 1000 ml culture of *E. coli* DH5α harboring pSBP4. Purified pro-

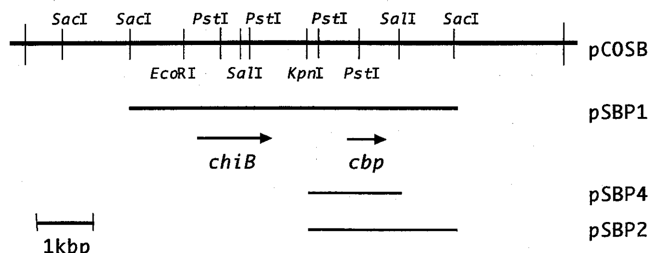

Fig. 3. Restriction Map of the Cloned DNA Fragments and Relative Location of the *chiB* and *cbp* Genes.

Arrows indicate positions of the *chiB* and *cbp* genes and direction of transcription.

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  1 GATCAAAGATCGTCATCATAATATTTTTCGTTAATAGCCGGTAAAACATTCCAATCTACG   60
 61 GCCAAACCAGAATGGTAAAAATAAAACAAGCCGACAAGAAGGGTTATTTCCAATTGCGTT  120
121 ATTAGTCTCTCATTTTGTCGGTGAAATACGCTCAACTTACAACATTATTTCAGTCAGGAG  180

181 TGACTTATGAACAAAACTTCCCGTACCCTGCTCTCTCTGGGCCTGCTGAGCGCGGCCATG  240
  1       M  N  K  T  S  R  T  L  L  S  L  G  L  L  S  A  A  M     18

241 TTCGGCGTTTCGCAACAGGCGAATGCTCACGGTTATGTCGAGTCGCCAGCCAGCCGCGCC  300
 19 F  G  V  S  Q  Q  A  N  A↑ H  G  Y  V  E  S  P  A  S  R  A     38

301 TACCAGTGCAAACTGCAGCTCAACACGCAGTGCGGCAGCGTGCAGTACGAACCGCAGAGC  360
 39 Y  Q  C  K  L  Q  L  N  T  Q  C  G  S  V  Q  Y  E  P  Q  S     58

361 GTCGAAGGCCTGAAAGGCTTCCCACAGGCCGGCCCGGCTGACGGCCACATCGCCAGCGCC  420
 59 V  E  G  L  K  G  F  P  Q  A  G  P  A  D  G  H  I  A  S  A     78

421 GACAAGTCCACCTTCTTCGAACTGGATCAGCAAACGCCGACGCGCTGGAACAAGCTCAAC  480
 79 D  K  S  T  F  F  E  L  D  Q  Q  T  P  T  R  W  N  K  L  N     98

481 CTGAAAACCGGCCCGAACTCCTTTACCTGGAAGCTGACCGCCCGTCACAGCACCACCAGC  540
 99 L  K  T  G  P  N  S  F  T  W  K  L  T  A  R  H  S  T  T  S    118

541 TGGCGCTATTTCATCACCAAGCCGAACTGGGACGCTTCGCAGCCGCTGACCCGCGCTTCC  600
119 W  R  Y  F  I  T  K  P  N  W  D  A  S  Q  P  L  T  R  A  S    138

601 TTTGACCTGACGCCGTTCTGCCAGTTCAACGACGGCGGCGCCATCCCTGCCGCACAGGTC  660
139 F  D  L  T  P  F  C  Q  F  N  D  G  G  A  I  P  A  A  Q  V    158

661 ACCCACCAGTGCAACATACCGGCAGATCGCAGCGGTTCGCACGTGATCCTTGCCGTGTGG  720
159 T  H  Q  C  N  I  P  A  D  R  S  G  S  H  V  I  L  A  V  W    178

721 GACATAGCCGACACCGCCAACGCCTTCTATCAGGCGATCGACGTCAACCTGAGCAAATAA  780
179 D  I  A  D  T  A  N  A  F  Y  Q  A  I  D  V  N  L  S  K  *    197

781 TCGGTCAAATGAGGAGATCGTTAAGGGTTCAGCCTGGCGCTGAACCCTTTTTTTGCAGG  840
                          ---------->      <----------
841 TGCCGCTATTGCGACATGTTGAGGATAGTGCGCACATTGAGCGCGCTGGTGGCGATAATG  900
901 TCGATGGCGCCGGTGCGCAAAGCGCCCAAAATCGCCAACGCCTTGGTATTCTCGGAAGCA  960
961 ATGGCGATCACGCAAGGGATTTTGCGCAGCTCATCAATGCTCAGCCCGATCACTCTGTCA 1020
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Fig. 4. Nucleotide Sequence of the Putative *cbp* Gene and Deduced Amino Acid Sequence.

A Shine-Dalgarno sequence is underlined and inverted repeat sequences are indicated by horizontal arrows. A vertical arrow indicates the cleavage site of the signal sequence.

tein gave a single band on SDS-PAGE as shown in Fig. 5. The N-terminal amino acid sequence of the purified protein was analyzed and found to be HGYVESPASRAYQCK. It perfectly matched the N-terminal amino acid sequence of CBP21 produced by *S. marcescens* 2170 and, therefore, the region from His-28 of the deduced amino acid sequence of the cloned gene.

Based on the observations described above, we conclude that the gene we have cloned is the gene (*cbp*) for CBP21.

Sequence similarity to other proteins

The *cbp* gene was identified using the open reading frame for "basic 21-kDa protein" of *S. marcescens* 27117 found in the EMBL data base (EMBL data base, L38484) as a guide. However, the amino acid sequences of "basic 21-kDa protein" and CBP21 did not match as well as we expected. Several distinctive differences were observed between the two translated sequences. First, "basic 21-kDa protein" (227 amino acids) is 30 amino acids longer than CBP21. Second, the region from Gly-37 to Gly-75 and Ser-141 to Asp-155 of CBP21 did not show any similarity to the corresponding regions of "basic 21-kDa protein".

A search of the SWISS-PROT protein data base

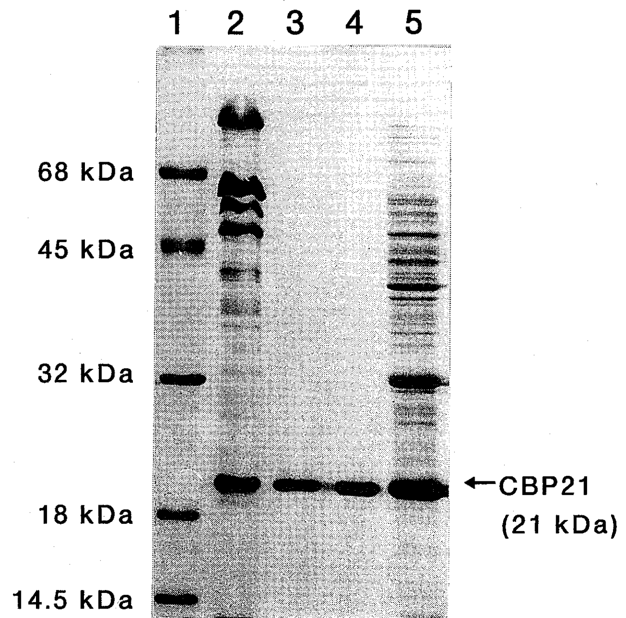

Fig. 5. SDS-PAGE Analysis of CBP21 Produced by *E. coli* DH5α Cells Harboring the Plasmid pSBP4.

Lane 1, size marker; lane 2, proteins in the culture supernatant of *S. marcescens* 2170 grown in the presence of colloidal chitin; lane 3, purified CBP21 from the culture supernatant of *S. marcescens* 2170; lane 4, purified CBP21 from periplasmic protein fraction of *E. coli* DH5α cells harboring pSBP4; lane 5, proteins in the periplasmic fraction of *E. coli* DH5α cells harboring pSBP4.

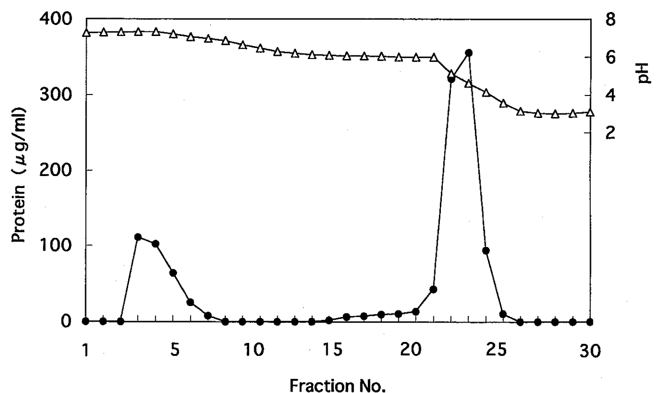

Fig. 6. Purification of CBP21 Produced by *E. coil* DH5α Cells Carrying the Cloned *cbp* Gene by Chitin Affinity Column Chromatography.

A periplasmic protein fraction was prepared from *E. coli* DH5α cells harboring the plasmid pSBP4 grown in LB medium containing ampicillin for 24 h, and put on the chitin affinity column. Symbols: ●, protein; △, pH.

found that CBP21 has apparent similarity (45.3% amino acid identity) to the CHB1 of *Streptomyces olivaceoviridis* reported by Schnellmann *et al.*[13] as shown in Fig. 7. CHB1 is an extracellular protein produced by *S. olivaceoviridis* that specifically binds to α-chitin. CBP21 (precursor: 197 amino acids, mature form: 170 amino acids) and CHB1 (precursor: 201 amino acids, mature form: 171 amino acids) are very similar in size. Three out of the four tryptophan residues of

CHB1 suggested to be involved in the interaction with chitin were also found in CBP21.

No other proteins with significant similarity to CBP21 were found by protein database search.

Properties of the purified CBP21

Binding properties of purified CBP21 produced by *E. coli* DH5α cells carrying pSBP4 were examined and compared with those of chitinase A. The purified chitinase A used in this experiment was prepared from culture supernatant of *E. coli* DH5α cells carrying cloned *chiA* gene of *S. marcescens* 2170.[10]

The binding of CBP21 and chitinase A to various insoluble substrates was examined and compared as shown in Fig. 8. The highest binding activity of CBP21 was observed when squid chitin (β-chitin) was used as a binding substrate, followed by regenerated chitin and colloidal chitin. The binding activity to cellulose and powdered prawn shell chitin was less significant and that to chitosan was almost negligible. On the other hand, chitinase A showed the highest binding activity toward colloidal chitin and regenerated chitin. Binding activity to squid chitin and powdered chitin was slightly lower than that toward colloidal chitin and regenerated chitin. Chitinase A also showed significant binding to cellulose and chitosan. Therefore, the binding specificity of chitinase A is wider than that of CBP21. Another striking difference between CBP21 and chitinase A was in the binding to squid chitin (β-chitin), squid chitin was the best substrate for CBP21 binding but not for chitinase A.

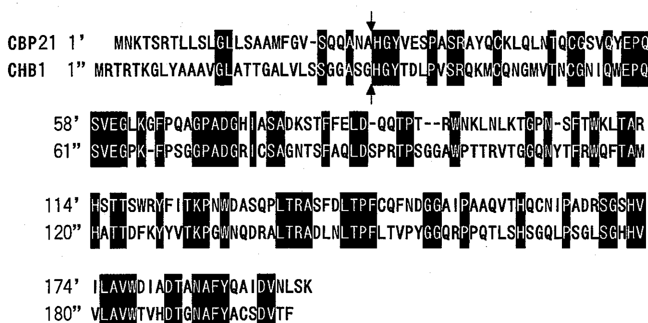

Fig. 7. Alignment of CBP21 with CHB1 of *Streptomyces olivaceoviridis*.

Identical amino acids are shown on a black background. Vertical arrow indicates the cleavage site of the signal sequence.

The effects of pH on the binding of CBP21 onto regenerated chitin were examined and compared with that of chitinase A (Fig. 9). Changes in pH affected binding of CBP21 significantly. Binding activity of CBP21 was maximized at a pH of around 7 and lower pH reduced binding activity markedly. In contrast, binding of chitinase A was relatively less sensitive to pH change, although it was reduced at pH 3. Binding of chitinase A was not affected by pH from pH 4 to 12. These results were consistent with the elution profile of CBP21 and chitinase A from chitin affinity column, that is, CBP21 was eluted with sodium acetate buffer at pH 5.5 and chitinase A was eluted at pH below 4.0 in the course of elution with a linear gradient of sodium acetate and 20 mM acetic acid.[10]

Both chitinase A and CBP21 are major proteins in culture supernatant when *S. marcescens* 2170 is grown in the presence of chitin, and both have chitin binding activity. Therefore, CBP21 may compete for chitin with chitinases. To study the effects of chitinase on the binding of CBP21 to chitin, the individual binding of chitinase A and CBP21 was assayed with a mixture containing two proteins (Fig. 10). Slightly more bound chitinase A was detected in the tested range of protein concentration. The protein concentration used in this assay was higher than that used in the above experiments, but even at the highest protein concentration, chitinase

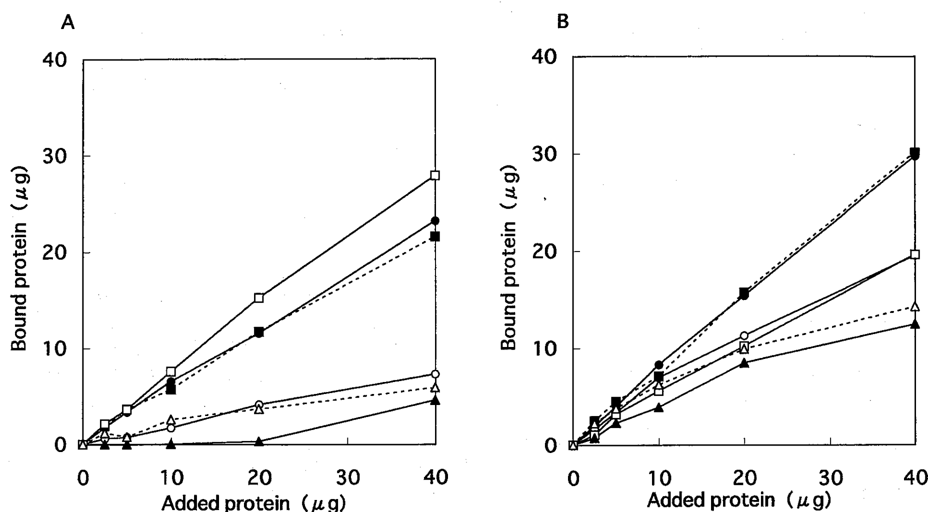

Fig. 8. Binding Specificity of CBP21 and Chitinase A for Various Insoluble Polysaccharides.

Various concentrations of CBP21 (panel A) or chitinase A (panel B) were mixed with 4 mg of an insoluble polysaccharide used as a binding assay substrate, allowed to adsorb for 1 h on ice with occasional mixing, and centrifuged. The amount of unadsorbed protein remaining in the supernatant was determined. The amount of adsorbed protein was estimated by subtracting the amount of unadsorbed protein from the total protein added to the tube. The insoluble polysaccharides used as the binding assay substrates were regenerated chitin (●), powdered chitin (○), colloidal chitin (■), squid chitin (□), chitosan (▲), and cellulose (△).

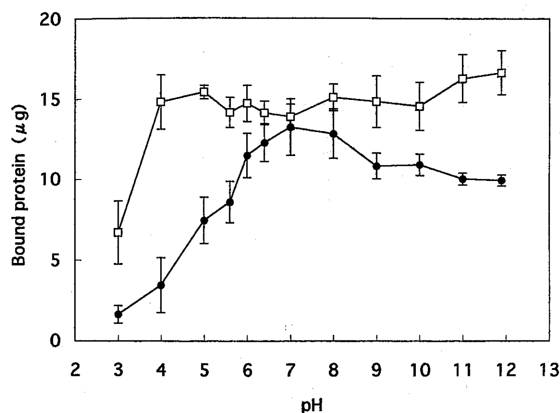

Fig. 9. Effects of pH on the Binding of CBP21 and Chitinase A to Regenerated Chitin.

CBP21 or chitinase A was mixed with regenerated chitin in buffer with various pHs, allowed to adsorb for 1 h on ice with occasional mixing, and centrifuged. The amount of adsorbed protein was estimated by subtracting the amount of unadsorbed protein from the total protein added to the tube. Symbols: ●, CBP21; □, chitinase A.

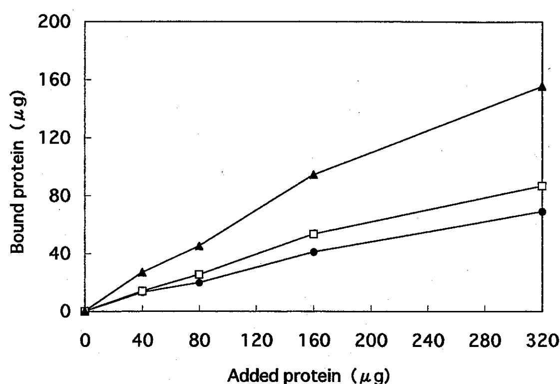

Fig. 10. Effects of Chitinase A and CBP21 on the Binding to Regenerated Chitin.

Equal amounts of chitinase A and CBP21 were added to the binding assay mixture containing 4 mg regenerated chitin and left for 1 h. After centrifugation to separate unadsorbed proteins and regenerated chitin, the amount of chitinase A and CBP21 remaining in the supernatant was measured separately by HPLC. Symbols: ●, CBP21; □, chitinase A; ▲, total.

A did not expel CBP21 from chitin or vice versa. Therefore, CBP21 may bind together with chitinases on the surface of chitinous substrates.

Discussion

Most microbial chitinases consist of multiple domains including catalytic domains, chitin binding domains, and other domains with unknown function.[29-33] The chitin binding domains of several chitinases have been demonstrated experimentally to be involved in the binding to chitin and others were deduced from sequence similarity to known chitin binding domains of chitinases or cellulose binding domains of cellulases.[11,12] The C-terminal domains of chitinase B of *S. marcescens* strains have significant amino acid sequence similarity to the chitin binding domains of several chitinases,[34] including chitinases Al and D of *Bacillus circulans* WL-12, demonstrated to be essential for binding of the enzymes to chitin.[12] On the other hand a weak sequence similarity was observed between the N-terminal domain of *S. marcescens* chitinase A and the cellulose binding domains of endo-β-1,4-glucanase CenA of *Cellulomonas fimi*. These similarities suggest that the C-terminal domain of chitinase B and the N-terminal domain of chitinase A are chitin binding domains. Chitinase A and B, as well as C1 and C2, were adsorbed on the chitin affinity column and therefore all chitinases detected in the culture supernatant of strain 2170 have chitin binding activity. However, no significant amino acid sequence similarity was observed between CBP21 and the possible chitin binding domains of chitinase A and B. The binding properties of these chitinases and CBP21 were also quite different. Squid chitin (β-chitin) was the best substrate for binding of CBP21 but not for chitinase A. The binding specificity of chitinase A was wider than that of CBP21. Binding of chitinase A was less sensitive to pH change than that of CBP21. The difference in the elution profile between the chitinases and CBP21, observed in the chitin affinity column chromatography, was consistent with the difference in the effects of pH on the chitin binding of these proteins.

A search of the protein data base revealed that CBP21 is highly similar to the CHB1 of *Streptomyces olivaceoviridis* reported by Schnellmann *et al.*,[13] with approximately 45% amino acid identity overall between the two polypeptides. CHB1 is a 20-kDa extracellular protein of *S. olivaceoviridis* which specifically binds to α-chitin, but not to chitosan, β-chitin, nor various types of cellulose.[13] Four tryptophan residues were found within a region of about 100 amino acids and the relative positions of these tryptophan residues in CHB1 were analogous to those of conserved tryptophan residues observed in family II cellulose binding domains (CBDs). The possible involvement of tryptophan residues in the interaction of CHB1 with crystalline substrate is also suggested by spectroscopical studies. CBDs are classified into 10 families of related amino acid sequences.[35] Family II CBDs are about 100 amino acids long and contain four highly conserved tryptophan residues. Three out of four conserved residues in the family II CBDs of *C. fimi* Cex are located on the surface of the polypeptide and possibly interact with cellulosic substrate.[36] The relative positions of three tryptophan residues in Cex correspond to that of Trp-57, 114, and 134 in CHB1 and therefore, may be important in the interaction with chitin. Three out of four tryptophan residues (Trp-57, 99, 114, and 134) of CHB1 were found in CBP21 (Trp-99, 114, and 134). Trp-57 in CHB1 was replaced by Tyr in CBP21. In spite of the high degree of amino acid sequence similarity, significant differences in binding properties were observed between CHB1 and CBP21. The most striking difference was observed in the binding to β-chitin. CBP21 preferentially bound to squid chitin (β-chitin) followed by regenerated chitin and colloidal chitin. In contrast, CHB1 specifically bound to α-chitin and weaker adsorption of CHB1 was observed on colloidal chitin.[13] In addition, binding of

CHB1 was independent of pH in the range 4.5-9, while binding of CBP21 was severely affected especially by low pH. Such differences in binding properties may be related to the different amino acid residues at position 57 (Trp for CHB1 and Tyr for CBP21), if the aromatic residues at this position are involved in the interaction with chitin.

Chitin binding proteins without chitinase activity have been isolated from various sources including plants and microorganisms.[37-41] All plant chitin binding proteins sequenced to date contain a common structural motif of 30-43 amino acids with several conserved amino acid residues.[41] In microorganisms, several chitin binding membrane proteins were reported in *Vibrio harvayi* and *Vibrio alginolyticus* which mediate attachment of bacterial cells to chitin containing substrates.[37,38,40] Attachment of bacterial cells to chitin-containing substrates is especially important in aquatic environments.[42] A strain of *Bacillus licheniformis* produces chitin binding lipopeptide CB-1.[39] CBP21 and CHB1 do not share amino acid sequence similarity with plant chitin binding proteins. CBP21 and CHB1 apparently differ from the bacterial chitin binding proteins previously reported in size, structural component, and localization, and probably in the roles they play.

The number of proteins detected in the culture supernatant is very limited when *S. marcescens* 2170 is grown in the presence of chitin, CBP21 is a major protein in addition to the chitinases. CBP21 binds to chitinous substrates and production of CBP21 and chitinases seemed to be regulated concurrently. The *cbp* gene encoding CBP21 was found in the vicinity of the *chiB* gene encoding chitinase B. All of these observations strongly suggest that CBP21 is involved in chitin degradation in this bacterium. The presence of similar chitin binding proteins in the distantly related microorganism, *Streptomyces* and *Serratia*, suggests a wide distribution of this type of chitin binding protein in chitinolytic microorganisms.

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