JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification, Characterization, and Gene Cloning of a Chitosanase from *Bacillus* Species Strain S65

CAIXIN SU, DONGMEI WANG, LIMING YAO, AND ZENGLIANG YU*

Ion Beam Bioengineering Key Lab, Institute of Plasma Physics, Chinese Academy of Sciences, 230031, Hefei 1126#, People's Republic of China

For the production of oligosaccharides from chitosan, a chitosanase-producing bacterium, S65, was isolated from soil. On the basis of phylogenetic analysis of the 16S rDNA gene sequence and phenotypic analysis, S65 was identified as a *Bacillus* sp. strain. This bacterium constitutively produced chitosanase in a culture medium without chitosan as an inducer. S65 chitosanase was homogeneously purified by DEAE Sepharose fast flow anion exchange followed by Superdex 75 size exclusion, and the molecular weight was 45 kDa according to SDS−PAGE. Enzyme analysis showed that the optimum pH and temperature of S65 were 6.0 and 65 °C, respectively. Catalytic activity was stable from pH 5.5−6.5 at temperatures below 40 °C, and the pI of chitosanase was about 6.0 as determined by a test tube method. S65 chitosanase degraded carboxymethyl cellulose (CMC) at the degree of about 5.3% relative to the value of soluble chitosan, but it cannot hydrolyze colloidal chitin and crystalline cellulose. Gene encoding was cloned and sequenced. The deduced amino acid sequence of the S65 exhibited the highest homology to those of family 8 glycanase, suggesting that the enzyme belonged to family 8.

KEYWORDS: Chitosan; chitosanase; purification; characterization; gene cloning

INTRODUCTION

Chitin, the β-(1−4)-linked homopolymer of *N*-acetyl-D-glucosamine, is the second most abundant polysaccharide with an annual production of 10^{10} to 10^{11} tons per annum, which is only next to that of cellulose. They are commercially obtained from shrimp and crab shell in the fishing industry (*1*). Despite its abundance in nature, the commercial utilization of chitin has remained undeveloped due to its insolubility in common solvents (*2*). Due to the shortage of the global resource and the crisis of environmental pollution, there is growing interest in exploiting renewable chitin for the production of biodegradable polymers.

Chitosan, a D-glucosamine polymer, is a totally or partially deacetylated derivative of chitin. Even chitosan has very strong functional properties in many areas, such as medicine, agriculture, and environmental treatment (*3*−*7*); its high molecular weight and high viscosity may restrict the use in vivo. In addition, there is no doubt that such properties will influence absorption in the human intestine.

Chitosan oligomers possess additional functional properties such as antifungal (*8*, *9*), antimicrobial (*10*−*12*), antitumor (*13*, *14*), immuno-enhancing, and weight-losing effects (*15*, *16*). Moreover, they have lower viscosities, lower molecular weights, and shorter chain lengths and are soluble in neutral aqueous solutions. Therefore, converting chitosan to water-soluble oligosaccharides has attracted an increasing attention, especially in the functional food and medicine industries.

* Corresponding author. Phone: 086-0551-5592189. Fax: 086-0551-5591310. E-mail: zlyu@ipp.ac.cn.

Traditionally, chitosan oligosaccharides were processed by chemical methods in industries. There are many problems existing in chemical processes, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides (*17*), high cost in separation, and also environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, and reproducibility, chitosanase hydrolysis becomes more and more popular in recent years.

Generally, chitosanases have been recognized as enzymes that attack chitosan but not chitin. In 2004, the Enzyme Commission amended the definition of chitosanase, and it is now defined as the enzyme performing endohydrolysis of β-1,4-linkages between D-glucosamine residues in a partly acetylated chitosan. Since the first report of chitosanase (*18*), chitosanases have been found in a variety of microorganisms, including virus (*19*, *20*), actinomycetes (*21*), fungi (*22*), and bacteria (*23*−*32*). Among them, *Bacillus* and *Streptomyces* strains are most often studied.

In an attempt to obtain a chitosanase that can be used for large-scale production of chitosan oligosaccharides, a bacterial strain with high chitosanase activity, *Bacillus* sp. strain S65, was isolated from soil on the bank of a lake. This bacterium, which has the technical advantage during fermentation, can produce chitosanase without chitosan as an inducer. This paper describes the purification, characterization, and gene cloning of the chitosanase from *Bacillus* sp. strain S65.

MATERIALS AND METHODS

Materials. Chitosan with different DDA (degree of deacetylation) and chitin were purchased from local suppliers in China. DEAE Sepharose FF, Superdex 75 pg (Hiload 16/60), and the AKTA explorer

10.1021/jf0600556 CCC: $33.50
Published on Web 05/17/2006

100 protein purification instrument were from Amersham-Biosciences. Cross-flow superfiltration 10 kDa membrane (Pellicon pack) and Amicon stirred cells instruments were from Millipore Corporation. D-Glucosamine was purchased from Sigma-Aldrich. All other reagents were of analytical grade.

Screening of Chitosanase-Producing Microorganisms. *Primary Screen.* The chitosanase-detection agar (CDA) plate with chitosan as the sole carbon source was used to screen the microorganisms. A volume of 1 L of medium for the CDA broth was prepared by mixing 10 g of chitosan (predissolved in 200 mL of 1% acetic acid) with the following salts: Na_2HPO_4 (1.3 g), KH_2PO_4 (3.0 g), NaCl (0.5 g), NH_4Cl (1.0 g), $MgSO_4$ (0.24 g), and $CaCl_2$ (0.01 g). For the CDA plate, 2% agar was added. Soil samples from the bank of a local lake were suspended in sterilized distilled water, spread onto the CDA plate, and incubated at 29−30 °C for 4−5 days. Chitosanase activity can be indicated by the clear zone formed on the CDA plate.

Secondary Screen. Strains with big clear zones were selected for the secondary screen. These strains were cultivated in CDA broth at 29−30 °C for 5 days on a rotary shaker at 120 rpm. At 1 day intervals, the microorganisms were removed from the culture broths by centrifugation (12 000 rpm, 10 min) and the supernatants were used for chitosanase assay.

Assay of Chitosanase Activity. Chitosanase activity was determined by measuring the reducing sugars liberated during the hydrolysis of the chitosan with the 3,5-dinitrosalicylic acid method. For the secondary screen of microorganisms, the assay was performed by mixing 2 mL of 1% chitosan (predissolved in 200 mL of 1% acetic acid) pH 6.0, with 2 mL of suitably diluted enzyme. After incubation for 10, 20, and 30 min, individually at 37 °C, hydrolysis reactions were terminated and analyzed by adding 4 mL of dinitrosalicylic acid reagent. The mixture was boiled for 10 min, chilled, and centrifuged to remove insoluble chitosan. The resulting adducts of reducing sugars were measured spectrophotometrically at 520 nm. One unit of chitosanase activity was defined as the amount of enzyme required to release 1 μmol of detectable reducing sugar in 1 min, with D-glucosamine as the standard. For the rest of the experiments, the incubation temperature was changed to 65 °C, and time was fixed at 10 min.

Analysis of Biochemical and Physiological Properties of Strain S65. The biochemical and physiological characteristics of the isolated strain were determined by *Bergey's Manual of Systematic Bacteriology* (*33*).

PCR Amplification of the 16S rDNA Gene. PCR was performed to amplify the 16S rDNA coding region, using two oligonucleotide primers, 5′-GAGAGTTTGATCCTGGCTCAG-3′ (relative to *E. coli* 16S rDNA positions 8−27) and 5′-GCCCCCGTCAATTCCTTTGAG-3′ (relative to *E. coli* 16S rDNA positions 910−931). The PCR program was 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Following the cycles is a final extension step at 72 °C for 10 min. The PCR products of the expected sizes were 900 bp. Following amplification, the PCR product was purified (DNA gel extraction kit, V-gene) and sequenced with an ABI PRISMTM 377XL DNA sequencer (Takara Biotechnology Co., Ltd.).

Purification of Chitosanase. *Bacillus* sp. S65 was cultivated by 50 mL of liquid medium containing 1% glucose, 0.5% yeast extract, and 0.2% Na_2HPO_4 in a 250 mL flask at 30 °C for 60−65 h. The speed of the rotary shaker is 180 rpm. After centrifugation of the culture broth (2 L) at 6000*g* for 10 min, the supernatant solution was purified by the following steps. Step 1: the crude enzyme solution was superfiltered in cross-flow by a 10 kDa membrane (Pellicon pack). Step 2: the superfiltered sample was applied to anion-exchange chromatography column (DEAE sepharose FF). Proteins were eluted with a linear gradient of 0−1 mol/L NaCl with 10 mmol/L PBS buffer (pH 6.5) at a flow rate of 1 mL/min. Fractions were collected according to the peaks at 280 nm. Step 3: fractions with chitosanase activity were pooled, concentrated (Amicon stirred cell), and applied to gel filtration on Superdex 75 pg preequilibrated with 10 mmol/L sodium acetate buffer (pH 5.6). Proteins were eluted with the same buffer at a flow rate of 1 mL/min.

SDS−PAGE. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out by using the method of Laemmli (*34*). Proteins were stained with 0.05% Coomassie brilliant blue R-250.

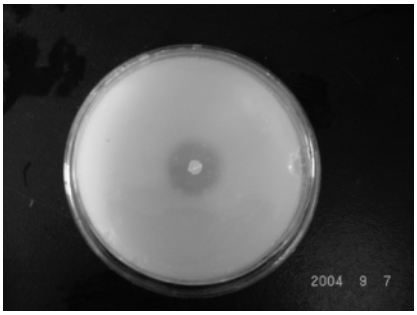

Figure 1. Strain S65 on a CDA plate with a clear zone.

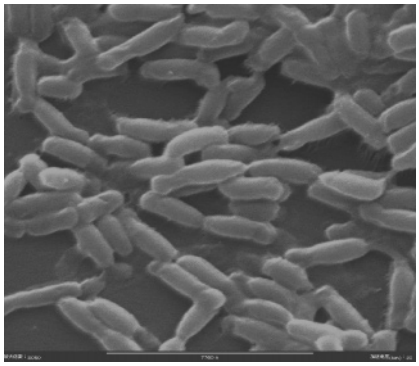

Figure 2. SEM image of S65 (×8050).

Test Tube Method to Determine the Isoelectric Point of Chitosanase. Put 1 mL of DEAE Sepharose ion exchanger into each of nine test tubes and equilibrate the media to different pH values, respectively, by washing 10 times with 10 mL of 0.02 mol/L buffer (pH 4−5.5 sodium acetate buffer, pH6−8 PBS buffer, with 0.5 pH unit intervals between tubes). Then add 2 mL of chitosanase sample (preequilibrated with the corresponding pH buffer) and mix the contents of the tubes for 5−10 min. At last, centrifuge the tubes and analyze the supernatants for chitosanase.

Cloning and Sequencing of the Gene Encoding Chitosanase. Chitosanase gene was amplified by PCR using chromosomal DNA isolated from S65 as a template. Two conserved regions selected from the published *Bacillus* chitosanase sequences were used to design two primers, and the sequences were listed as the following: primer 1, 5′-TTAATTATCGTATCCTTCATAG-3′, and primer 2, 5′-GCATTT-TAAAAGGAGCTGACAAACC-3′. The PCR product was prepared in pBST vector and transformed into *E. coli* top10 with the TA cloning method. The transformants were cultivated on LB medium containing ampicillin. Then, the nucleotide sequence of the chitosanase from *E. coli* top10 was determined by the ABI Prism model 377.

RESULTS

Isolation and Initial Identification of Bacterial Strain S65. The soil sample was selected from the bank of a local lake. After the primary screen, more than 10 kinds of strains with clear zones on the CDA plates were obtained, including fungi, actinomyces, and bacteria. Among these microorganisms, strain S65 (**Figure 1**) was ultimately selected as a chitosanase producer because it showed the highest chitosanase activity. **Figure 2** shows the scanning electron microscopy (SEM) image of S65.

The taxonomic identification was performed on the basis of the following facts. The organism was Gram positive; it was in rod shape (diameter, 0.9−1.1 μm); it could form round end spore; it was positive in the following tests: catalase reaction, Voges−Proskauer test, casein hydrolysis, gelatine liquefaction, starch hydrolysis, nitrate reduction, lecithinase activity; it could hydrolyze tyrosine; it could convert glycerol to dihydroxy acetone; it was negative by indole production. It resembled *Bacillus cereus* in morphological and biochemical characteris-

Table 1. Purification of Chitosanase from *Bacillus* Species S65

step	total vol (mL)	total activity (units)	total protein (mg)	specific activity (units·mg^{-1} protein)	purification (fold)	recovery (%)
crude	800	1162	144.3	8.05	1	
cross-flow superfiltration	100	1058	67.8	15.6	1.94	91
DEAE Sepharose FF	56	905.5	30.6	29.6	3.68	77.9
Superdex 75 gel filtration	48	664.3	7.1	93.6	11.63	57.2

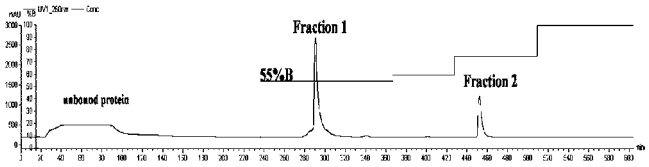

Figure 3. Separation of chitosanase by anion-exchange chromatography.

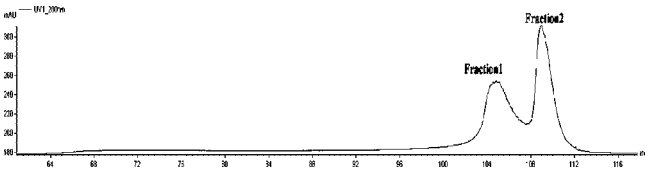

Figure 4. Separation of chitosanase by gel chromatography.

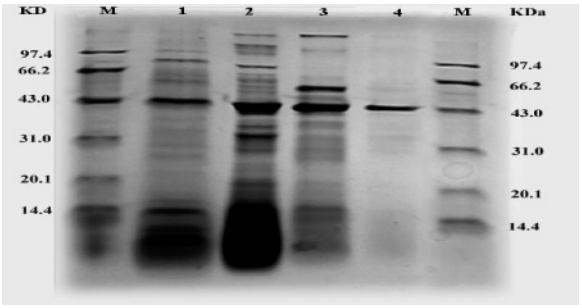

Figure 5. SDS–PAGE of purified chitosanase: M, standard marker proteins; 1, fermentation supernatant; 2, cross-flow superfiltration; 3, DEAE Sepharose FF chromatography; 4, Superdex 75 gel filtration.

tics. The strain was tentatively designated as belonging to *Bacillus* sp. and named *Bacillus* sp. strain S65.

Purification of Chitosanase. Crude enzyme solution prepared by cross-flow superfiltration was applied to an anion-exchange chromatography column (DEAE Sepharose FF). As shown in **Figure 3**, chitosanase (fraction 1) was eluted by 55% 1 mol/L NaCl and separated completely with unbound protein and other proteins in fraction 2. Proteins with chitosanase activity were then concentrated (Amicon stirred cell) and applied to Superdex 75 pg (**Figure 4**), and chitosanase of the eluted active fraction (fraction 1) was almost homogeneous on SDS−PAGE (**Figure 5**). The molecular weight was estimated to 45 000 Da. The purification steps from culture broth are summarized and shown in **Table 1**.

Effects of pH and Temperature on Chitosanase. Chitosanase activity was measured at various pHs under the stated condition, using sodium acetate (pH 4−5.6) and PBS (pH 6−8) buffers. **Figure 6** shows the effects of pH on S65 chitosanase. The optimum pH was about 6, and the stable pH for the enzyme activity in the incubation at 28 °C for 60 min was 5.5−6.5. As shown in **Figure 7**, the optimum temperature was around 65 °C and enzyme activity was relatively stable below 40 °C in the incubation at pH 6 for 1 h.

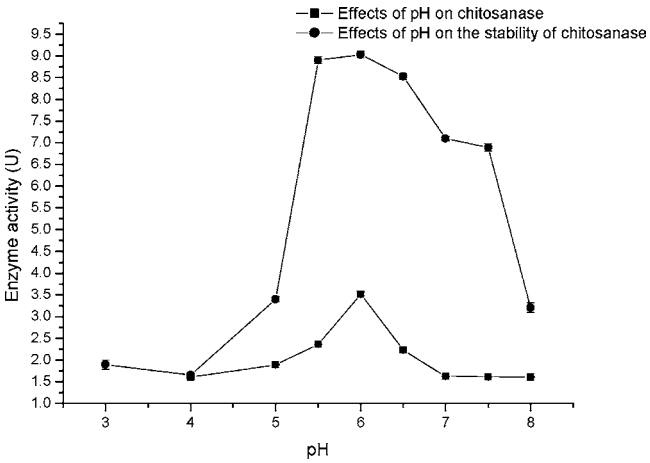

Figure 6. Effects of pH on chitosanase and stability of chitosanase.

Table 2. Effects of Solvent on Chitosanase Activity

solvent	enzyme activity (U)
1% acetic acid	10.81
1% citric acid	1.59
1% hydrochloric acid	9.18
1% lactic acid	8.9

Effect of Solvents on Chitosanase. Solvents were prepared as follows: 1 g of chitosan was predissolved in 20 mL of different solvents (**Table 2**), then 80 mL of water was added. The chitosanase was incubated in the solvent, and the activity was assayed. As shown in **Table 2**, acetic acid was the best solvent for chitosanase activity.

Effect of Metal Ions and Other Reagents on Chitosanase Activity. Chitosanase activity was assayed after the addition of metal ions Co^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, Ba^{2+}, Ni^{2+}, Sr^{2+}, Zn^{2+}, and Fe^{2+} to the reaction mixtures at a final concentration of 1, 5, and 10 mmol/L, respectively (**Table 3**). When the concentration ranged from 1 to 10 mmol/L, Mn^{2+} could significantly improve the enzyme activity, while Fe^{3+} could significantly inhibit the enzyme activity, and Co^{2+}, Ba^{2+}, Ni^{2+}, and Sr^{2+} showed different inhibition to the enzyme activity. Cu^{2+} and Zn^{2+} could improve the enzyme activity at the concentration of 1 mmol/L but significantly inhibited it when the concentration was more than 1 mmol/L. Ca^{2+} could improve the enzyme activity at the concentration of 5 mmol/L and inhibited it when the concentration was higher or lower than 5 mmol/L. For Fe^{2+}, 10 mmol/L could improve the enzyme activity, and when the concentration was lower than 10 mmol/L, the activity was inhibited markedly.

Substrate Specificity. Of 90% DDA chitosan, 85% DDA chitosan, 80% DDA chitosan, colloidal chitin, crystalline cellulose, and CMC, the chitosanase showed the highest activity for 85% DDA chitosan. The enzyme could not hydrolyze

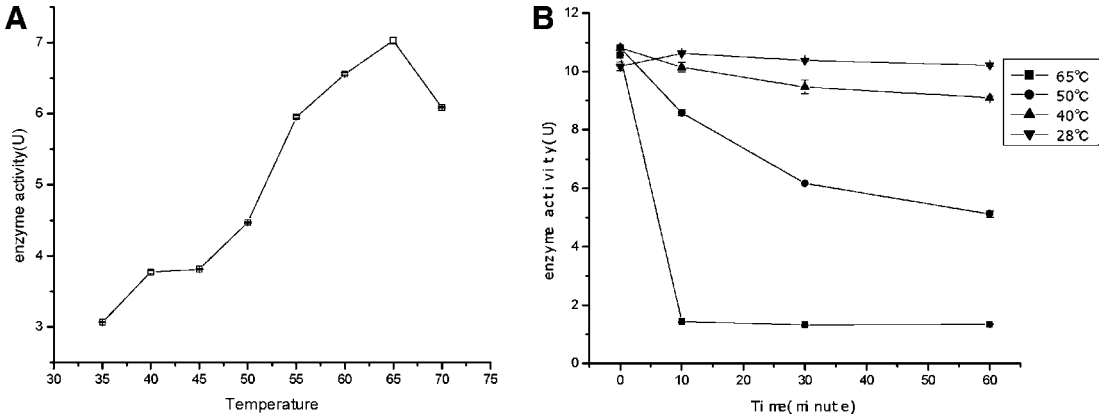

Figure 7. (**A**) Effects of temperature on chitosanase. (**B**) Effects of temperature on the stability of chitosanase.

Table 3. Effect of Metal Ions on the Activity of S65 Chitosanase

	relative activity (%)				relative activity (%)		
reagent	10 mmol/L	5 mmol/L	1 mmol/L	reagent	10 mmol/L	5 mmol/L	1 mmol/L
none	100	100	100	$FeCl_2$	119.3	58.2	19.3
$CoCl_2$	67.2	82.7	102.9	$BaCl_2$	96.2	88.5	86.3
$CaCl_2$	51.4	115	90.0	$NiCl_2$	92.3	87.9	81.2
$MgCl_2$	102.9	99	101.8	$SrCl_2$	95.6	83.5	98.8
$MnCl_2$	126.4	137.7	141.2	$ZnCl_2$	57.2	98.3	124.3
$CuCl_2$	31.1	67.1	119	$FeCl_3$	18.4	16.9	15.2

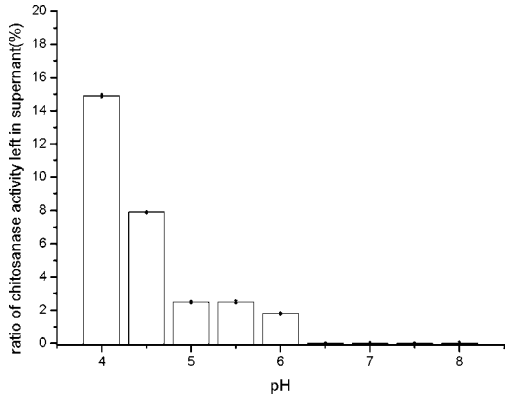

Figure 8. Test tube method to determine the pI of chitosanase.

colloidal chitin or crystalline cellulose, and it hydrolyzed CMC only by 5.3% of the activity for soluble chitosan.

Test Tube Method To Determine the pI of Chitosanase. As shown in **Figure 8**, the chitosanase bound completely at pH 6.5 and above, which indicated that the pI of chitosanase was about 6−6.5.

Analysis of *Bacillus* Species S65 Chitosanase Gene. The nucleotide sequence of *Bacillus* sp. S65 chitosanase is shown in **Figure 9**. Analysis with DNAman indicated an open reading frame of 1359 bp and encoded a 453-amino acid precursor enzyme. The promoter-like sequence and typical Shine−Dalgarno (SD) sequence were observed upstream from the possible ATG start codon.

DISCUSSION

To obtain a chitosanase that can be used for large scale production of chitosan oligosaccharides, *Bacillus* sp. strain S65 was isolated from the soil on the bank of a lake. With three simple separation and purification steps, chitosanase was purified to homogeneity, and its molecular mass was 45 kDa on SDS−PAGE.

Since the first report of *Bacillus* chitosanase (*35*), more than 14 *Bacillus* strains have been reported to produce chitosanase (*35*−*40*), which indicates the ubiquity of *Bacillus* chitosanase in the environment. *Bacillus* species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular medium, and the GRAS (generally regarded as safe) status with the Food and Drug Administration for species, such as *B. subtilis* and *B. licheniformis* (*41*). In addition, much is now known about the biochemistry, physiology, and genetics of *B. subtilis* and other species, which facilitates further development and greater exploitation of these organisms in industrial processes.

In most cases, bacterial chitosanases are inducible by the substrate chitosan. Yet for S65 chitosanase, we found that it was produced in the absence of the substrate chitosan in the medium, and exogenously added chitosan could not be used efficiently as a carbon source. Moreover, in fermentation condition, chitosan added to the medium inhibited the cell growth to some degree. In comparison to an inducible enzyme, a constitutive one has more advantage in fermentation condition and commercial cost, which has gained general acceptance (*31*, *32*).

According to sequence-based classification of glycosyl hydrolases by Henrissat and Bairoch (*42*), S65 chitosanase belongs to family 8. Until now, gene coding and three-dimensional (3D) structures have been reported for chitosanases from *Streptomyces* sp. N174 (family 46) (*43*), *Bacillus circulans* (family 46) (*44*), and *Bacillus* sp. K17 (family 8) (*45*). These 3D structures make it feasible to elucidate the precise reaction mechanism in bacterial chitosanases. The glutamic and aspartic acid residues, previously identified as essential for catalytic activity in the family 8 endo-glucanase K from *Bacillus* sp. KSM-330 (*46*), are conserved in S65 chitosanase at positions 122 and 183, respectively. The deduced amino acid sequence of S65 chitosanase showed identity of 90% to K17 chitosanase. Comparison of the deduced amino acid sequences of *Bacillus* sp. K17 and S65 chitosanase reveals nine different amino acids, among which 212Lys-Gln, 229Arg-Gln locate near the glycosyl hydrolases family 8 domain, ATDGDLDIAYSLLLAHKQW (*42*), and the

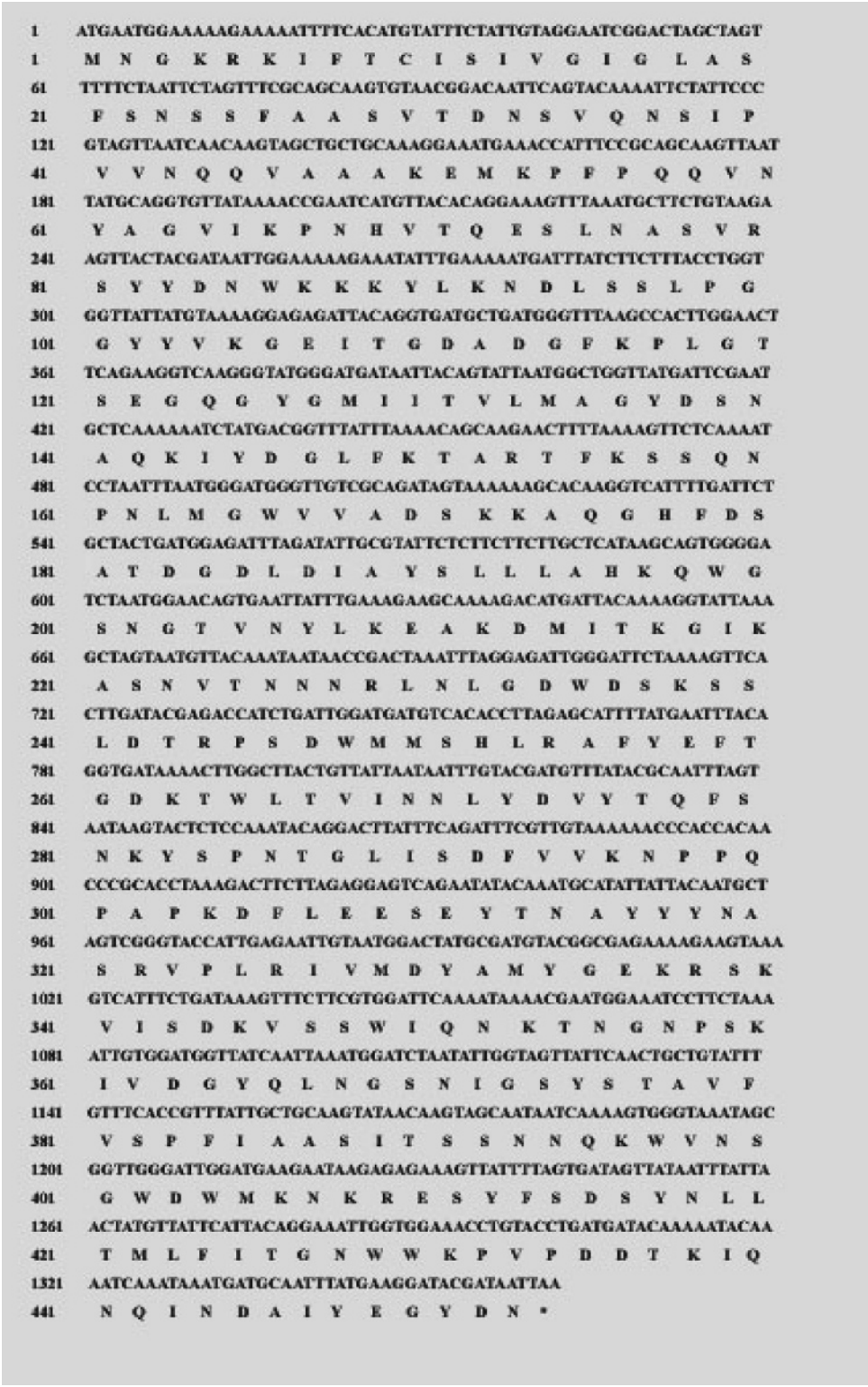

Figure 9. Nucleotide and deduced amino acid sequence of S65.

other four changes locate in the c terminal of the enzyme. The essential amino acid residues for catalytic function and reaction are not clear. Further data on the 3D structure and functional domains are required to define the catalytic center and minimal region required for the activity. Protein engineering study and mutation analysis are now in progress to elucidate the catalytic mechanism and to modify the enzymatic characteristics such as substrate specificity.

In the study of the metal ions' effect, we found a peculiar characteristic of S65 chitosanase which has not been found in other chitosanase. A concentration of 1 mmol/L Fe^{2+} can significantly inhibit the activity of chitosanase (80.7%), while with the increase of the concentration of Fe^{2+}, the inhibition was reduced, and even 10 mmol/L Fe^{2+} can improve the activity by 19.3%.

It is noteworthy that the level of chitosanlytic activity of *Bacillus* bacterial (*Bacillus* KCTC 0377BP, 100 U/mL) (*32*) was fairly high as compared to fungal chitosanase (*Aspergillus* CJ22-326, 3.61 U/mL; *Aspergillus oryzae* IAM2660, 0.05 U/mL; *Fusarium solani*, 0.0015 U/mL) (*47*), which verifies the importance and advantages of *Bacillus* in the applied microbi-

ology. The coding sequence of S65 is different from that of KCTC 0377BP with exclusively one amino acid (212Lys-Gln), yet the yield of S65 chitosanase is lower than that of KCTC 0377BP, 16 U/mL. Besides the activity difference induced by the amino acid change (212Lys-Gln), the difference of the promoter sequence is a potential factor for the high yield of KCTC 0377BP, which needs more exploration to test and will provide a new method to improve the yield of the enzyme.

In conclusion, we have purified and characterized a *Bacillus* chitosanase S65 as a potential candidate for biotechnological applications in the industry.

ACKNOWLEDGMENT

We thank Professor Renrui Pan for his technical assistance and Dr. Hourong Xiao, Hefei University, for his critical reading of the manuscript.

LITERATURE CITED

(1) Felse, P. A.; Panda, T. Studies on applications of chitin and its derivatives. *Bioprocess Eng.* **1999**, *20*, 505−512.

(2) Austin, P. R.; Brine, C. J.; Castle, J. E.; Zikakis, J. P. Chitin: new facets of research. *Science* **1981**, *212*, 749−753.

(3) Knorr, D. Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.* **1991**, *45*, 114−122.

(4) Somashekar, D.; Joseph, R. Chitotanase-properties and applications: a review. *Bioresour. Technol.* **1986**, *55*, 35−45.

(5) Ueno, H.; Yamada, H.; Tanaka, I.; Kaba, N.; Matsuura, M.; Okumura, M.; Kadosawa, T.; Fujinaga, T. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. *Biomaterials* **1999**, *20*, 1407−1414.

(6) Rhoades, J.; Roller, S. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Appl. Environ. Microbiol.* **2000**, *66*, 80−86.

(7) Ben-Shalom, N.; Ardi, R.; Pinto, R.; Aki, C.; Fallik, E. Controlling gray mould caused by Botrytis cinerea in cucumber plants by means of chitosan. *Crop Prot.* **2003**, *22*, 295−290.

(8) Hirano, S.; Nagao, N. Effect of chitosan, pectic acid, lysozyme and chitinase on the growth of several photopathogens. *Agric. Biol. Chem.* **1989**, *53*, 3056−3061.

(9) Kobayashi, M.; Watanabe, T.; Suzuki, S.; Suzuki, M. Effect of *N*-acetylchitohexaose against *Candida albicans* infection of tumor-bearing mice. *Microbiol. Immunol.* **1990**, *34*, 413−426.

(10) Amako, K.; Shimodori, S.; Imoto, T.; Miake, S.; Umeda, A. Effect of chitin and its soluble derivatives on the survival of *Vibrio cholerae* O1 at low temperature. *Appl. Environ. Microbiol.* **1987**, *53*, 603−605.

(11) Tokoro, A.; Kobayashi, M.; Tatewaki, N.; Suzuki, K.; Okawa, Y.; Mikami, T.; Suzuki, S.; Suzuki, M. Protective effect of *N*-acetylchitohexaose on *Listeria monocytogenes* infection in mice. *Microbiol. Immunol.* **1989**, *33*, 357−367.

(12) Jeon, Y. J.; Park, P. J.; Kim, S. K. Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydr. Polym.* **2001**, *44*, 71−76.

(13) Suzuki, K.; Mikami, T.; Okawa, Y.; Tokoro, A.; Suzuki, S.; Suzuki, M. Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydr. Res.* **1986**, *151*, 403−408.

(14) Tokoro, A.; Tatewaki, N.; Suzuki, K.; Mikami, T.; Suzuki, S.; Suzuki, M. Growth-inhibitory effect of hexa-*N*-acetylchitohexaose against Meth-A solid tumor. *Chem. Pharm. Bull.* **1988**, *36*, 784−790.

(15) Suzuki, K.; Tokoro, A.; Okawa, Y.; Suzuki, S.; Suzuki, M. Enhancing effect of *N*-acetyl-chitooligosaccharides on the active oxygen-generating and microbicidal activities of peritoneal exudates cells in mice. *Chem. Pharm. Bull.* **1985**, *33*, 886−888.

(16) Tokoro, A.; Suzuki, K.; Matsumoto, T.; Mikami, T.; Suzuki, S.; Suzuki, M. Chemotactic response of human neutrophilis to *N*-acetyl chitohexaose in vitro. *Microbiol. Immunol.* **1988**, *32*, 387−395.

(17) Rupley, J. A. The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme. *Biochim. Biophys. Acta* **1964**, *83*, 245−255.

(18) Monaghan, R. L.; Douglas, E. E. Chitosanase, a novel enzyme. *Nature (London), New Biol.* **1973**, *245*, 78−80.

(19) Takashi, Y.; Shingo, H.; Puttaporn, S.; Makoto, F. Alternative expression of a chitosanase gene produces two different proteins in cells infected with *Chlorella* virus CVK2. *Virology* **1997**, *230*, 361−368.

(20) Liangwu, S.; Byron, A.; Gurnon, J. R.; Yen, Y.; Van Etten, J. L. Characterization of two chitinase genes and one chitosanase gene encoded by *Chlorella* virus PBCV-1. *Virology* **1999**, *263*, 376−387.

(21) Boucher, I.; Dupuy, A.; Vidal, P.; Neugebauer, W. A.; Brzezinski, R. Purification and characterization of a chitosanase from *Streptomyces* N174. *Appl. Microbiol. Biotechnol.* **1992**, *38*, 188−193.

(22) Fenton, D. M.; Eveleigh, D. E. Purification and mode of action of a chitosanase from *Penicillium islandicum*. *J. Gen. Microbiol.* **1981**, *126*, 151−65.

(23) Yabuki, M.; Uchiyama, A.; Suzuki, K.; Ando, A.; Fujii, T. Purification and properties of chitosanase from *Bacillus circulans* MH-K1. *J. Gen. Appl. Microbiol.* **1988**, *34*, 255−270.

(24) Seino, H.; Tsukud, K.; Shimasue, Y. Properties and action pattern of a chitosanase from *Bacillus* sp. 7I-7S. *Agric. Biol. Chem.* **1991**, *55*, 2421−2423.

(25) Izume, M.; Nagae, S.; Kawagishi, H.; Mitsutomi, M.; Ohtakara, A. Action pattern of *Bacillus* sp. no. 7-M chitosanase on partially *N*-acetylated chitosan. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 448−453.

(26) Yoshihara, K.; Hosokawa, J.; Kubo, T.; Nishiyama, M.; Koba, Y. Purification and properties of a chitosanase from *Pseudomonas* sp. H-14. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 972−3.

(27) Yamasaki, Y.; Hayashi, I.; Ohta, Y.; Nakagawa, T.; Kawamukai, M.; Matsuda, H. Purification and mode of action of chitosanolytic enzyme from *Enterobacter* sp. G-1. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 444−449.

(28) Kurakake, M.; Shou, Y.; Nakagawa, K.; Sugihara, M.; Komaki, T. Properties of chitosanase from *Bacillus cereus* S1. *Curr. Microbiol.* **2000**, *40*, 6−9.

(29) Aktuganov, G. E.; Shirokov, A. V.; Melent'ev, A. I. Isolation and characterization of chitosanase from the strain *Bacillus* sp. 739. *Appl. Biochem. Microbiol.* **2003**, *39*, 469−474.

(30) Chiang, C.-L.; Chang, C.-T.; Sung, H.-Y. Purification and properties of chitosanase from a mutant of *Bacillus subtilis* IMR-NK1. *Enzyme Microb. Technol.* **2003**, *32*, 260−267.

(31) Pyoung Il Kim; Kang Tae Heung Kang; Kyoung Jin Chung; In Sean Kim; Ki-chul Chung. Purification of a constitutive chitosanase produced by *Bacillus* sp. MET 1299 with cloning and expression of the gene. *FEMS Microbiol. Lett.* **2004**, *240*, 31−39.

(32) Choi, Y. J.; Kim, E. J.; Piao, Z; Yun, Y. C.; Shin, Y. C. Purification and characterization of chitosanase from *Bacillus* sp. strain KCTC 0377BP and its application for the production of chitosan oligosaccharides. *Appl. Environ. Microbiol.* **2004**, *70*, 4522−4531.

(33) Holt, J. G.; Sneath, H. A. *Bergey's Manual of Systematic Bacteriology*; The Williams and Wilkins Co.: Baltimore, MD, 1986; Vol. 2, pp 1104−1139.

(34) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(35) Tominaga, Y.; Tsujisaka, Y. Purification and some enzymatic properties of the chotosanase from*Bacillus* R-4 which lyses *Rhizopus* cell walls. *Biochim. Biophys. Acta* **1975**, *410*, 145−155.

(36) Ho-geun, Y.; Hee-Yun, K.; Young-Hee, L.; Hye-Kyung, K.; Dong-Hoon, S.; Bum-Shik, H.; Hong-Yon, C. Thermostable chitosanase from *Bacillus* sp. strain CK4: cloning and expression of the gene and characterization of the enzyme. *Appl. Environ. Microbiol.* **2000**, *66*, 3727−3734.

(37) Piza, F. A. T.; Siloto, A. P.; Carvalho, C. V.; Franco, T. T. Production, characterization and purification of chitosanase from *Bacillus cereus*. *Braz. J. Chem. Eng.* **1999**, *16*, 185−192.
(38) Pelletier, A.; Sygusch, J. Purification and characterization of three chitosanase activities from Bacillus megaterium P1. *Appl. Environ. Microbiol.* **1990**, *56*, 844−848.
(39) Uchida, Y.; Ohtakara, A. Chitosanase from *Bacillus* species. *Methods Enzymol.* **1988**, *161*, 501−505.
(40) Davis, B.; Eveleigh, D. E., Chitosanases: occurrence, production and immobilization. In *Chitin, Chitosan and Related Enzymes*; Zikakis, J. P., Ed.; Academic Press: New York, 1984; pp 161−179.
(41) Schallmey, M.; Singh, A.; Ward, O. P. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* **2004**, *50*, 1−17.
(42) Henrissat, B.; Bairoch, A. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* **1996**, *316*, 695−696.
(43) Tamo, F.; Andre, H. J.; Hans, J. V.; Yuji, H.; Hugo, T.; Isabelle, B.; Witold, A. N.; Ryszard, B. Theoretical calculation of pK_a reveals an important role of Arg205 in the activity and stability of *Streptomyces* sp. N174 chitosanase. *J. Biol. Chem.* **2000**, *275*, 25633−25640.
(44) Tamo, F.; Satoko, A.; Kei, Y.; Tomoye, Y.; Tomomi, K.; Junichi, S.; Michihiko, S.; Kunio, M.; Yoshiho, N.; Akikazu, A. *Bacillus circulans* MH-K1 chitosanase: amino acid residues responsible for substrate binding. *J. Biochem.* **2005**, *138*, 563−569.
(45) Wataru, A.; Yuri, S.; Shinji, S.; Tomoko, S.; Tetsuya, F.; Mamie, S.; Rie, Y.; Satoshi, N.; Akio, T. Crystal structure of family GH-8 chitosanase with subclass II specificity from *Bacillus* sp. K17. *J. Mol. Biol.* **2004**, *343*, 785−795.
(46) Ozaki, K.; Sumitomo, N.; Hayashi, Y.; Kawai, S.; Ito, S. Site-directed mutagenesis of the putative active site of endoglucanase K from *Bacillus* sp. KSM-330. *Biochim. Biophys. Acta* **1994**, *1207*, 159−164.
(47) Chen, X.; Xia, W.; Yu, X. Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326. *Food Res. Int.* **2005**, *38*, 315−322.

Received for review January 6, 2006. Revised manuscript received April 2, 2006. Accepted April 4, 2006. Financial support for this study from the Chinese Academy of Sciences is highly appreciated (Project KSCZ2-SW-324).

JF0600556