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Purification and Properties of Chitosanase from Chitinolytic β-*Proteobacterium* KNU3

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Abstract A bacterial strain concurrently producing extracellular chitosanase and chitinase was isolated from soil and identified as a member of the β-subgroup of *Proteobacteria* through its 16S rRNA analysis and some biochemical analyses. The newly discovered strain, named as KNU3, had 99% homology of its 16S rRNA sequence with chitinolytic β-*Proteobacterium* CTE108. Strain KNU3 produced 34 kDa of chitosanase in addition to two chitinases of 68 kDa and 30 kDa, respectively. The purified chitosanase protein (ChoK) showed activity toward soluble, colloidal, and glycol chitosan, but did not exhibit any activity toward colloidal chitin. The optimum pH and temperature of ChoK were 6.0 and 70°C, respectively. The chitosanase was stable in the pH 4.0 to 8.0 range at 70°C, while enzyme activity was relatively stable at below 45°C. MALDI-TOF MS and N-terminal amino acid sequence analyses indicated that ChoK protein is related to chitosanases from *Matsuebacter* sp. and *Sphingobacterium multivorum*. HPLC analysis of chitosan lysates revealed that glucosamine tetramers and hexamers were the major products of hydrolysis.

Key words: β-*Proteobacterium*, chitosanase, chitinase, purification, MALDI-TOF MS

Chitosanases (EC3.2.1.132), which are member of glycosyl hydrolase family 46, are hydrolytic enzymes acting on chitosan, a polymer composed of β-(1-4)-linked glucosamine residues [4, 14]. Chitosanolytic enzymes have been found in a variety of microorganisms, including bacteria and fungi [1, 2, 3, 14, 17, 21]. Oligomeric chitosans obtained by enzymatic depolymerization have many potential biotechnological applications, including medical and food materials, antifungal agents, and elicitors of pathogenesis-related proteins in higher plants [14].

Over the past decade, several microbial chitosanases have been purified [6, 7, 8, 14, 18] and characterized with gene cloning [5, 10, 11, 19, 20]. Most of the bacterial chitosanases are induced by the substrate chitosan and degrade chitosan. Their molecular weight is low, being in the range of 10–50 kDa. The optimum pH and temperature of chitosanase activity are in the range of 4.0–8.0 and 30–60°C, respectively [15]. Chitosanases can be divided into three classes according to their specificity for hydrolysis of the β-glycosidic linkages in partially N-acetylated chitosan molecules. Class I degrades chitosan by recognizing a GlcNAc-GlcN bond, class II recognizes both GlcNAc-GlcN and GlcN-GlcN bonds, and class III is specific to the GlcN-GlcN bond [22].

Recently, in the course of screening microorganisms that produce a novel chitinase, we isolated a bacterium belonging to *Beta Proteobacteria*, from soil and named it KNU3. This strain secreted both chitinase and chitosanase when colloidal chitosan or soluble chitosan was added to the medium. Until now, two kinds of chitosanase have been purified from the bacteria that belong to β-*Proteobacteria* and characterized by their gene cloning [11, 13]. *Matsuebacter chitosanotabidus* 3001, a newly classified genus belonging to the β-subclass of *Proteobacteria*, produces 34-kDa size of chitosanase (ChoA), whereas *Bukholdria gladioli* strain CHB101 produces 28 kDa of chitosanase with two chitinases, 30 kDa and 37 kDa [12].

In this study, in order to investigate the enzymatic response of β-*Proteobacterium* KNU3 to the degradation of chitinous compounds, a chitosanase (ChoK) was first purified, and its enzymatic properties were then characterized.

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Materials and Methods

Bacterial Strain, Growth Conditions, and Phylogenetic Analyses

The bacterial strain (KNU3) used in this work was initially isolated from soil for its chitinolytic activity on colloidal chitin plate. The screening medium for chitinase-producing strains was composed of 0.4% colloidal chitin, 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4 \cdot 5H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$. This chitinolytic strain was tentatively assigned to the β-subgroup of *Proteobacteria*, besed on 16S rRNA sequencing and other morphological or biochemical analyses. It was later discovered that this strain showed a chitosanolytic activity in addition to its chitinolytic activity. For purifying chitosanase, cells were grown aerobically in nutrient broth medium containing 0.4% colloidal chitosan at 30°C. Soluble chitosan (95% deacetylated) and crude chitosan were obtained from Korea Chitosan (Youngdeok, Korea). Crude chitosan was used for colloidal chitosan preparation. An API 20NE bacterial identification kit (Biomerieux, France) was used for bacterial identification. The morphological characteristics were studied using SEM and TEM (data not shown). For the analysis of the 16S rRNA gene, two universal primers (fD1 and rP2) were used to amplify the partial 16S rRNA gene [16].

Purification of Chitosanase

All the steps in the chitosanase purification were performed at 4°C. The chitosanase present in the supernatant of 1-l culture was salted out by adding ammonium sulfate to 80% saturation. The precipitated protein which was separated by centrifugation was suspended in 10 ml of 50 mM sodium phosphate buffer (pH 6.8) and dialyzed against the same buffer. The crude enzyme suspension was applied onto CM-sepharose CL-6B (Sigma, U.S.A.) column chromatography. The column was extensively washed with 50 mM sodium phosphate buffer (pH 6.8) to remove contaminated proteins and eluted with a linear gradient of 0 to 1.0 M NaCl solution. Fractions (4 ml each) with chitosanase activity were checked by activity staining and were used for further enzymatic characterizations.

Chitosanase Assay

Chitosanase activity was assayed by using soluble chitosan as a substrate. The reaction mixture consisted of 0.55 ml of 1% soluble chitosan and 0.05 ml of the enzyme suspension, and the mixtures were incubated for 30 min at 70°C. Reactions were terminated by boiling it for 10 min. The reaction mixtures were centrifuged, and the supernatants were retained. The amount of reducing sugars produced was determined at $Abs_{575\,nm}$ by the modified Schales method [11]. One unit of chitosanase activity was defined as the amount of enzyme required to produce 1 μmole of reducing sugars (glucosamine) per minute.

Amino Acid Sequencing and MALDI-TOF Analyses

The N-terminal amino acid sequence of the purified chitosanase was determined by an automated protein sequencer (HP241, Hewlett-Packard, U.S.A.). MALDI-TOF mass analysis of purified chitosanase was conducted at Dongil-Shimazdu Co. Proteome Facility (Daejeon, Korea) using Shimazdu AXIMA CFR mass analyzer (Shimadzu, Japan), and the data were analyzed by MS-Fit program.

HPLC Analysis of Chitosan Lysates

HPLC analysis was carried out with a μ-Bondapak™ NH_2 column (Waters, U.S.A.). The products were eluted with an acetonitrile-water mixture (60:40) at a flow rate of 0.8 ml/min and detected with a refractive index (RI) detector. Chitosan dimer, trimer, tetramer, pentamer, and hexamer (Seikagaku Co., Tokyo, Japan) were used as standards.

Gel Electrophoresis and Activity Staining

The SDS-PAGE was performed in 12% SDS polyacrylamide gel containing 0.05% glycol-chitosan or 1% glycol-chitin [9]. The samples were heat-treated for 5 min at 80°C before being loaded and run at 40 mA for 40 min using Bio-Rad Mini-Protein III Gel Kit. After the electrophoresis, the gels were washed for 1 h at room temperature with shaking in 50 mM sodium phosphate buffer (pH 6.8) with 1% Triton X-100. The protein gels were stained with Coomassie brilliant blue G, while the activity gels were stained with 0.1% Congo red solution for 10 min and destained with 1 M NaCl solution.

Effect of pH on Enzyme Activity and Stability

The enzyme activity was measured by incubating the reaction mixture at different pHs for 30 min, as described under the section of Chitosanase Assay. To measure the pH stability, the enzyme was incubated at room temperature for 30 min in different pH buffers, and the residual activity was then measured as described in Chitosanase Assay. The buffer solutions used were McIlvaine (pH 3 to 8), Tris-HCl (pH 9 to 10), and phosphate buffer (pH 10 to 11).

Effect of Temperature on Enzyme Activity and Stability

The effect of temperature was measured by incubating the reaction mixture at different temperatures for 30 min, as described for the enzyme assay. For the thermostability measurement, the enzyme was incubated at different temperatures for 30 min in a 50 mM sodium phosphate buffer (pH 6.8), and the residual activity was measured.

Nucleotide Sequence Accession Number

The nucleotide sequences of the 16S rRNA gene of the strain KNU3 reported in this article have been assigned GenBank accession number AY299334.

RESULTS

Taxonomic Analysis of Strain KNU3

A Gram-negative, rod-shaped strain KNU3 was tentatively identified as a member of the group β-*Proteobacterium* by brief taxonomic tests, morphological analyses, and 16S rRNA gene sequencing (Table 1). Because the results of an API NE20 bacterial identification kit did not match with any genus of conventional classification, the partial nucleotide sequence of 16S rRNA gene from strain KNU3 was compared to sequences available in GenBank databases. Figure 1 shows a phylogenetic tree of strain KNU3 with other related β-*Proteobacterium*. The 16S rRNA gene sequencing of 1.4-kb PCR product (GenBank No. AY299334) showed 99% homology with that of chitinolytic β-*Proteobacterium* CTE108 (GenBank No. AJ496446).

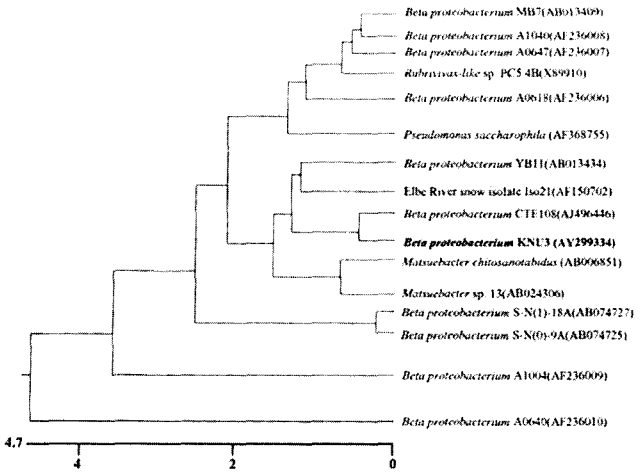

Fig. 1. Phylogenetic tree of β-*Proteobacterium* KNU3, based on 16S rRNA sequences.

Chitosanase Purification and Chitinase Activity of Strain KNU3

Crude enzyme suspension prepared by salting out with ammonium sulfate and dialysis was applied to gel chromatography on CM-Sepharose (Table 2). The chitosanase activities in the eluted active fractions were confirmed by activity staining, and it was almost homogeneous on SDS-PAGE (Fig. 2A). The molecular weight of the purified chitosanase (ChoK) was estimated to be about 34 kDa. Furthermore, the strain KNU3 produced two chitinases

Table 1. Physiological properties and biochemical characteristics of β-*Proteobacterium* KNU3 and *M. chitosanotabidus* 3001.

Morphological characteristics	β-*Proteobacterium* KNU3	*M. chitosanotabidus* 3001*
Form	rods	rods
Gram strain	negative	negative
Flagella	positive	positive
Physiological properties		
Growth Temp.	15-40°C	20-30°C
Growth pH	4-9	5-9
Catalase test	+	+
Oxidase test	+	+
Urease test	-	-
Indole production test	-	-
H_2S production test	-	+
Citrate utilization test	-	NT
Methyl red test	-	-
Voges-Proskauer test	-	-
Nitrate reduction test	+	+
Oxidation-Fermentation test	+	no action on carbohydrate
Carbon sources for growth		
D-Glucose	+	+
Sucrose	-	-
Galactose	-	-
Fructose	-	-
Arabinose	-	-
Xylose	+	-
Mannose	-	-
Maltose	+	+
Sorbitol	-	-

Symbols: +, positive; -, negative; NT, not tested.
*: *M. chitosanotabidus* 3001 data were from reference [9].

Table 2. Purification of chitosanase from β-*Proteobacterium* KNU3 culture supernatant.

Purification procedure	Protein (mg)	Total units (U)	Specific activity (U/mg)	Recovery (%)	Fold
Culture broth	11.32	594.18	261.92	100	1
Ammonium sulfate precipitation	1.91	443.62	1146.31	74.66	4.38
CM-sepharose	0.14	127.45	4441.87	21.45	16.96

ranging from 68 kDa to 30 kDa in the presence of colloidal chitin (Fig. 2B).

Enzymatic Characterization of ChoK

Chitosanase activity was measured at various pHs and temperatures. The optimum temperature of the purified enzyme under standard assay conditions was 70°C. Chitosanase activity was found to be stable at below 45°C (Fig. 3A). The purified ChoK had an optimal pH of 6.0 for chitosan hydrolysis. When the enzyme was kept at 25°C for 30 min, the enzyme was stable at pH 4–8 (Fig. 3B).

Amino Acid Sequence Analyses of ChoK by N-Terminal Determination and MALDI-TOF MS Identification

In order to identify ChoK protein, the N-terminal region of the purified chitosanase and several peptides resulting from tryptie digestions were determined (Fig. 4). The N-terminal amino acid sequence of purified KNU3 chitosanase was AAAAGVI. The partial amino acid sequences of ChoK protein obtained from MALDI-TOF mass analyses were VYGNVFDK (941.4972 m/z), LLPVLDALK (981.6639 m/z), NAYAPYVLGK (1095.6-204 m/z), QAQUVMSHYAHIDSDK (1892.9004 m/z), and NWGVQVTGIAAADMK-ADDTR (1560.8586 m/z). These data combined with its 34 kDa molecular mass were compared with amino acid sequences of other chitosanases reported [10, 11]. There were many conserved amino acid sequences between KNU3 chitosanase and other chitosanases (GenBank numbers, AB010493 and AB030253) from *Matsuebacter chitosnotabidus* 3001 and *Sphingobacter multivorum* KST109 (data not shown). These results suggest that ChoK protein might be related to the chitosanases of these newly identified bacteria [10, 11].

Substrate Specificity

Various derivatives of chitin and chitosan were used to determine the specificity of chitosanolytic activity of KNU3 chitosanase (Table 3). Soluble chitosan and colloidal chitosan showed 100% and 19.1% susceptibilities to KNU3 chitosanase (ChoK), respectively. Chitin derivatives

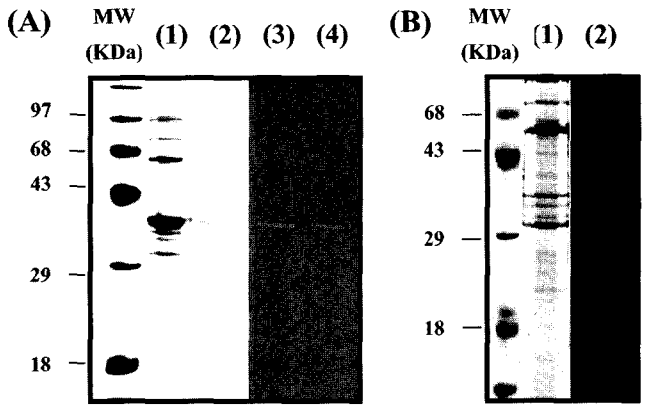

Fig. 2. Detection of chitosanolytic and chitinolytic activities in *Beta Proteobacterium* KNU3 by activity stainings.
(A) Chitosanase activity of strain KNU3. MW: Protein size markers, (1) & (2): Protein bands of Crude extract and purified ChoK protein; (3) & (4): Activity staining bands of crude extract and purified ChoK. (B) Chitinase activity of strain KNU3. (1): Protein bands of crude extract (2): Activity staining of crude extract.

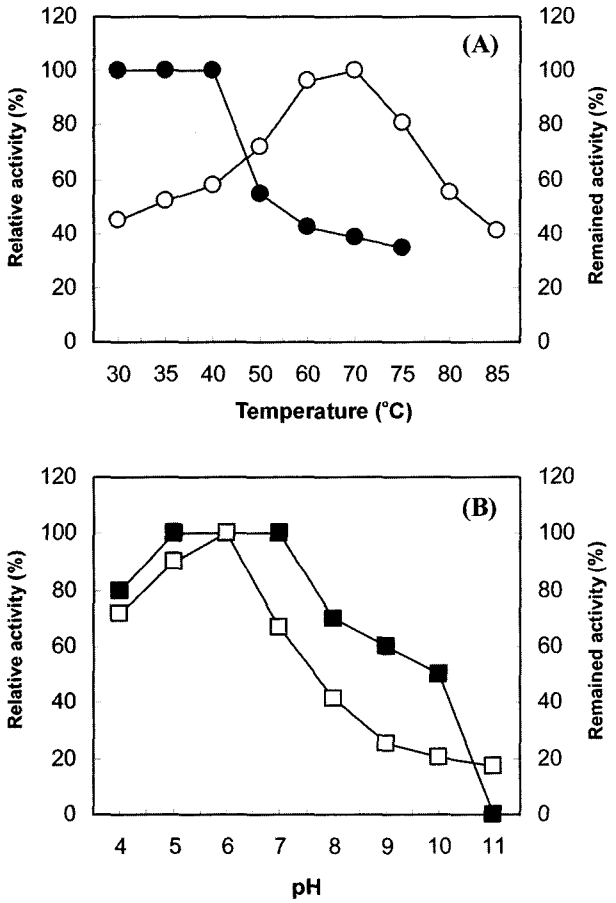

Fig. 3. Enzymatic properties of purified ChoK protein.
(A) Temperature effect (○) and thermostability (●), (B) pH effect (□) and pH stability (■).

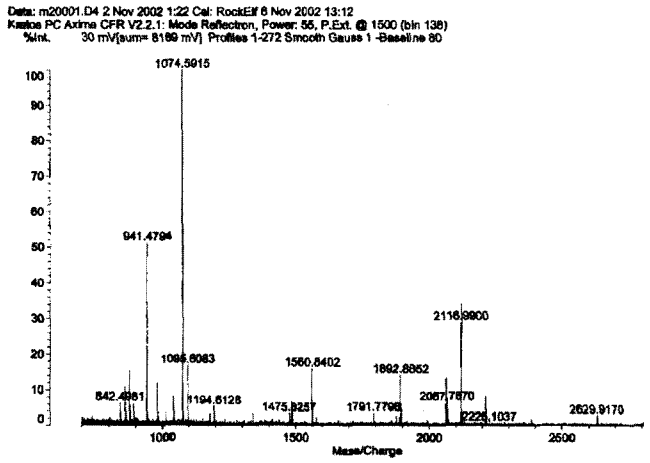

Fig. 4. MALDI-TOF mass fingerprinting analysis of purified ChoK protein.

and carboxymethyl cellulose were not degraded by the purified enzyme. The existence of separate chitinases of KNU3 could be responsible for chitin degradation.

Effects of Metal Ions on Chitosanase Activity

The effect of several metal ions on the activity of the purified chitosanase was tested. Initially, the dependence of chitosanase activity on metal ions was determined by chelating with EDTA, and only a small faction of the activity (=15%) remained after EDTA addition, indicating the dependence of the enzyme reaction on metal ions. Chitosanolytic activity was further assayed after adding Co^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Zn^{+}, or Ca^{2+}. As shown in Table 4. the chitosanase was greatly activated by the addition of Co^{2+} and Cu^{2+}, but inhibited when Zn^{+} and Ca^{2+} were added.

Analysis of Hydrolysis Products

The hydrolysis products of chitosan by the purified enzyme were analyzed by HPLC chromatography (Fig. 5). When soluble chitosan was used as a substrate, tetramers and hexamers of glucosamine were detected as major products after 24 h of incubation at 37°C. These data suggest that the chitosanlolytic pattern of ChoK might be an endo-splitting type.

Table 3. Effects of substrate specificity on the chitosanase activity.

Substrate	Relative activity (%)
Soluble chitosan	100
Colloidal chitosan	19.1
Glycol chitosan	2.4
CM- cellulose	ND
Colloidal chitin	ND
Glycol chitin	ND

ND: not detected.

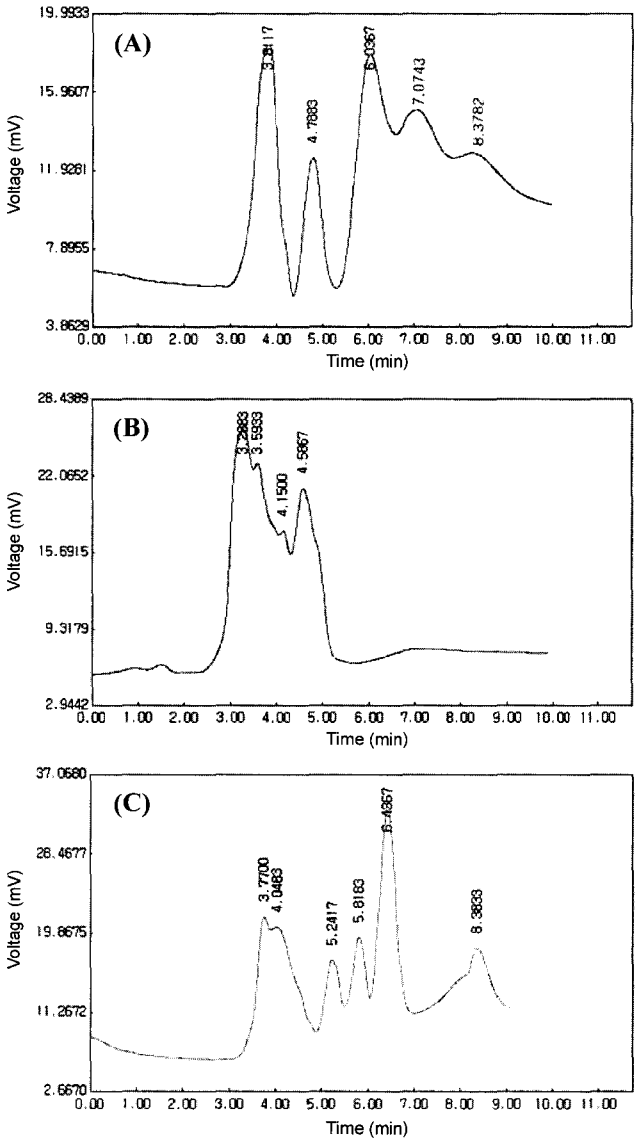

Fig. 5. HPLC chromatogram of chitosan hydrolysates by the chitosanase of β-*Proteobacterium* KNU3.
(A) Standards: N-acetylglucosamine oligomers from dimers $(GlcN)_2$ to hexamers $(GlcN)_6$, (B) Negative control: soluble chitosan without enzyme addition. (C) Enzymatic hydrolysates for 24 h incubation.

Table 4. Effects of metal ions on the activity of chitosanase (ChoK) from β-*Proteobacterium* KNU3.

Metal ion	Relative activity (%)
None	100
EDTA	15
Co^{2+}	185
Cu^{2+}	159
Fe^{2+}	119
K^{+}	98
Mg^{2+}	79
Zn^{+}	69
Ca^{2+}	67

Discussion

We have described here the characterization of a chitosanase-producing chinitinolytic bacterium isolated from soil in Korea. This strain was classified as the β-subgroup of *Proteobacteria* by virtue of its morphological and physiological properties and by phylogenetic studies based on analysis of the 16S rRNA gene sequences. The 16S rRNA sequence of strain KNU3 showed high similarity (99%) to that of chitinolytic β-*Proteobacterium* CTE108 (GenBank accession number, AJ496446). At present, however, it is not clear whether the two strains are related evolutionarily. Nevertheless, the chitinolytic activity of CTE108 (unpublished results) further supports its phylogenetic classification as β-*Proteobacterium*.

The purified mature ChoK protein was identified by N-terminal amino acid sequencing and MALDI-TOF MS analyses. The high similarity in both N-terminal amino acid sequence and internal partial peptide sequence suggests that ChoK protein is evolutionarily related to chitosanases from *Matsuebacter chitosanotabidus* and *Sphingobacter multivorum* in amino acid sequence level (data not shown). We tried to clone an unknown chitosanase gene from KNU3 by degenerated PCR primers based on two related known genes described above. However, none of the degenerated primers resulted in any PCR products (data not shown). These results suggest that the nucleotide sequence of ChoK might be somewhat different from those of *Matsuebacter* sp. or *Sphingobacter* sp. To eliminate any potential errors in the phylogenic analysis, API bacterial identification kit NE20 was used, and its results clearly demonstrated that KNU3 did not belong to *Sphingobacter* sp. (data not shown). In contrast, Table 1 shows that the two β-*Proteobacteria*, KNU3 and *Matsuebacter* sp., are almost similar in biochemical analyses, except for H_2S production and xylose utilization. The 98% identity of the 16S rRNA gene between KNU3 and *Matsuebacter* sp. further supports the conclusion that these bacteria are related with each other (Fig. 1). It was known that the *S. multivorum* mature chitosanase had high homology (87%) with chitosanase obtained from *M. chitosanotabidus*, and both enzymes were classified as a family-80 chitosanase group [10]. These data suggest that the newly identified ChoK protein is related to two chitosanases at the amino acid level.

When the enzymatic properties of purified ChoK were compared with that of *M. chitosanotabidus* 3001, the optimum pH and optimum temperature were 6 and 70°C, respectively (Fig. 3). Even though the optimum temperature of ChoK was unusually high compared to other chitosanases, its thermostability was low (Fig. 3A). Unfortunately, we were unable to compare the thermostability or pH stability of ChoK with other related chitosanases [10, 11] due to lack of comparable data.

Chitosanase purified from KNU3 had high activity toward 95% deacetylated soluble chitosan and also hydrolyzed colloidal chitosan, but it could not degrade the colloidal chitin or carboxymethyl cellulose, indicating that ChoK belongs to the Class III chitosanase family, because its range of substrates is limited to chitosan [22]. The HPLC analysis of the hydrolysis products of soluble chitosan revealed that tetramers and hexamers were major products. The results of HPLC analysis on chitosan oligomers and its genetic similarities with previously reported chitosanases [10, 11] suggest its enzymatic mechanism to be endochitosanase.

In conclusion, we isolated a new chitosanolytic bacterium, belonging to β-*Proteobacteria*, with chitinolytic activity. The chitosanase of strain KNU3 was induced by the substrate chitosan, and the purified chitosanase (ChoK) degraded only chitosan derivatives. We are now in a process to purify the two chitinases in strain KNU3. The characterization of the chitinases will help to elucidate the enzymatic degradation mechanism of chitin and chitosan in this strain.

Acknowledgments

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References

1. Fenton, D. M. and E. D. Eveleigh. 1981. Purification and mode of action of a chitosanase from *Penicillium islandicum*. *J. Gen. Microbiol.* **126:** 151-165.
2. Hong, I. P., H. K. Jang, S. Y. Lee, and S. G. Choi. 2003. Cloning and Characterization of a bifunctional cellulase-chitosanase gene from *Bacillus licheniformis* NBL420. *J. Microbiol. Biotechnol.* **13:** 35-42.
3. Isabelle, B., A. Dupuy, and P. Vidal. 1992. Purification and characterization of a chitosanase from *Streptomyces* N174. *Appl. Microbiol. Biotechnol.* **38:** 188-193.
4. Japanase Society for Chitin and Chitosan. 1990. *Application of Chitin and Chitosan*, pp. 71-98. Kibodang Publisher. Tokyo. Japan.
5. Jo, Y. Y., K. J. Jo, Y. L. Jin, W. J. Jung, J. H. Kuk, K. Y. Kim, T. H. Kim, and R. D. Park. 2003. Characterization of endochitosanases-producing *Bacillus cereus* P16. *J. Microbiol. Biotechnol.* **13:** 960-968.
6. Kazutoshi, Y., J. Hosokawa, T. Kubo, M. Nishiyama, and Y. Koba. 1992. Purification and properties of a chitosanase from *Pseudomonas* sp. H-14. *Biosci. Biotech. Biochem.* **56:** 972-973.

7. Kurakake, M., S. You, K. Nakagawa, M. Sugihara, and T. Komaki. 2000. Properties of chitosanase from *Bacillus cereus* S1. *Curr. Microbiol.* **40:** 6-9.
8. Lee, H. W., J. W. Choi, D. P. Han, N. W. Lee, S. L. Park, and D. H. Yi. 1996. Purification and characteristics of chitosanase from *Bacillus* sp. HW-002. *J. Microbiol. Biotechnol.* **6:** 19-25.
9. Masato, I., S. Nagae, H. Kawagishi, M. Mitsuromi, and A. Ohtakara. 1992. Action pattern of *Bacillus* sp. No. 7-M chitosanase on partially N-acetylated chitosan. *Biosci. Biotech. Biochem.* **56:** 448-453.
10. Matsuda, Y., Y. Iida, T. Shinogi, K. Kakutani, T. Nonomura, and H. Toyoda. 2001. *In Vitro* suppression of mycelial growth of *Fusarium oxysporum* by extracellular chitosanase of *Sphingobacterium multivorum* and cloning of the chitosanase gene *csn*SM1. *J. Gen. Plant Pathol.* **67:** 318-323.
11. Park, J. K., K. Shimono, N. Ochiai, K. Shigeru, M. Kurita, Y. Ohta, K. Tanaka, H. Matsuda, and M. Kawamukai. 1999. Purification, characterization, and gene anlaysis of a chitosanase (choA) from *Matsuebacter chitsanotbidus* 3001. *J. Bacteriol.* **181:** 6642-6649.
12. Shimosaka, M., Y. Fukumori, T. Narita, X. Y. Zhang, N. J. He, R. Kodaira, M. Nogawa, and M. Okazaki. 2001. The bacterium *Burkholderia gladioli* strain CHB101 produces two different kinds of chitinases belonging to families 18 and 19 of the glycosyl hydrolases. *J. Biosci. Bioeng.* **91:** 103-105.
13. Shimosaka, M., Y. Fukumori, X. Y. Zhang, N. J. He, R. Kodaira, and M. Okazaki. 2000. Molecular cloning and characterization of a chitosanase from the chitosanolytic bacterium *Burholderia gladioli* strain CHB101. *Appl. Microbiol. Biotechnol.* **54:** 354-360.
14. Somashekar, D. and R. Joseph. 1996. Chitosanase-properties and applications: a review. *Bioresource Technol.* **55:** 35-45.
15. Tanabe, T., K. Morinaga, T. Fukamizo, and M. Mitsutomi. 2003. Novel chitosanase from *Streptomyces griseus* HUT6037. *Biosci. Biotech. Biochem.* **67:** 354-364.
16. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173:** 697-703.
17. Yamasaki, Y., I. Hayashi, Y. Ohta, T. Nakagawa, M. Kawamukai, and H. Matsuda 1993. Purification and mode of action of chitosanolytic enzyme from *Enterobacter* sp. G-1. *Biosci. Biotech. Biochem.* **57:** 444-449.
18. Yasushi, U. and A. Ohtakara. 1988. Chitosanase from *Bacillus* species. *Methods in Enzymol.* **161:** 501-505.
19. Yoon, H. G., H. Y. Kim, Y. H. Lim, H. K. Kim, D. H. Shin, B. S. Hong, and H. Y. Cho. 2000. Thermostable chitosanase from *Bacillus* sp. Strain CK4: Cloning and expression of the gene and characterization of the enzyme. *Appl. Environ. Microbiol.* **66:** 3727-3734.
20. Yoon, H. G., K. H. Lee, H. Y. Kim, H. K. Kim, D. H. Shin, B. S. Hong, and H. Y. Cho. 2002. Gene cloning and biochemical analysis of thermostable chitosanase (TCH-2) from *Bacillus coagulans* CK108. *Biosci. Biotechnol. Biochem.* **66:** 986-995.
21. Yoshihara, K., J. Hosokawa, T. Kubo, M. Nishiyama, and Y. Koba. 1992. Purification and properties of chitosanase from *Pseudomonas* sp. H-14. *Biosci. Biotech. Biochem.* **56:** 972-973.
22. Zhu, X. F., X. Y. Wu, and Y. Dai. 2003. Fermentation conditions and properties of a chitosanase *Acinetobacter* sp. C-17. *Biosci. Biotechnol. Biochem.* **67:** 284-290.