Novel Chitosanase from *Streptomyces griseus* HUT 6037 with Transglycosylation Activity

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*Streptomyces griseus* HUT 6037 inducibly produced two chitosanases when grown on chitosan. To elucidate the mechanism of degradation of chitinous compound by this strain, chitosanases I and II of *S. griseus* HUT 6037 were purified and characterized. The purified enzymes had a molecular mass of 34 kDa. Their optimum pH was 5.7, and their optimum temperature was 60°C. They hydrolyzed not only partially deacetylated chitosan, but also carboxymethylcellulose. Time-dependent 1H-NMR spectra showing hydrolysis of (GlcN)_6 by the chitosanases were obtained for identification of the anomeric form of the reaction products. Both chitosanases produced the β-form specifically, indicating that they were retaining enzymes. These enzymes catalyzed a glycosyltransfer reaction in the hydrolysis of chitooligosaccharides. The N-terminal and internal amino acid sequences of chitosanase II were identified. A PCR fragment corresponding to these amino acid sequences was used to screen a genomic library for the entire gene encoding chitosanase II. Sequencing of the chioII gene showed an open reading frame encoding a protein with 359 amino acid residues. This is the first report of a family 5 chitosanase with transglycosylation activity.

**Key words:** chitosanase; *Streptomyces griseus*; family 5; transglycosylation

Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the degradation of chitosan. Chitosanases are produced by various bacteria and fungi and are useful for the preparation of biofunctional chitooligosaccharides. Chitosanases sequenced so far have been put into four families of glycosyl hydrolases on the basis of their sequence similarity. The chitosanases of *Bacillus circulans* MH-K1, *Streptomyces* sp. strain N174, and *Nocardioles* sp. strain N106 belong to family 46. Those of *Bacillus circulans* WL-12 and *Paenibacillus fukuinensis* strain D-2 have been classified into family 8. Recently, the chitosanases of *Fusarium solani* and *Matsuebacter chitosanotabidus* 300 have been categorized as families 75 and 80, respectively. The chitosanases from *Streptomyces* sp. strain N174 and *Bacillus circulans* MH-K1 have had their three-dimensional structure analyzed. On the other hand, chitosanases can be divided into three classes according to their specificity for the hydrolysis of the β-glycosidic linkages in partially N-acetylated chitosan molecules: class I chitosanases split GlcNAc-GlcN and GlcN-GlcN bonds, class II chitosanase split only the GlcN-GlcN bonds, and class III chitosanases split GlcN-GlcNAc and GlcN-GlcN bonds. The family 46 chitosanases degrade only chitosan, but, in contrast, the family 8 chitosanases hydrolyze both chitosan and CM-cellulose. However, no simple relationship between the structure and substrate specificity of chitosanases has been found. More knowledge about the structures and functions of chitosanases might clarify the role of chitosanases in the degradation of chitinous compounds in nature.

*Streptomyces griseus* HUT 6037 was first of interest as an organism with strong chitosanolytic activity when grown on a medium containing chitosan. The strain also secretes multiple chitinases when grown on chitin. We have already reported about the purification, characterization, and gene cloning of the chitinase system of this strain. Recently, we found that *S. griseus* HUT 6037 produced two chitosanases, I and II, secreting them into the culture medium, the enzymes had both chitosanase and β-1,4-glucanase activities. In this study, we set out to purify and characterize the chitosanases from *S. griseus* HUT 6037 to discover their role in the degradation of β-1,4-glycan. We purified the chitosanases, and found that they were retaining...
enzymes and that they catalyzed glycosyltransfer reaction in the hydrolysis of chitooligosaccharides. There has been no previous report of a chitosanase with transglycosylation activity. This observation led us to suspect that these enzymes were unusual among chitosanases in terms of their catalytic mechanism and structure. In a check of this, we studied the mode of action of chitosanases I and II, and identified the complete nucleotide sequence of the chitosanase II gene. Comparison of the deduced amino acid sequence of chitosanase II with the sequences of other glycosyl hydrolases showed sequence similarity to the catalytic domain of family 5 glucanases. This result indicated that chitosanase II belonged to family 5 of glycosyl hydrolases. This is the first report of a family 5 chitosanase with transglycosylation activity.

Materials and Methods

Materials. Powdered chitosan (96% deacetylated) was purchased from Katakura Chikkarin Co., Ltd. (Tokyo, Japan). Colloidal chitosan was prepared from powdered chitosan by the method of Yabuki et al. Chitosan 7B (70% deacetylated chitosan), 8B (80% deacetylated chitosan), 9B (90% deacetylated chitosan), 10B (100% deacetylated chitosan), and powdered chitin (Chitin EX) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Colloidal chitin was prepared from powdered chitin by the method of Jeuniaux. CM-cellulose was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CM-Sephadex C-25 and Sephadex G-75 were products of Amersham Pharmacia Biotech. Bio-Gel P-2 was purchased from Bio-Rad Laboratories. 2H2O was purchased from MSD Isotopes. Immobilon P (Millipore) was used as the polyvinylidene difluoride membrane for electrophoretic blotting. Restriction endonucleases were obtained from Toyobo Co., Ltd. (Osaka, Japan) and Takara Shuzo Co., Ltd. (Kyoto, Japan). HyBond-N+ (Amersham Pharmacia Biotech) was used as a nylon transfer membrane for Southern hybridization. Other reagents were of analytical grade.

Microorganisms, plasmids, media, and culture conditions. S. griseus HUT 6037 was used as the source of chitosanase and chromosomal DNA for cloning of the gene encoding chitosanase II. Escherichia coli DH5α and JM109 were used as the host organisms for cloning of chitosanase II gene and PCR products. Plasmid pUC19 and pUC119 were used as vectors for chitosanase II gene cloning. S. griseus HUT 6037 was maintained as described previously. The strain was cultured in a 5-liter conical flask containing 1 liter of a medium consisting of 0.2% colloidal chitosan, 0.05% KCl, 0.1% KH2PO4, 0.05% MgSO4, and 0.001% FeSO4 (pH 7.0) at 30°C for 3 d on a circular shaker for chitosanase production. The culture broth was centrifuged for 15 min at 10,000 g and the supernatant obtained was used for enzyme purification. A seed culture medium was used for chromosomal DNA preparation. E. coli cells carrying vectors and its derivatives were grown at 37°C on Luria-Bertani medium containing 100 μg of ampicillin per milliliter.

Enzyme assay. Chitosanase activity was assayed with soluble chitosan as the substrate. The reaction mixture consisted of 0.9 ml of 0.55% chitosan solution in 0.1 M sodium acetate buffer (pH 5.7) and 0.1 ml of enzyme solution. The mixture was incubated for 10 min at 37°C. The reaction was stopped by the addition of 2 ml of 0.5 M sodium carbonate containing 0.05% potassium ferricyanide. The final volume of reaction mixture was adjusted to 1.5 ml by addition of 0.5 ml of water. The amount of reducing sugar liberated was measured by the modified Shales' method described by Imoto and Yagishita. One unit of chitosanase activity was defined as the amount of enzyme that produced reducing sugar corresponding to one μmol of D-glucosamine per minute.

Protein measurement. During column chromatography, the protein concentration in each fraction was monitored in terms of the absorbance at 280 nm. The protein concentration was measured also by the method of Hartree with bovine serum albumin as a standard.

Electrophoresis. SDS-PAGE was done by the method of Laemmli, with 12.5% polyacrylamide gel containing 0.1% SDS. Native PAGE was done by the method of Reisfeld et al.

Enzyme purification. All steps of chitosanase purification were performed at 4°C. The culture filtrate (3.76 liters) was mixed with 0.2 M phenylmethylsulfonyl fluoride in acetone to give a final concentration of 0.001 M phenylmethylsulfonyl fluoride. The culture filtrate was concentrated to about one-third of its original volume by dialysis against ammonium sulfate and brought to saturation by the addition powdered ammonium sulfate. The precipitate obtained was dissolved in distilled water. The concentrated enzyme solution was filtered on a Bio-Gel P-2 column (2.6 × 98 cm) equilibrated with 0.02 M sodium phosphate buffer (pH 6.0), and eluted with the same buffer. The active fractions were collected. The desalted enzyme solution was put on a CM-Sepahdex C-25 column (2.6 × 27 cm) equilibrated with 0.02 M sodium phosphate buffer (pH 6.0) at the flow rate of 40 ml/h. After the column was washed with the same buffer, the enzyme was eluted with a linear sodium chloride gradient from 0 to 0.4 M. The active fractions were pooled and concentrated by an ultrafiltra-
NMR spectroscopy and identification of the anomeric form of the hydrolytic products. $^1$H-NMR spectra were obtained with a 5-mm probe on an EX-270 instrument (JEOL Ltd.). Chitohexaose [(GlcN)$_6$, 5 mg] as the substrate was lyophilized three times from $^2$H$_2$O, and then dissolved in 0.6 ml of $^2$H$_2$O. The substrate solution was placed in a 5-mm NMR tube, and the enzyme dissolved in $^2$H$_2$O was added (chitosanase I, 30 µg; chitosanase II, 120 µg). The NMR tube was immediately set into the NMR probe, which was thermostatically controlled at 30°C. After an appropriate time, accumulation of $^1$H-NMR spectra was started. The accumulation took 3 min.

TLC. The products of the enzymatic hydrolysis of substrate were analyzed by TLC. The reaction mixture (50 µl) consisted of 0.2% (GlcN)$_6$, 0.01 M sodium acetate buffer (pH 5.7), and 0.0875 unit of purified enzyme, incubated for various times at 37°C. After the mixtures were boiled for 5 min to stop the reaction, 2 µl of the samples were applied to TLC. Sugars were developed on a silica gel plate (Silica gel 60, Merck) with a n-propanol-water-30% ammonia (70:15:15) mixture as the developing solvent. The spots were made visible with diphenylamine-aniline reagent.

HPLC and MS. N-Acetychloritosaccharides were separated by HPLC and detected by monitoring of the absorbance at 210 nm. The analysis of the oligosaccharides was done by a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Voyager Elite, PerSeptive Biosystems, MA, USA).

Preparation of reduced S-pyr-dylethylated chitosanase II (PE-CholI). PE-CholI was prepared by the method of Cavins and Friedman. Three hundred micrograms of chitosanase II was dissolved in 500 µl of reducing buffer (0.5 M Tris-HCl, 7 M guanidine hydrochloride, and 0.01 M EDTA, pH 8.5). One milligram of dithiothreitol was added to the solution. The mixture was left for 5 h at 25°C in the dark. After reduction, 2 µl of 4-vinylpyridine was added, and the mixture was left for 30 min at 25°C in the dark. The reaction mixture was dialyzed against 0.02 M Tris-HCl buffer (pH 8.3). The dialyzed solution was concentrated to 20 µl under reduced pressure.

Proteolytic digestion. V8 protease (Sigma) was used for digestion of PE-CholI. To a PE-CholI solution was added 200 µl of 0.1 M sodium bicarbonate buffer (pH 7.8) and 40.5 µl of V8 protease. The molar ratio of V8 protease to PE-CholI was 1:20. Digestion was at 25°C for 20 h.

Peptide separation. The digested fragments of PE-CholII were separated by SDS-PAGE in slabs with a 15% polyacrylamide gel. Peptide bands were electrophoretically blotted on a polyvinylidenedifluoride membrane, stained with 0.25% Coomassie brilliant blue R-250 in 60% methanol and 7.5% acetic acid, and decolorized with 60% methanol. Visible peptide bands were cut out and their amino acid residues were sequenced after decolorization of the bands.

Amino acid sequencing. The N-terminal and internal amino acid sequences of the purified enzyme were analyzed on a 491C protein sequencer (Applied Biosystems). The amino acid sequence was compared with sequences available in the SWISS-PROT protein sequence database (Release 40.0+/08-07, Aug 02).

DNA manipulation. Chromosomal DNA of S. griseus HUT 6037 was extracted from mycelia by the method of Hopwood et al. A part of the gene encoding chitosanase II of S. griseus HUT 6037 was amplified by PCR as described below. A sense primer (5’-TTCTGCAGBGBAACGGHCAGCT-3’) and an antisense primer (5’-ATAAGCTTGACTCTGTCVGCTA-3’) were designed basis of the N-terminal and internal amino acid sequences of chitosanase II. These primers had a PstI or HindIII cleavage site at their 5’ end, shown by the underlining. PCR was done in a 100-µl reaction mixture containing 110 ng of genome template DNA, 4 µM each primer, 400 µM each deoxy nucleoside triphosphate, 2 U of Takara LaTaq DNA polymerase (Takara Shuzo), and a 10× concentration of the reaction buffer (GC buffer I). After a cold start (42°C, 10 min), denaturation of the template was done (98°C, 5 min). Amplification was with 30 cycles of denaturation at 98°C for 30 sec, annealing at 40°C for 30 sec, and polymerization at 72°C for 30 sec. General cloning techniques were done as described by Sambrook et al. The PCR products and pUC119 were doubly digested by HindIII and PstI and ligated. They were used to transform E. coli JM109. Plasmids were extracted and purified by the GENECLEAN III kit (Q-Biogene).

Southern hybridization and construction of genomic libraries. The PCR-amplified 460-bp DNA fragment containing a part of the chitosanase II gene was used as a probe. The digoxigenin-labeled probe was synthesized with a DIG DNA Labeling and Detection Kit (Roche). For Southern hybridization,
 restriction enzyme-digested genomic DNA was electrophoresed on a 1% agarose gel and transferred onto a Hybond-N+ and hybridized with the probe. Staining and detection of hybridized DNA were done by the manual of the manufacturer. For construction of a genomic library, the total chromosomal DNA from S. griseus HUT 6037 was completely digested with SalI and separated on a 1% agarose gel. Parts of the gel containing fragments of about 2 kb were cut out, and the DNA fragments in the gel were recovered with a GENE CLEAN III kit according to the supplier’s manual. The DNA was ligated to SalI-digested pUC19 and used to transform E. coli DH5α cells. The libraries were screened by colony hybridization with the probe described above.

**Nucleotide sequence analysis.** Various deletion mutants of pUC19 inserted in the 2-kb DNA fragment were prepared with a deletion kit (Nippon Gene Co., Ltd., Tokyo, Japan). The resulting plasmids were sequenced. The reaction for DNA sequencing was done with the dideoxy DNA sequencing system of a BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) as instructed by the supplier. Fluorescence-labeled DNA fragments were analyzed on an ABI PRISM 310 Genetic Analyzer by the manufacturer’s procedure. Nucleotide sequences were compared with those in the GenBank database. The DNA sequence of the chitosanase II gene was deposited in the DDBJ/EMBL/GenBank database with the accession number AB088201.

**Results**

**Purification of chitosanases**

With colloidal chitosan in the medium as the sole carbon source, S. griseus HUT 6037 produced chitosanases and secreted them into the culture fluid. These enzymes were purified from the culture filtrate. The chitosanase activity was separated into two peaks, 1 and 2, by gel filtration on Sephadex G-75 (Fig. 1). The chitosanolytic proteins that eluted in peaks 1 and 2 were designated chitosanases I and II, respectively. The enzymes were purified by rechromatography on Sephadex G-75. The purification procedure and the results are summarized in Table 1. Chitosanases I and II were found as a single band in SDS-PAGE and native PAGE (Fig. 2). The molecular weights of both enzymes were estimated to be 34,000 by SDS-PAGE.

**Effects of pH on activity and stability**

The effects of pH on the enzymes are shown in Fig. 3. Both chitosanases had maximum activity at pH 6.0. The enzymes were stable and retained more than 80% of their original activity at from pH 6.0 to 9.0.

**Effects of temperature on activity and stability**

The optimum temperature for the activity of these chitosanases was 60°C (Fig. 4A). The thermal stability of the enzymes was examined after incubation in McIlvaine’s buffer (pH 6.8) at various temperatures for 15 min. The enzyme was stable up to 40°C (Fig. 4B).

**Substrate specificity**

The activities of the chitosanases on chitosan-related compounds are shown in Table 2. Both chitosanases hydrolyzed not only chitosan but also CM-cellulose; they did not attack colloidal chitin or

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### Table 1. Purification of Chitosanases from S. griseus HUT 6037

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>560</td>
<td>6620</td>
<td>11.8</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate ppt.</td>
<td>54.2</td>
<td>2820</td>
<td>52.0</td>
<td>4.41</td>
<td>42.6</td>
</tr>
<tr>
<td>CM-Sephadex C-25</td>
<td>22.0</td>
<td>1980</td>
<td>90.0</td>
<td>7.63</td>
<td>29.9</td>
</tr>
<tr>
<td>1st Sephadex G-75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>1.70</td>
<td>130</td>
<td>76.5</td>
<td>6.48</td>
<td>2.0</td>
</tr>
<tr>
<td>Peak 2</td>
<td>8.18</td>
<td>710</td>
<td>86.8</td>
<td>7.36</td>
<td>10.7</td>
</tr>
<tr>
<td>2nd Sephadex G-75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>0.56</td>
<td>40</td>
<td>71.4</td>
<td>6.05</td>
<td>0.6</td>
</tr>
<tr>
<td>Peak 2</td>
<td>4.29</td>
<td>370</td>
<td>86.2</td>
<td>7.31</td>
<td>5.6</td>
</tr>
</tbody>
</table>
crystalline cellulose (Avicel). Hydrolysis was maximum for chitosan 7B. The enzymes had 40.5–47.1% of the maximum hydrolysis rate against a highly deacetylated substrate, chitosan 10B. CM-cellulose was hydrolyzed at about 30% of the maximum rate.

Both enzymes hydrolyzed GlcN oligomers larger than tetramers by endo-splitting manner but did not hydrolyze oligomers of GlcNAc (not shown). These results indicated that the chitosanases did not cleave the glycosidic linkages of GlcNAc-GlcNAc in partially N-acetylated chitosan.

**Analysis of anomeric configuration of hydrolysis products**

A time-dependent profile of the ¹H-NMR spectra showing hydrolysis of (GlcN)₆ by chitosanase II was obtained (not shown). After addition of the enzyme solution, changes in the signals derived from the anomeric protons of the reducing-end GlcN residue (α- or β-form) and the other internal GlcN residues were monitored. Each of the peak areas of these signals relative to the total peak area of anomeric proton signals was calculated and plotted against reaction time. Figure 5 shows the results for chitosanase II. In the early stages of the reaction, the peak area of the H1/β-signal-derived β-form rapidly increased. Thus, the anomeric form of the hydrolytic products was β. The results for chitosanase I were similar to those of chitosanase II. The results indicated that the catalytic reaction of chitosanases I and II takes place through a retaining mechanism.

**Transglycosylation activity of the chitosanases**

The hydrolysis products from (GlcN)₅ by the purified enzyme are shown in Fig. 6. After incubation of the reaction mixture at 37°C for 24 h, (GlcN)₂, (GlcN)₃, and (GlcN)₄ were detected as the hydrolysis products. But GlcN was not released in the hydrolysis of (GlcN)₅. The results suggested that the purified enzymes catalyzed transglycosylation in the hydrolysis of (GlcN)₅. To identify the transfer products, the reaction mixture (50 μl) used consisted of 10% (GlcN)₅ as the donor, 1% (GlcN)₃ as the acceptor, 0.0625 unit of the chitosanase II, and 0.01 M sodium acetate buffer (pH 5.7); the mixture was incubated for 8 h at 37°C. The results of HPLC and MS are in Fig. 7. In HPLC, two peaks, A and B, were eluted later than (GlcN)₃. In the MS of peak A, [M+Na]⁺ ions and [M+K]⁺ ions were observed at
Fig. 4. Effects of Temperature on the Activity (A) and Stability (B) of the Chitosanases.

(A) The enzyme activities were assayed under standard conditions at various temperatures from 30 to 70°C. (B) The enzymes were incubated for 15 min at various temperatures and the residual activities were assayed under standard conditions. Symbols: ♂, chitosanase I; ○, chitosanase II.

Table 2. Substrate Specificity of Chitosanases I and II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chitosanase I</th>
<th>Chitosanase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan 7B</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chitosan 8B</td>
<td>79.6</td>
<td>84.8</td>
</tr>
<tr>
<td>Chitosan 9B</td>
<td>77.9</td>
<td>79.3</td>
</tr>
<tr>
<td>Chitosan 10B</td>
<td>40.5</td>
<td>47.1</td>
</tr>
<tr>
<td>Colloidal chitin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Avicel</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>34.7</td>
<td>29.2</td>
</tr>
</tbody>
</table>

The reactions were done under standard conditions with shaking. The amounts of reducing sugars liberated were measured as described in Materials and Methods.

![Fig. 5. Course of (GlcN)_6 Degradation and Anomer Formation Catalyzed by Chitosanase II from S. griseus HUT 6037.](image)

Fig. 5. Course of (GlcN)_6 Degradation and Anomer Formation Catalyzed by Chitosanase II from S. griseus HUT 6037.

H1α and H1β indicate the signals derived from the anomeric protons of the reducing-end residue. H1 indicates the signals derived from the other GlcN residues. The peak areas of H1α, H1β, and H1 relative to the total peak area of the anomeric protons (H1α/H1β/H1) were calculated as a percentage from the time-dependent 1H-NMR spectra, and plotted against reaction time.

Chitosanase II is the major chitosanase in the culture supernatant of S. griseus HUT 6037 grown on chitosan. To clone the chitosanase II gene, some partial amino acid sequences of the enzyme were analyzed. The N-terminal amino acid sequence of chitosanase II was AATPLAANGQLSVCGRQLCNASQXVALNG. The fragments of PE-ChoII digested by V8 protease were separated into eight bands by SDS-PAGE; the bands were named V8-1 to V8-8 from high to low molecular mass. The N-terminal amino acid sequences of V8-1, V8-4, and V8-7 were GGYETDPAGFTARAQKFIDAAHXGMYAVI, IANEPSGVSXAIKXYAEQIIP, and FGTQNYAGEGANDFTMSQRYLDDMKRKKI, respectively. The N-terminal amino acid sequence of V8-5 was the same as that of V8-1. The N-terminal amino acid sequence of S. griseus HUT 6037 was determined to be AATPLAANGQLSVCGRQLCNASQXVALNG.
acid sequences of V8-2, V8-3, V8-6, and V8-8 were the same as that of the N-terminus of chitosanase II. These sequences had high identity to the internal region in *S. lividans* 66 endoglucanase CELA[27] and *Thermomonospora fusca* endoglucanase E-5[30] belonging to family 5.

On the basis of the N-terminal amino acid sequence of chitosanase II and V8-4, two PCR primers were synthesized as described above. A 460-bp DNA fragment was obtained by PCR. The DNA sequence of this fragment was similar to that of the *S. lividans* 66 endoglucanase CELA gene. Using this fragment as a DIG-labeled probe, we did Southern hybridization with the total chromosomal DNA from *S. griseus* HUT 6037 digested with *Sal*I. One strong signal at a position near 2 kb was found. For this reason, 2-kb fragments were collected, ligated with pUC19, and used to transform *E. coli* DH5α cells. Colony hybridization was done for the transformant colonies with the same DIG-labeled probe, and positive clones were detected. These clones contained a plasmid identical to the 2-kb inserted DNA, and the plasmid was designated pChitoM2. The physical map of the inserted DNA of pChitoM2 is shown in Fig. 8. Deletion mutants were constructed and their nucleotide sequences were identified.

Nucleotide sequence

The nucleotide sequence of the 2-kb fragment is shown in Fig. 9. One open reading frame of 1076 nucleotides starting from the ATG initiation codon at position 302 was identified in the sequenced region. A probable ribosome-binding sequence, AAGGGAG, was found upstream from the initiation codon. The overall G+C content of the reading frame was 69.0%. This open reading frame would code for a 359-amino-acid polypeptide. The deduced N-terminal 53 amino acids were suggested to be a signal sequence region because of their similarity to the signal region of endoglucanase E-5 of *T. fusca*. Analysis of the N-terminal sequence of purified mature chitosanase II suggested that the signal sequence was 15 residues long and ended with a glutamate residue.
from the culture filtrate of chitosanases I and II were purified to homogeneity to discover the role of the enzymes in the degradation of chitinous compounds in nature, and to study the chitinolytic enzymes of S. griseus HUT 6037. We are growing on colloidal chitin and colloidal chitosan, and the chitosanases I and II were separated by Sephadex G-75, and their molecular masses were estimated to be 17,300 and 10,400, respectively. These results suggest that the affinities of the enzymes against dextran may differ. In SDS-PAGE of peptides obtained from proteolysis of chitosanase I by V8 protease, the electrophoresis pattern was the same as that of chitosanase II (not shown). Chitosanase I seemed to be a derivative of chitosanase II.

The cleavage specificity against partially N-acetylated chitosan of chitosanase from S. griseus HUT 6037 has been investigated earlier. The chitosanase corresponded to chitosanase II, according to its elution position on Sephadex G-75. The chitosanase from S. griseus HUT 6037 belongs to class III chitosanases, which can split GlcN-GlcN and GlcN-GlcNac bonds in partially N-acetylated chitosan. In this study, chitosanase I and II degraded partially N-acetylated chitosan faster than fully deacetylated chitosan. The results suggested that GlcNac residues in partially N-acetylated chitosan were important for effective hydrolysis of chitosan by the enzymes. As mentioned before, chitosanases can be classified into two groups: the enzymes that hydrolyze only chitosan and the enzymes that hydrolyze both chitosan and CM-cellulose. We found that chitosanases I and II hydrolyzed not only chitosan but also CM-cellulose. Among the chitosanases so far sequenced, family 8 chitosanases from Bacillus sp. No. 7-M (GenBank AB051575) and Bacillus circulans WL-12 degraded CM-cellulose. Furthermore, B. circulans WL-12 chitosanase splits β-1,4-glycosidic linkages in partially N-acetylated chitosan by a mode of action similar to that of the chitosanase from S. griseus HUT 6037. However, the amino acid sequence of chitosanase II was similar to that of the endoglucanase E-5 of T. fusca, and not similar to amino acid sequences of chitosanases belonging to families 8, 46, 75, and 80.

Family 46 chitosanases from Streptomyces sp. strain N174 and Bacillus pumilus BN-262 hydrolyze chitoosaccharides with inversion of the anomeric configuration. To clarify the anomeric specificity of chitosanases I and II, we identified the anomeric form of hydrolysis products by 'H-NMR spectroscopy. The enzymes produced the β-anomer, so they were retaining enzymes. The hydrolytic mechanism of the chitosanases from S. griseus HUT 6037 was different from the mechanisms of chitosanases from Streptomyces sp. strain N174 and B. pumilus.
Fig. 9. Nucleotide Sequence of the choII (DDBJ/EMBL/GenBank AB088201) Gene of S. griseus HUT 6037 and Deduced Amino Acid Sequence of the Gene Product. A possible ribosome-binding sequence is underlined with dots. Amino acid sequence regions matched to the sequenced N-terminal amino acid residues of chitosanase II and peptide fragments obtained by proteolysis with V8 protease are boxed and underlined, respectively. The asterisk marks the stop codon.

Fig. 10. Alignment of Chitosanase II with the Catalytic Domain of the Endoglucanase E-5 of T. fusca (GenBank L01577). Amino acids identical in the two enzymes are shown on a black background.
Endoglucanase from *A.* are classified now as family 5 glycosyl hydrolases. 1,4-glucanases, formerly known as cellulase family differ into family 5 and has glycosyltransfer activity. It will be interesting to find whether family 5 endoglucanases have chitosanase activity or not.

Chitinous compounds are present as copolymers of N-acetyl-D-glucosamine and D-glucosamine, with different degrees of N-acetylation, in nature. *S. griseus* HUT 6037 may secrete multiple chitinases and chitosanase to efficiently degrade and use the copolymers as a carbon and energy source. On the other hand, chitooligosaccharides produced by the hydrolysis of chitin and chitosan have many bioactivities, such as elicitor, antifungal, antibacterial, and antitumor activities. Chitosanases with a glycosyltransfer activity should be useful for enzymatic synthesis of novel oligosaccharides with bioactivity.

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