Biochemical and Genetic Properties of *Paenibacillus* Glycosyl Hydrolase Having Chitosanase Activity and Discoidin Domain*

Received for publication, September 7, 2001, and in revised form, February 11, 2002
Published, JBC Papers in Press, February 19, 2002, DOI 10.1074/jbc.M108660200

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Cells of “*Paenibacillus fukuinensis*” D2 produced chitosanase into surrounding medium, in the presence of colloidal chitosan or glucosamine. The gene of this enzyme was cloned, sequenced, and subjected to site-directed mutation and deletion analyses. The nucleotide sequence indicated that the chitosanase was composed of 797 amino acids and its molecular weight was 85,610. Unlike conventional family 46 chitosanases, the enzyme has family 8 glycosyl hydrolase catalytic domain, at the amino-terminal side, and discoidin domain at the carboxyl-terminal region. Expression of the cloned gene in *Escherichia coli* revealed β-1,4-glucanase function, besides chitosanase activity. Analyses by zymography and immunoblotting suggested that the active enzyme was, after removal of signal peptide, produced from inactive 81-kDa form by proteolysis at the carboxyl-terminal region. Replacements of Glu115 and Asp176, highly conserved residues in the family 8 glycosylase region, with Gln and Asn caused simultaneous loss of chitosanase and glucanase activities, suggesting that these residues formed part of the catalytic site. Truncation experiments demonstrated indispensability of an amino-terminal region spanning 425 residues adjacent to the signal peptide.

Chitosanase (EC 3.2.1.132) catalyzes endohydrolysis of β-1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partly acetylated chitosan. However, only four species (1–4) have been registered, and such an enzyme as Bacillus sp. number 7-M chitosanase hydrolyzes the linkages between N-glucosamine residues alone (5). Chitosanase is more restrictively defined as the enzymes capable of hydrolyzing chitosan but not chitin (6).

According to sequence-based classification of glycosyl hydrolases by Henrissat and Bairoch (7), chitosanase belongs to family 8 glycosyl hydrolase group, rather than family 46. Although family 46 chitosanases are without glucanase function, be-

EXPERIMENTAL PROCEDURES

Materials—Chitosan 10B (less than 10% acetylated chitosan) was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Glycol chitosan, glycol chitin, and lichenan were purchased from Sigma, carboxymethyl (CM)-1-cellulose sodium salt, Congo red, and n-glucosamine hydrochloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Coomassie Brilliant Blue R-250 was purchased from Bio-Rad Laboratories (Richmond, CA). Restriction enzymes were products from Takara Shuzo Co., Ltd. (Kyoto, Japan), Toyobo Co., Ltd. (Osaka, Japan), or Nippon gene (Toyama, Japan). Ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and isopropyl-1-thio-β-D-galactopyranoside were obtained from Nippon gene (Toyama, Japan). Yeast extract, tryptone, and beef extract were purchased from Difco Laboratories (Detroit, MI). All other chemicals were reagent grade or molecular biological grade.

**Bacterial Strains, Plasmid, and Culture Conditions**—*P. fukuinensis* D2, was isolated from soil collected in Fukui, Japan, and selected for its ability to form clear zones on agar plates containing 0.5% (w/v) colloidal chitosan, 0.5% tryptone, 1.0% beef extract, 0.1% KH2PO4, 0.1% MgSO4, and 1.5% agar at pH 7.0. Apparently, this bacterium was an active chitosanase producer and was chosen for further studies of this enzyme. *P. fukuinensis* D2 was identified as a novel bacterium of genus Paeni-

**Agar Plate Assay for Chitosanase and Glucanase Activity**—Cells were plated on agar media containing 0.5% (w/v) colloidal chitosan, 0.5% glycol chitosan, 0.5% CM-cellulose, or 0.4% glycol chitin. **Experimental Details**—Obvious differences were noted in the ability of *P. fukuinensis* D2 to degrade various chitosanases and glucanases are required for appropriate definition and classification of the enzyme groups. The present report deals with a chitosanase, newly found in *Paeni-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB006819.

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**The Journal of Biological Chemistry Vol. 277, No. 17, Issue of April 26, pp. 14695–14702, 2002**

Printed in U.S.A.

This paper is available on line at http://www.jbc.org
was incubated at 37 °C for 2 days and P. fukuinensis D2 was incubated at 30 °C for 4 days. After incubation, the agar plate was flooded with Congo red solution (1 mg/ml) for 15 min at room temperature followed by further treatment with 1 μM NaCl (15–17).

**Chitosanase-glucanase of P. fukuinensis D2** was assayed using soluble chitosan as a substrate (10). The reaction mixture, consisting of 0.5 ml of soluble chitosan (50 mg/ml sodium acetate buffer (pH 5.0)), 0.5 ml of enzyme solution, was incubated for 30 min at 37 °C, and the reducing sugars released were determined (19). One unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of the reducing sugar per min, using glucosamine as the standard. The endoglucanase activity was assayed using lichenan as a substrate. The reaction mixture, consisting of 0.5 ml of 0.5% lichenan in 50 mM sodium acetate buffer (pH 6.0) and 0.5 ml of enzyme solution, was incubated for 60 min at 37 °C. The amount of reducing sugars released was measured by the 3,5-dinitrosalicylic acid method (20). One unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of the reducing sugar per min using glucose as the standard.

**Immunoblot Analysis—Antiserum was raised against chitosanase by injecting, into rabbits, a fragment of the enzyme deficient in the NH₂-terminal 41-amino acid region and COOH-terminal 372-amino acid region (3). Culture supernatant of P. fukuinensis D2 was subjected to SDS-PAGE (10% total acrylamide), and proteins were transferred onto PVDF membrane (Bio-Rad) by electroblotting. After transfer, the membrane was blocked with 5% skim milk and 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 1 h. The membrane was washed with PBST, and the blotted protein was cut out from the membrane, and sequenced by automated Edman degradation with a PE Bio-Rad model 491 Procise protein sequencing system (PE Applied Biosystems, Foster City, CA).**

**Northern Blot Analysis—Overnight culture of the P. fukuinensis D2 was diluted 1:20 in 20 ml of chitosanase production medium containing 0.05% lichenan and grown to an A₆₀₀ of 0.25 (5 h), corresponding to mid-logarithmic growth phase. Overnight culture of the E. coli XLI-Blue harboring pCG2 or pUC19 was diluted 1:50 in 10 ml of LB broth and grown to an A₆₀₀ of 0.6 (2.5 h), corresponding to mid-logarithmic growth phase.**

**Site-directed Mutagenesis and Deletion Analysis—Oligonucleotide-
mediated mutagenesis was performed using a commercial kit, TaKaRa LA PCR in vitro Mutagenesis Kit (Takara Shuzo Co., Ltd.). The plasmid pCG2, carrying the chitosanase gene, was used as a template for site-directed mutagenesis. The two mutagenesis primers used were as follows: CT-MUT-3, 5′-GGGGCACTCGTCCAGGGGCAAGG-3′ (E115Q) and CT-MUT-4, 5′-CTCTGACACCTCACTTCCACCAgTTgCCCgTAATgAAC-3′ (D176N). The gene encoding full-length or truncated chitosanase was amplified by PCR, using specific primer sets listed in Table I. The primers were designed according to the sequence of chitosanase gene and contained modifications to add appropriate restriction enzyme sites for insertion into the vector. XbaI site in the forward primer and the KpnI site in the reverse primer were underlined, respectively. The sequence of all PCR products were confirmed by DNA sequencing.

RESULTS

Characterization of the Chitosanase of P. fukuinensis D2—

Colony of P. fukuinensis D2 formed lytic halo on the plate containing colloidal chitosan. Chitosanase activity was detected extracellularly, when the bacilli were incubated with colloidal chitosan or glucosamine which did not sustain the bacterial growth. Glucose was feeble in production of the chitosanase (Table II). These results suggest that D2 chitosanase is an exoenzyme inducible with chitosan or glucosamine. In addition to the chitosanase, strain D2 exhibited chitinase and glucanase activities on plates containing glycol chitin or CM-cellulose (Fig. 1). The chitosanase protein precipitated from D2 culture supernatant with ammonium sulfate was partially purified by chromatography on SP-Sephadex C-50, Bio-Gel A, and DEAE-Toyopearl columns. Upon SDS-polyacrylamide gel electrophoresis of the enzyme preparation, two bands (67 and 40 kDa) were discerned as the major components (Fig. 2). Amino-terminal sequence of the two protein bands was identical (Fig. 3), suggesting derivation of the bands by cutting at different positions in the carboxyl region of a common precursor.

Cloning of the Chitosanase Gene from P. fukuinensis D2—

Based on the amino-terminal sequence, primers were designed and the chitosanase gene was cloned from P. fukuinensis D2 genome, using PCR. Sequence of 3031-bp region around the gene and the amino acid sequence deduced therefrom are shown in Fig. 3. The nucleotides 325 to 330 corresponded to the consensus Shine-Dalgarno sequence (AGGAGG) and the trinucleotides 340 to 342 (TTG) 10 bp downstream the ribosome-binding site was inferred to be the initiation codon. Putative −10 promoter (TAAACT, nucleotide 289 to 294) and −35 promoter (TTGGCT, nucleotide 267 to 272) were present, about 50 and 70 bp upstream from the initiation codon. TGA (nucleotide 2731 to 2733) was regarded as the termination codon and an inverted repeat sequence (nucleotide 2777–2807) was seen 50 bp downstream from the codon. Free energy change value for the inverted repeat sequence was calculated, using GENETYX-MAC computer software described in “DNA Sequencing and Sequence Analysis.”

This putative transcriptional terminator had a ΔG° of −23 kcal/mol (−95 kJ/mol). This inverted repeat was directly followed by a nonanucleotide composed of T, like ρ-independent terminator of E. coli (23). Using the primers CT9 and -10, a 2857-bp region of D2 genomic DNA including the putative promoter and terminator was amplified, subcloned into the Smal site of pUC19, and introduced into E. coli XL1-Blue. Cells of E. coli (pCG2), in which the chitosanase gene had been inserted in the reverse direction to lac promoter, formed lytic halo on plates containing chitosan or CM-cellulose (Fig. 1). This result indicates that D2 chitosanase promoter functions in E. coli, and the chitosanase gene encodes glucanase activity as well.

Prediction of Amino Acid Sequence of the P. fukuinensis D2 Chitosanase—From the nucleotide sequence, the chitosanase of P. fukuinensis D2 was deduced to be a 85,610 Da protein composed of 797 amino acid residues (Fig. 3). A signal peptide sequence composed of 41 residues was present at the amino terminus, and its cleavage site was coincident with that of Trichoderma reesei. A tandem repeat of discoidin domain (27) at the carboxyl terminus (Fig. 4) was composed of 130 amino acid residues (from Asn530 to Gly659), whereas the C2 domain was from Asn666 to Gly796 (131 residues). Homology between C1 and C2 was 71.8%, and 135 residues were conserved. Alignment of C1 and C2 is compared with typical discoidin domain in Fig. 5.

Molecular weight of the D2 chitosanase protein was the highest among family 8 glycosyl hydrolas. Homology to the
Chitosanase-glucanase of *P. fukuinensis* D2

Between glycol chitosan gel and CM-cellulose gel was corrected from the mobility of prestained protein standards. As shown in Fig. 6, five lytic bands (67, 58, 51, 44, and 42 kDa) were detected from the culture supernatant, on CM-cellulose gel as well as on glycol chitosan gel. The 67- and 42-kDa bands probably corresponded to the protein species used for the initial amino-terminal sequencing. As to the lysate of the recombinant *E. coli* cells, four bands (62, 57, 53, and 44 kDa) were discerned, not only on glycol chitosan gel but also on CM-cellulose gel. The major product newly expressed in the *E. coli* transformant was 62 kDa, suggesting that 57-, 53-, and 44-kDa bands were possibly derived by proteolysis in *E. coli*. In plasmid pCG1, the chitosanase gene was inserted downstream from the lac promoter in tandem, whereas in pCG2 the gene was situated in reverse direction. For unknown reasons, however, expression of the chitosanase (glucanase) was lower in pCG1 than pCG2. Both in the D2 culture supernatant and the *E. coli* extract, the zymogram pattern on the chitosan gel closely resembled that on the CM-cellulose gel, indicating that the chitosanase protein *per se* was endowed with glucanase activity. Efficiency of this enzyme was, however, higher for glycol chitosan than for CM-cellulose in the *E. coli* extract as well as in D2 culture supernatant, as evidenced by thickness of the lytic band in zymogram.

**Immunoblot Analysis**—Time course of the chitosanase production, from *P. fukuinensis*, into the culture supernatant was followed by immunoblotting. As shown in Fig. 7, several protein bands were detected, besides five molecular species found in zymogram. The faint 90-kDa band was undetectable within the cells (data not shown), and probably unrelated to the chitosanase. The 75-kDa protein, 8% smaller than the putative full-length molecule of 81 kDa deduced from the nucleotide sequence, was regarded as the inactive precursor. The protein bands of 54, 33, and 32 kDa might be produced by proteolysis of the precursor. The 51-kDa band found in the zymogram was resolved into 51- and 50-kDa components. The 44-kDa species in the zymogram was very weak in the immunoblot profile, indicating its higher specific activity. Interestingly, the 58-kDa active form was prominent in the supernatant of the 5-h culture (corresponding to mid-log growth phase), whereas after 10 h the inactive 75-kDa species became the major antigenic protein in the culture supernatant.

**Northern Blot Analysis**—To elucidate whether variety of D2 chitosanase proteins detected in immunoblotting was produced at the transcriptional level, the bacillar RNA was isolated for Northern blot. Although colloidal chitosan was most effective for production of the D2 chitosanase (Table II), chitosan formed in the zymogram was very weak in the immunoblot profile, indicating its higher specific activity. Interestingly, the 58-kDa active form was prominent in the supernatant of the 5-h culture (corresponding to mid-log growth phase), whereas after 10 h the inactive 75-kDa species became the major antigenic protein in the culture supernatant.

**Site-directed Mutagenesis and Deletion Analysis**—Expression of the D2 chitosanase gene in *E. coli* yielded dual activities of chitosanase and glucanase. Chitinase activity found in the D2 culture supernatant was, however; not detected in the transformant, showing that this enzyme activity was unrelated to the chitosanase-glucanase protein. Functional domain of enzyme protein having chitosanase and glucanase activities

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### FIG. 1. Detection of chitosanase, chitinase, and glucanase activities on agar plates

Cells of *P. fukuinensis* D2 and *E. coli* XL1-Blue harboring pCG2 or pUC19 were spotted onto the plates containing colloidal chitosan, glycol chitosan, CM-cellulose, or glycol chitin and incubated at 30°C for 4 days (*P. fukuinensis* D2) or at 37°C for 2 days (*E. coli* XL1-Blue). After incubation, each plate was stained with 0.1% (w/v) Congo red. Appearance of the clear zone surrounding the colony manifests glycosyl hydrolase activity.

### FIG. 2. Analysis of the purified *P. fukuinensis* D2 chitosanase by SDS-PAGE

Lanes 1 and 6, high molecular weight calibration kit proteins (Amersham Bioscience UK Ltd.); lanes 2 and 5, low molecular weight calibration kit proteins (Amersham Bioscience UK Ltd.); lane 3, the purified enzyme from *P. fukuinensis* D2; lane 4, crude proteins precipitated with ammonium sulfate from culture supernatant of *P. fukuinensis* D2. Proteins were electrophoresed and stained with Coomassie Brilliant Blue R-250.

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family members were: *Bacillus* sp. KSM-330 (28), 71.0%; *B. circulans* WL-12 (29), 43.1%; *Clostridium josui* (30), 30.6%; *Clostridium cellulolyticum* (31), 29.2%; *Clostridium thermocellum* (32), 27.9%; *Cellulomonas uda* CB4 (33), 21.1%; *Erwinia chrysanthemi* (34), 20.0%; *Acetobacter xylinum* (35), 19.8%. On the other hand, significant homology was not detected between the D2 enzyme and chitosanases thus far reported.

**Zymogram and SDS-polyacrylamide Gel Electrophoresis—**Glycosyl hydrolase activities in culture supernatant of *P. fukuinensis* D2 and lysate of the recombinant *E. coli* XL1-Blue were analyzed by zymography. Difference in electrophoretic mobility

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*P. fukuinensis* D2  
*E. coli* (pCG2)  
*E. coli* (pUC19)

Colloidal chitosan  
Glycol chitosan  
CM-cellulose  
Glycol chitin

FIG. 1. Detection of chitosanase, chitinase, and glucanase activities on agar plates. Cells of *P. fukuinensis* D2 and *E. coli* XL1-Blue harboring pCG2 or pUC19 were spotted onto the plates containing colloidal chitosan, glycol chitosan, CM-cellulose, or glycol chitin and incubated at 30°C for 4 days (*P. fukuinensis* D2) or at 37°C for 2 days (*E. coli* XL1-Blue). After incubation, each plate was stained with 0.1% (w/v) Congo red. Appearance of the clear zone surrounding the colony manifests glycosyl hydrolase activity.

![Image](image_url)
has not been analyzed thus far. To define the active region of the D2 chitosanase-glucanase, site-directed mutagenesis and deletion experiments were performed, using the recombinant E. coli system. Correction was made on amount of enzyme protein expressed in each clone, referring to immunoblot data.

In the endoglucanase K of Bacillus sp. KSM-300, Glu130 and Asp191 were inferred to be the active site (36), whereas in the endoglucanase CelA of Clostridium thermocellum, Glu 95 was regarded as the proton donor and either Asp152 or Asp278 was the possible general base catalyst (37). As shown in Fig. 9, however, the D2 residue corresponding to Asp278 of CelA was Trp, whereas D2 Glu115 and Asp176 were conserved in the other

![Fig. 3. Nucleotide sequence of the chitosanase gene of P. fukuinensis D2 and deduced amino acid sequence.](image-url)

* has been inferred to be the active site (36), whereas in the endoglucanase CelA of Clostridium thermocellum, Glu(35) was regarded as the proton donor and either Asp(152) or Asp(278) was the possible general base cata-

Fig. 3. Nucleotide sequence of the chitosanase gene of P. fukuinensis D2 and deduced amino acid sequence. The ~35 and ~10 regions of a putative promoter sequence and a possible Shine-Dalgarno sequence for the ribosome-binding site are underlined. Broken underlined amino acids were determined by NH₂-terminal amino acid sequencing of the purified chitosanase. The glycosyl hydrolases family 8 domain is boxed. Solid underline indicates the repeats in the amino acid sequence (discoidin domains). Horizontal arrows indicate inverted-repeat sequence.

* *, termination codon.
Conversion of either D2 Glu115 to Gln or Asp176 to Asn by site-directed mutagenesis resulted in simultaneous loss of chitosanase and glucanase activities (Table IV). These results suggest that the two residues probably form part of the active site of the D2 enzyme.

Since several truncated molecular species were detected in zymogram, deletion analysis was carried out to determine minimal active region of the D2 enzyme. To each D2 deletion mutant, ATG initiation codon was attached, and the insert was put under lacP control. The amino-terminal sequence of 67- and 42-kDa bands in the zymogram was identical and lacking a signal peptide region composed of 41 amino acid residues. As presented in Table IV, clone /H9004 N-41, from which the signal peptide region had been removed, retained both chitosanase and glucanase activities comparable with those of pCG2 having the full-length insert. Further deletion of four residues caused significant decrease in the activities (/H9004 N-45), and curtailing of the amino-terminal 53 residues (12 residues from the signal peptidase cut end) resulted in total loss of the activities (/H9004 N-53).

The minimal active protein detected in the culture supernatant was the 42-kDa species, and a distinct homology between the D2 enzyme and endoglucanase K was confined to the region including 425 residues from the signal peptidase cut end.

Based on these results, truncation of the carboxyl-terminal residues was genetically performed, and expressed in E. coli using inherent promoter. In clone /H9004 C-372 deleted in the carboxyl-terminal side 372 residues (from the Thr 426 residue on), the activity level of chitosanase and glucanase was almost the same as that of the wild-type strain (Table IV).
deletion reached the Pro$^{425}$ residue, the activities reduced to less than 50% of the wild type ($\Delta$C-373), and further removal of the next Lys$^{424}$ residue caused total loss of chitosanase and glucanase activities ($\Delta$C-374). These results indicate that the region ranging from the Ala$^{41}$ residue to the Pro$^{425}$ residue is essential for the enzymatic activities. Chitosanase and glucanase activities of $P. fukuinensis$ D2 are probably catalyzed by the same active center. The smaller active proteins of 58 to 42 kDa might be formed by proteolysis at carboxyl side from the Thr$^{426}$ residue on.

**DISCUSSION**

Cloning of the chitosanase gene of $P. fukuinensis$ D2 demonstrated that the enzyme bears glucanase function, but not chitinase activity. Thus, the chitinase activity in D2 culture supernatant is due to an enzyme other than the chitosanase-glucanase. Halo formed on the colloidal chitosan plate was more clear around the $P. fukuinensis$ D2 colony than the transformant $E. coli$ (pCG2). This might result from simultaneous action of the chitosanase and chitinase on the substrate holding some acetylated region. On the other hand, halo on glycol chitosan plate was rather turbid around the D2 colony than $E. coli$ (pCG2) cells. The reason for this inefficient lysis is unknown at present.

Expression of the chitosanase gene resulted in production of the 62-kDa protein probably deficient in a carboxyl-terminal region. Upon Northern blot analysis, full-length 2.5-kb transcript was detected in the recombinant $E. coli$ as well as in $P. fukuinensis$, although degradation of the mRNA was extensive in the Gram-negative bacterium. Among the smaller RNA species, 1.5-kb molecule might code active chitosanase fragment detected in zymography. It seems also possible that the enzyme fragment was formed by intracellular proteolysis. Despite the presence of the 2.5-kb mRNA, immunoreactive protein with molecular weight higher than 62,000 was absent in the recombinant $E. coli$ extract (data not shown). Thus, subtle difference in mechanism of translation, rather than transcription or post-translational modification, probably interrupted production of the 81- or 75-kDa chitosanase protein in $E. coli$. In the Gram-positive $P. fukuinensis$, a putative antitermination factor might prevent termination of peptide elongation at the potential hairpin region (nucleotide 2314–2331) with $\Delta G^\circ$ of $-25$ Kcal/mol, corresponding to p-independent terminator in $E. coli$.

Arrangement of D2 discoidin domain C1 and C2 is similar to that of blood coagulation factors V (38) and VIII (39). Interestingly bacterial proteins having discoidin domain are generally pathogenic factors (30). Deletion analysis indicates that the D2 discoidin domain is, under the experimental conditions, dispensable for chitosanase and glucanase activities. Thus, the role of this peculiar domain in the enzymatic function remains to be elucidated.

The glucanases CenA and Cex of Cellulomonas fimi are cut by its own protease at the carboxyl side of the Pro-Thr repeat (Pro-Thr box), and this proteolysis is prevented by glycosylation of the glucanase proteins (40–42). The chitosanase-glucanase of $P. fukuinensis$ D2, although lacking in Pro-Thr box, contains five Pro-Thr (PT) sequences outside the region required for the catalytic activity. If proteolysis took place behind the PT sequences in the 81-kDa precursor, molecular species of 76, 61, 52, 44, and 43 kDa would be produced, which closely resembled the bands detected in the zymogram and immuno-

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**FIG. 8. Northern blot analysis of the $P. fukuinensis$ D2 and $E. coli$ transcripts.** RNA was extracted from $P. fukuinensis$ D2 and $E. coli$ at a midlogarithmic growth phase. Ten micrograms of total RNA purified from $P. fukuinensis$ D2 and $E. coli$ were separated on a 1.0% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with the $^{32}$P-labeled chitosanase gene fragment. Sizes of RNA are indicated in kilobases. Lanes: 1, total RNA from $P. fukuinensis$ D2; 2, total RNA from $E. coli$ XL1-Blue harboring pCG2; 3, total RNA from $E. coli$ XL1-Blue harboring pUC19 (vector only).

**TABLE IV**

| Mutation     | Chitosanase activity | Glucanase activity | Glycol chitosan | CM-cellulose |
|--------------|----------------------|--------------------|-----------------|--------------|
| None (pCG2, 85.6 kDa)* | 100                  | 100                | +++             | +++          |
| Glu$^{115}$→Gln | <0.01                | <0.01              | -               | -            |
| Asp$^{176}$→Asn | <0.01                | <0.01              | -               | -            |
| $\Delta$N-41 (81.4 kDa) | 69.6                | 88.7               | +++             | +++          |
| $\Delta$N-45 (80.9 kDa) | 27.6                | 64.5               | ++              | +            |
| $\Delta$N-49 (80.6 kDa) | 1.9                 | 25.8               | +               | +            |
| $\Delta$N-53 (80.1 kDa) | <0.01               | <0.01              | -               | -            |
| $\Delta$C-372 (46.8 kDa) | 89.0 (100)           | 90.8 (100)         | +               | +++          |
| $\Delta$C-374 (46.6 kDa) | 21.8 (22.2)         | 28.0 (31.9)        | +               | +            |

* Predicted molecular size.
Chitosanase-glucanase of P. fukuinensis D2

blot analysis. On the other hand, proteolysis of the 81-kDa molecule at the PT sequence near the carboxyl end would yield, besides a 6-kDa fragment, a 75-kDa species equivalent to the inactive 75-kDa molecule found in the immunoblot experiment. The two PT sequences in the discoidin domain C1 and C2 are followed by NWTVY, which might serve a preferred recognition site for a putative D2 protease, to yield the 58-kDa protein at log phase of growth. Glycation of the precursor at stationary phase might protect the protein from hydrolysis.

Molecular weight of the D2 chitosanase-glucanase is double than the other members of the family 8 glycosyl hydrolases. The minimal region required for the enzymatic activities, however, resides in the amino-terminal part, including the family 8 domain. Moderate disparity in sensitivity of chitosanase and glucanase activities to amino acid deletion may be due to differences in affinity for the substrate or assay methods.

According to Marcotte et al. (43) active site of chitosanase is electron-negative, whereas that of chitinase is neutral. Generally, chitosanase is devoid of chitinase and glucanase activities, and this might be caused by a steric hindrance of the chitin acetyl group to active site, or weakness of hydrogen bond between the CM-cellulose and active site (43). In this regard, analysis of tertiary structure of the D2 chitosanase-glucanase protein is an interesting and important subject.

The β-1,3-1,4-glucanase of B. circulans WL-12 has chitosanase activity and is induced by chitosan, but not by β-1,3-1,4-glucan (12). Similarly, P. fukuinensis D2 was induced by colloidal chitosan or glucosamine, but neither by CM-cellulose nor glucose (Table II). Moreover, CM-cellulose and lichenan were hydrolyzed efficiently by the D2 enzyme, but did not serve as the carbon source for the cellular growth (data not shown). Thus, the D2 enzyme, a member of family 8 glycosylases, has distinct chitosanase activity to supply the carbon source from chitosan. Further studies are in progress to elucidate catalytic properties, role of the discoidin domain, and production mechanism of this enzyme.

Acknowledgments—We are grateful to Dr. Akira Yokota University of Tokyo, Japan, for his suggestion in identification of the D2 strain. We also thank Shouji Yasuoka for his contribution in the early phase of this study.

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