Journal of Metals, Materials and Minerals. Vol. 15 No. 1 pp. 27-32, 2005

Purification and Properties of a Chitosanase from *Bacillus cereus* P16

Woo-Jin JUNG[1], Ju-Hee KUK[1], Yu-Lan JIN[1] and Ro-Dong PARK*

[1]Glucosamine Saccharide Materials-National Research Laboratory (GSM-NRL), Department of Agricultural Chemistry, and Institute of Agricultural Science and Technology, Chonnam National University Graduate School, Gwangju, 500-757, Korea

Abstract

Bacillus cereus P16 secreted six endo-type chitosanase isozymes, two major (38 and 45 kDa) and four additional (54, 65, 82 and 96 kDa). Among them, 45-kDa endochitosanase was purified and characterized. The N-terminal sequence of the protein was found to be AAKEMKPFFQQVNYA-. The purified enzyme showed an optimum pH of 5.5 and optimum temperature of 60°C, and was stable between pH 4.5-10.0 and under 50°C. N-Bromosuccinimide, 2-hydroxy-5-nitrobenzylbromide and chloramine T severely inhibited the enzyme activity at 1 mM. The K_m and V_{max} were measured to be 0.52 mg/ml and 7.71×10^{-6} mol/sec/mg protein, respectively. The chitosanase digested partially N-acetylated chitosans, with maximum activity for the chitosan of D.A. 15-30%. The chitosanase hydrolyzed $(GlcN)_7$ in an endo-splitting manner producing a mixture of $(GlcN)_{2\text{-}5}$. Time course studies showed a decrease in the rate of substrate degradation from $(GlcN)_7$ to $(GlcN)_6$ to $(GlcN)_5$, as indicated by the apparent first order rate constants, k_1 values, of 4.98×10^{-4}, 2.3×10^{-4}, and 9.3×10^{-6} sec^{-1}, respectively. It is suggested that the 45-kDa chitosanase belongs to a member of family 8 and subclass III, based on products analysis, N-terminal amino acid sequence, and its substrate specificity.

Introduction

Compared to the numerous reports on the primary structure and function of chitinases, information on chitosanases is quite limited (Nanjo *et al.*, 1990). The complete amino acid sequences have been reported for prokaryotic chitosanases from *Bacillus circulans* MH-K1 (Ando *et al.*, 1992), *Nocardioides* sp. N106 (Masson *et al.*, 1995), *Streptomyces* sp. N174 (Fukamizo *et al.*, 1995), and *B. circulans* WL-12 (Mitsutomi *et al.*, 1998) and for fungus one from *Fusarium solani*. Among them, the former three chitosanases show structural similarity, but the one from *F. solani* does not. *B. circulans* WL-12 chitosanase has no similarity to the three and the fungal chitosanases (Mitsutomi *et al.*, 1998). The ones of *Streptomyces* sp. N174 (Fukamizo *et al.*, 1995), and *B. circulans* MH-K1 (Ando *et al.*, 1992), are the only chitosanases the tertiary structures of which have been analyzed. Recently, chitosanase genes have been cloned from *Bacillus subtilis* (Parro *et al.*, 1997), *Bacillus ehimensis* EAG1 (Akiyama *et al.*, 1999), *Bacillus sp.* strain CK (Yoon *et al.*,2000), *Burkholderia gladioli* CHB101 (Shimasaka *et al.*, 2000), *Amycolatopsis* sp. CsO-2 (Ando *et al.*, 2000), and *Matsuebacter chitosanotabidus* 3001 (Park *et al.*, 1999). These, except *M. chitosanotabidus* 3001, showed sequence similarities to the chitosanase from *B. circulans* MH-K1. But, little is known about how many different types of chitosanases exist in nature. In this study, we purified and characterized a 45-kDa chitosanase, a major and inducible enzyme, from *Bacillus cereus* P16, which has shown a strong endochitosanolytic activity toward chitosan with a broad range of degree of acetylation (D.A.).

*Corresponding author: Prof. Ro-Dong Park
Address: Department of Agricultural Chemistry, Chonnam National University, Gwangju, 500-757, Korea..
E-mail: rdpark@chonnam.ac.kr Phone: 82-62-530-2133 Fax: 82-62-530-2139

Materials and Methods

Bacterial strain and culture conditions for chitosanase production:

The bacterial strain used for chitosanase production was *Bacillus cereus* P16, which was isolated and characterized from a coastal area of the south-western part of Korea (Park *et al.* 1999). The medium used throughout this study contained 0.5% chitosan, 1% tryptone, and 1% NaCl (pH 5.5). The culture supernatant was obtained after centrifugation (10,000×g), concentrated using $(NH_4)_2SO_4$ precipitation and used for a chitosanase assay after dialysis against 50 mM sodium acetate buffer (pH 5.5).

Purification of chitosanase:

The dialyzed crude enzyme was put on a DEAE-cellulose column (2.8×75 cm). The column was eluted with a step-wise gradient of 0, 0.05, 0.1, 0.2, 0.3, and 0.4 M NaCl (100 ml each) in a 50 mM sodium acetate buffer (pH 5.5). The active fractions were put on a CM-Sephadex column (2.2×75 cm) and eluted with the sodium acetate buffer. The resulting active preparation was finally put on Sephacryl S-300 column (1.5×50 cm). Active fractions were pooled and considered as the purified enzyme. All purification steps were done at 4°C. Chitosanase activity was assayed with chitosan (D.A. of 15.4%) as the substrate in a standard method. A viscometric chitosanase assay was performed by using a Brookfield synchrolectric viscometer.

Analytical methods:

Protein contents were measured with a Bradford assay kit (Bio-Rad). The purity of the enzyme was measured by SDS-PAGE according to the method of Laemmli (1970). Active staining of the enzyme in 10% polyacrylamide gel containing 0.01% soluble chitosan was done by the Trudel and Asselin method (1989). The N-terminal sequence of the purified protein was analyzed by Edman degradation with an Applied Biosystems 476A protein sequencer (Foster City, U.S.A.). The reaction products were analyzed by TLC on Merck TLC silica gel G-25 and also by HPLC with an RI detector.

Results and Discussion

Screening and identification of the chitosan-assimilating bacterium:

Bacterial isolate P16 showed a strong endochitosanase activity, when tested with precipitation by alkalization of enzymatic reaction mixtures, reduction in viscosity of chitosan solutions, and TLC and HPLC analysis of enzymatic reaction products. P16 was identified as a member of the genus *Bacillus* according to Bergey's Manual of Systematic Bacteriology. In assimilation tests of the Biolog system, P16 was identified as *Bacillus cereus* (with similarity index of 0.64). A profile of cellular fatty acid composition was compared with that of the *B. cereus* group (with similarity index of 0.95). When the nucleotide sequence of 16S rRNA gene of P16 was sequenced and compared, no base difference between P16 (ID AY048782) and *B. cereus* 16S rRNA sequences (ID AF176322) was detected. Therefore, isolate P16 was designated as *Bacillus cereus* P16.

Properties of the crude chitosanase:

The culture supernatant showed a strong liquefying activity on soluble chitosan. The viscosity of 1% chitosan solution, when incubated with the supernatant, was rapidly decreased upon the days of cultivation. The supernatant also cleaved the insoluble chitosan powder but the hydrolysis rate was much lower. The culture fluids showed roughly 100-times higher activity on soluble chitosan compared with insoluble chitosan powder. But, no differences in the chitooligosaccharides composition of the hydrolysis products were observed when identified by TLC and HPLC. The chitooligosaccharide composition was affected with the chitosan concentration. When the higher concentration of chitosan was applied, more production in the high DP of chitooligosaccharide was resulted. What this means is that it is desirable to incubate the crude enzyme with a higher concentration of chitosan for producing high DP of chitooligosaccharides.

Purification of a chitosanase:

Six endo-type chitosanases, two major (38 and 45 kDa) and four additional (54, 65, 82, and 96 kDa), were found in the crude preparation. Among them, the 45-kDa endochitosanase was purified for further study. After three subsequent chromatographic steps, the enzyme was purified 11.7-fold, with a 45% recovery and a specific activity of 47.8 U/mg protein. It was assumed to be homogeneous as

estimated from a SDS-PAGE and from N-terminal amino acid sequencing. The N-terminal sequence of the purified protein was found to be A-A-K-E-M-K-P-F-F-Q-Q-V-N-Y-A-. The molecular weight of the chitosanase was estimated to be 45 kDa by SDS-PAGE. The enzyme was eluted from Sephacryl S-300 chromatography at the position of molecular weight of 42 kDa, indicating its monomeric conformation. The active staining profile of the crude and purified preparations suggested that the monomeric enzyme is active, homogeneous, and endochitosanolytic. The purified enzyme showed an optimum pH of 5.5 and optimum temperature of 60°C, and was stable between pH 4.5-10.0 and under 50°C.

Effects of ions and inhibitors:

The P16 chitosanase was completely blocked by mercury and lead at 10 mM. A large number of microbial chitosanases were inactivated by these heavy metal ions. The chitosanases from *Bacillus* sp. PI-7S (Seino *et al.*, 1991), *Myxobacter* AL-1 (Hedges and Wolfe, 1974), and *Streptomyces griceus* (Ohthakara *et al.*, 1984) were sensitive to $AgNO_3$ but the P16 chitosanase was only mildly sensitive. N-Bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide, a tryptophan residue modifiers, and chloramine T, a methionine residue modifier, severely inhibited the enzyme activity at 1 mM, suggesting that tryptophan and methionine residues are involved in the catalytic action of the enzyme. This is the first case of the inhibition of a chitosanase by chloramine T. The chitosanases from *Rhodotorula gracilis* CFR-1 (Somashekar and Joseph, 1992) and *Amycolatopsis* sp. CsO-2 (Okajima *et al.*, 1994) were inhibited by N-bromosuccinimide. It has been suggested that two invariant carboxylic amino acid residues (e.g., Asp 40 and Glu22 of *Streptomyces* sp. N174 chitosanase) (Fukamizo *et al.*, 1995) conserved in N-terminal segments of microbial chitosanases are essential for its catalytic activity. Interestingly, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, a carboxylic residue modifier, has no effect on P16 chitosanase.

Substrate specificity:

The enzyme was specific for soluble chitosan and glycol chitosan. Soluble chitosan was more rapidly hydrolyzed than glycol chitosan. The chitosanase also showed hydrolysis activity for carboxymethylcellulose (CMC), but much lower (5.2%) compared to that for soluble chitosan. No hydrolysis of chitins (powder, colloidal, swollen, regenerated, glycol, and carboxymethyl), amylose, amylopectin, curdlan, alternan, cyclodextrin, levan, dextran T40, pullulan, lichenan, laminarin, p-nitrophenyl-D-glucosaminide, or p-nitrophenyl-N-acetyl-D-glucosaminide was observed. The relationship between enzyme activity and the D.A. of the chitosan substrate was tested on a series of chitosans. P16 chitosanase was able to efficiently hydrolyse chitosans in the D.A. range of 2.6-60.8% tested. The maximal rate of hydrolysis was observed for chitosans of D.A. 15-30%, and 60% of the maximum rate for chitosans of D.A. 2.6-10%. Chitosan with D.A. over 61% was barely degraded by the chitosanase. This means that the P16 chitosanase has specificity to the linkages of GlcN-GlcN and GlcNAc-GlcN and/or GlcN-GlcNAc, and N-acetylglucosamine residues are important in the recognition and reaction mechanism of the substrate by the enzyme.

Kinetic parameters:

The purified enzyme (5.8 mU) was incubated with various concentrations of soluble chitosan (D.A 20.2%) for 15 min at 37°C and a Lineweaver-Burk plot was constructed. Kinetic parameters were measured as follows; V_{max} of 7.71×10^{-6} mol/sec/mg protein, K_m of 0.52 mg/ml, k_{cat} of 3.4×10^{2} sec^{-1}, and k_{cat}/K_m of 6.5×10^{2} ml/mg/sec.

Cleavage pattern:

Chitobiose, chitotriose, and chitotetraose were not degraded by the enzyme even after prolonged reactions. The smallest size of substrates which P16 chitosanase could recognize and fit for hydrolytical cleavage was the pentamer. P16 chitosanase hydolyzed chitopentaose into $(GlcN)_{2-3}$, chitohexaose into $(GlcN)_{2-4}$, and chitoheptaose into $(GlcN)_{2-5}$, in an endo-splitting manner. To measure the rate constants for $(GlcN)_n$ (n=5-7), reaction mixtures containing 10 mg/ml chitooligosaccharides and 5.8 mU of enzyme in 0.1 M sodium acetate buffer (pH 5.5) were incubated at 37°C, and the residual amount of the substrate was measured using HPLC. When kinetic plots were constructed, concentration of the substrate decreased exponentially with time, indicative of a first-order reaction. The apparent first order rate constant was found to be $k_1 = 4.98\times10^{-4}$ sec^{-1} for heptamer. The constant for heptamer was 2.1- and 53.4- times higher than that for hexamer and pentamer, respectively, indicating that the rate constants increased with a decrease in the substrate size from $(GlcN)_7$ to $(GlcN)_6$ to $(GlcN)_5$. No appreciable rate of degradation for

smaller oligosaccharides than pentamer was detected. To date, this is the first report on the rate constants of a chitosanase. The chitosanase from *B. gladioli* CHB101 could not hydrolyze $(GlcN)_{2-4}$ but could $(GlcN)_5$ (Shimosak *et al.*, 2000). The bifunctional chitosanase-cellulase from *Myxobacter* sp. AL-1 degraded only the six-unit or larger chitosan oligomers (Hedges and Wolfe 1974). It has been known that the shortest oligomer to be attacked is the tetramer for most microbial endochitosanases. The purified chitosanase catalyzed an endo-type of cleavage reaction, as judged from rapid reductions in the viscosity of chitosan solutions but the lag-phase production of reducing sugars. The final products from chitosan after a prolonged reaction with the purified enzyme were a mixture of chitooligosaccharides, $(GlcN)_{2-7}$, suggesting an endo-type glycohydrolase. No glucosamine has been detected from the reaction mixture. The enzyme retained no activity of N-acetylhexosaminidase, supporting no glucosamine production. P16 chitosanase didn't show transglycosylation activity, even with a higher concentration of chitooligosaccharides $(GlcN)_{2-7}$ in the presence or absence of various salts, such as ammonium sulfate. It has been reported that a chitosanase from *Aspergillus fumigatus* KH-94 showed transglycosylation activity (Kim *et. al.*, 1998).

Classification of the glycohydrolytic enzyme:

According to the glycosyl hydrolases classification based on the amino acid sequence similarity proposed by Henrissat *et al.* (1996), chitosanases from *B.circulans* MH-K1 (Ando *et al.*, 1992), *Nocardioides* sp. N106 (Masson *et al.*, 1995), *Streptomyces* sp. N174 (Masson *et al.*, 1994), *B. subtilis* (Parro *et al.*, 1997) and *B. pumilus* BN-262 (Fukamizo *et al.*, 1994) have been classified into family 46, and those from *B. circulans* WL-12 (Mitsutomi *et al.*, 1998), *Bacillus* sp. 928 (Seki *et al.*, 2001), and *Bacillus* sp. 7-M (Izume *et al.*, 1992) into family 8. Based on the following characteristics, P16 chitosanase was assumed to belong to family 8. First, N-terminal amino acid sequence of P16 chitosanase, AAKEMKPFFQQVNYA-, was very similar (only one different out of 15 residues) to those of *B. circulans* WL-12 (Mitsutomi *et al.*, 1998), Bacillus sp. 928 (Seki *et al.*, 2001), and *Bacillus* sp. 7-M. (Izume *et al.*, 1992) (Figure 1). Second, P16 chitosanase showed substantial CMCase activity, which is a criterion for family 8. Third, the enzyme split both GlcN-GlcN and GlcN-GlcNAc and/or GlcNAc-GlcN linkages in the endowise manner. Chitosanases are again subclassified according to their specificity for the hydrolysis of the β-glycosidic linkages in partially N-acetylated chitosan (Fukamizo *et al.*, 1994). Subclass II chitosanases cleave only GlcN-GlcN, and subclass III chitosanases cleave GlcN-GlcN and GlcN-GlcNAc. Subclass I chitosanases cleave GlcN-GlcN and GlcNAc-GlcN and have no activity on CM-cellulose (Seki *et al.*, 2001). P16 chitosanase retains CM-cellulase activity, similar to subclass III chitosanases from *B. circulans* WL-12 (Mitsutomi *et al.*, 1998). The enzyme showed highest activity toward D.A. 15-30% chitosan, but little activity on chitin (D.A. of 97.6%). Thus, it can be suggested that the purified 45 kDa chitosanase from *Bacillus* sp. P16 belongs to family 8 and subclass III, even though more information is needed on the composition and sequences of sugar units in the enzymatic hydolysis products of partially N-acetylated chitosan.

		1				5					10					15					20
Bacillus cereus P16		A-	A-	K-	E-	M-	K-	P-	F-	F-	Q-	Q-	V-	N-	Y-	A-					
Bacillus sp. 928		A-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-	-	-A	-G	-V	-I	-I
Bacillus sp. GM44	A-	A-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-	-	-A	-G	-V	-I	-I
Bacillus sp. 7-M	A-	A-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-	-	-A	-G	-V	-I	-I
Bacillus sp. KSM-330	A-	V-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-	-	-S	-G	-I	-L	-L
Bacillus circulans WL-12	A-	F-	A-	A-	P-	N-	K-	P-	F-	P-	Q-	H-	T-	T-	Y-	T	-S	-G	-S	-I	-I
Bacillus cereus ATCC 14579	A-	A-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-		-A	-G	-V	-I	-I
Bacillus cereus KUNC51	A-	A-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-		-A	-G	-V	-I	-I
Bacillus sp. KCTC 0377BP	A-	A-	A-	K-	E-	M-	K-	P-	F-	P-	Q-	Q-	V-	N-	Y-		-A	-G	-V	-I	-I
Paenibacillus fukuinensis		A-	A-	G-	E-	M-	M-		F-	P-	Q-	Q-	V-	S-	Y-		-S	-G	-I	-I	-I

Figure 1. Sequence homology of N-terminal amino acids in family 8 microbial chitosanases.

Acknowledgements

This work was supported by the National Research Laboratory (NRL) Program of the Ministry of Science and Technology (MOST), Korea.

References

Akiyama, K., Fujita, T., Kuroshima, K., Sakane, T., Yokota, A. and Takata, R. 1999. Purifiaction and gene cloning of a chitosanase from *Bacillus ehimemsis* EAG1. *J. Biosci. Bioeng.* **87**: 383-385.

Ando, A., Noguchi, K., Yanagi, M., Shinoyama, H., Kagawa, Y., Hirata, H., Yabuki, M. and Fujii, T. 1992. Primary structure of chitosanase produced by *Bacillus circulans* MH-K1. *J. Gener. Appl. Microbiol.* **38**: 135-144.

Fukamizo, T., Honda, Y., Goto, S., Boucher, I. and Brzezinski, R. 1995. Reaction mechanism of chitosanase from *Streptomyces* sp. N174. *Biochem. J.* **311**: 377-383.

Fukamizo, T., Ohkawa, T., Ikeda, Y. and Goto, S. 1994. Specificity of chitosanase from *Bacillus pumilus*. *Biochim. Biophys. Acta.* **1205**: 183-188.

Hedges, A. and Wolfe, R.S. 1974. Extracellular enzyme from *Myxobacter* AL-1 that exhibits both β-1,4-glucanase and chitosanase activities. *J. Bacteriol.* **120**: 844-853.

Henrissat, B. and Bairoch, A. 1996. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* **316**: 695-696.

Izume, M., Nagae, S., Kawagishi, H., Mitsutomi, M. and Ohtakara, A. 1992. Action pattern of *Bacillus* sp. No. 7-M chitosanase on partially *N*-acetylated chitosan. *Biosci. Biotechnol. Biochem.*. **56**: 448-453.

Kim, S.Y., Shon, D.H. and Lee, K.H. 1998. Purification and characterization of two types of chitosanases from *Aspergillus fumigatus* KH-94. *J. Microbiol. Biotechnol.* **8**: 568-574.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of *bacteriophage* T4. *Nature*. **227**: 680-685.

Masson, J.Y., Boucher, I., Neugebauer, W.A., Ramotar, D. and Brzezinski, R. 1995. A new chitosanase gene from a *Nocardioides* sp. Is a third member of glycosyl hydrolase family 46. *Microbiology*. **141**: 2629-2635.

Masson, J. Y., Denis, F., and Brezinski, R. 1994. Primary sequence of the chitosanase from *Streptomyces* sp. strain N-174 and comparison with other endoglycosidases. *Gene*. **140**: 103-107.

Mitsutomi, M., Isono, M., Uchiyama, A., Nakaido, N., Ikegami, T. and Watanabe, T. 1998. A chitosanase activity of the enzyme previously reported as β-1,3-1,4-glucanase from *Bacillus circulans* WL-12. *Biosci. Biotechnol. Biochem.* **62**: 2107-2114.

Nanjo, F., Ishikawa, M., Katsumi, R. and Sakai, K. 1990. Purification, properties and transglycosylation reaction of *N*-acetylhexosaminidase from *Nocardia orientalis*. *Agric. Biol. Chem.* **54** : 899-906.

Ohtakara, A., Ogata, H., Taketomi, Y., and Mitsutomi, M. 1984. Purification and characterization of chitosanase from *Streptomyces griceus*. In: *Zikakis, J. P. Orlando,* F.L. *(eds) Chitin, Chitosan and Related Enzyme.* New York, Academic Press: 147-159.

Okajima, S., Ando, A., Shinoyama, H. and Fuji, T. 1994. Purification and characterization of an extracellular chitosanase produced by *Amycolatopsis* sp. CsO-2. *J. Ferment. Bioeng.* **77**: 617-620.

Park, J. K., Shimono, K., Ochiai, N., Shigeru, K., Kurita, M., Ohta, Y., Tanaka, K., Matsuda, H. and Kawamukai, M. 1999. Purification, characterization, and gene analysis of a chitosanase (choA) from *Matsuebacter chitosanotabidus* 3001. *J. Bacteriol.* **181**: 6642-6649.

Park, R. D., Jung, M. R., Jo, Y. Y. and Chi, Y. T. 1999. Optimization of culture conditions of chitosanase-producing *Bacillus* sp. P16. *J. Korean Society Agric. Chem. Biotechnol.* **42**: 193-198.

Parro, V., San Roman, M., Galindo, I., Purnelle, B., Bolotin, A., Sorokin, A. and Mellano, R. P. 1997. A 23911 bp region of the *Bacillus subtilis* genome comprising genes located upstream and downstream of the *lev* operon. *Microbiology.* **143** : 1321-1326.

Seino, H., Tsukuda, K. and Shimasue, Y. 1991. Properties and action pattern of a chitosanase from *Bacillus* sp. PI-7S. *Agric. Biol. Chem.* **55** : 2421-2423.

Seki, K., Nakamura, T., Shimoda, H., Mitsutomi, M. and Uchida, Y. 2001. Family 8 chitosanase from *Bacillus* sp. No. 928. In: *Uragami, T., Kurita, K., and Fukamizo, T. (eds.) Chitin and Chitosan.* Tokyo, Kodansha Scientific : 452-453.

Shimosaka, M., Fukumori, Y., Zhang, X. Y., He, N. J., Kodaira, R. and Okazaki, M. 2000. Molecular cloning and characterization of a chitosanase from the chitosanolytic bacterium *Burkholderia gladioli* strain CHB101. *Appl. Microbiol. Biotechnol.* **54** : 354-360.

Somashekar, D. and Joseph, R. 1992. Partial purification and properties of a novel chitosanase secreted by *Rhodotorula gracilis. Lett. Appl. Microbiol.* **14**: 1-4.

Trudel, J. and Asselin, A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* **178** : 362-366.

Yoon, H.G., Kim, H.Y., Lim, Y.H., Kim, H.K., Shin, D.H., Hong, B.S. and Cho, H.Y. 2000. Thermostable chitosanase from *Bacillus* sp. strain CK4: cloning and expression of the gene characterization of the enzyme. *Appl. Environ. Microbiol.* **66**: 3727-3734.