APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1990, p. 844–848
0099-2240/90/040844-05$02.00/0

Vol. 56, No. 4

Purification and Characterization of Three Chitosanase Activities from *Bacillus megaterium* P1

A. PELLETIER AND J. SYGUSCH*

Département de Biochimie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

Received 22 May 1989/Accepted 3 January 1990

***Bacillus megaterium* P1, a bacterial strain capable of hydrolyzing chitosan, was isolated from soil samples. Chitosan-degrading activity was induced by chitosan but not by its constituent D-glucosamine. Extracellular secretion of chitosanase reached levels corresponding to 1 U/ml under optimal conditions. Three chitosan-degrading proteins (chitosanases A, B, and C) were purified to homogeneity. Chitosanase A (43 kilodaltons) was highly specific for chitosan and represented the major chitosan-hydrolyzing species. Chitosanases B (39.5 kilodaltons) and C (22 kilodaltons) corresponded to minor activities and possessed comparable specific activities toward chitosan, chitin, and cellulose. Chitosanase A was active from pH 4.5 to 6.5 and was stable on the basis of activity up to 45°C. The optimum temperature for enzymatic chitosan hydrolysis was 50°C. Kinetic studies on chitosanase A suggest that the enzyme is substrate inhibited. The apparent K_m and V_{max} determined at 22°C and pH 5.6 were 0.8 mg/ml and 280 U/mg, respectively. End products of chitosan hydrolysis by each of the three chitosanases were identified as glucosamine oligomers, similar to those obtained for previously reported chitosanase digestions.**

Chitosan is a linear polysaccharide composed of β-1,4-linked D-glucosamine residues. In nature, the polymer is partially acetylated, and the name chitosan in fact describes a wide range of polymers corresponding to various proportions of D-glucosamine and *N*-acetyl-D-glucosamine residues. Chitosan was first identified as a minor component of cell walls of *Phycomyces blakesleeanus* (7), and further investigation established its presence throughout the *Zygomycetes* fungi (4).

Chitosan is currently obtained by deacetylation of chitin, an *N*-acetylglucosamine polymer extracted from shellfish exoskeletons. Conservative estimates of the amount of crustacean shells produced worldwide fall in the range of 150×10^3 metric tons per annum (1), most of which is being disposed of into the sea, creating a potential environmental problem. Transformation of this unused biomass in chitosan and/or chitosan oligomers could be advantageous, since chitosan derivatives have numerous applications (11).

In an attempt to find chitosanases that are capable of depolymerizing chitosan commercially, new bacterial strains with chitosanase activity were sought. A *Bacillus megaterium* strain that was capable of hydrolyzing chitosan rapidly was isolated from soil samples. The enzymes responsible for this hydrolysis were purified and characterized and are described here. The chitosanase A enzyme was active over a wide range of pHs and possessed a higher catalytic efficiency than previously isolated chitosanases.

MATERIALS AND METHODS

Microorganisms were collected from compost and soil samples and screened for chitosanase activity after initial growth in liquid medium (0.5% yeast extract, 0.5% Casamino Acids, 0.1% glucose, 0.11 M NaCl, 11 mM KCl, 10 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 0.4 mM $CaCl_2$) containing 0.025% sodium propionate and 30 U of nystatin per ml. Soil was eliminated by gentle centrifugation, and the supernatant was centrifuged to harvest the suspended cells. The cell pellet was washed, suspended in saline buffer, and plated on complete medium (to evaluate the total heterotrophic bacteria) as well as on minimal medium that was selective for chitosanase activity (4). Colonies capable of hydrolyzing chitosan were purified by repeated alternate amplification and selection. Purified chitosan-hydrolyzing colonies were identified by the Institut Armand-Frappier (Ville de Laval, Québec, Canada).

Media. *B. megaterium* was plated on nutrient agar (Difco Laboratories) and grown on Trypticase soy broth (no. 11768; BBL Microbiology Systems). Liquid induction was done by using a selective medium (4) modified by omitting both yeast extract and agar and with chitosan flakes (10 g/liter) as the sole carbon source. The growth temperature was 28°C.

Chitosanase activity was assayed as follows. Each activity assay contained 500 μl of acetate buffer (100 mM, pH 5.6), 300 μl of a 1% (wt/vol) chitosan solution, 10 to 50 mU of enzyme, and water to a final volume of 1.5 ml. Activity was assayed by incubation for 30 min at 37°C. The reaction was terminated by the addition of 35 μl of 1 N NaOH, followed by chilling the reaction mixture on ice for 10 min to precipitate chitosan. Insoluble chitosan was eliminated by centrifugation, and soluble reducing sugars were measured by the Nelson-Somogyi assay (12). One unit of enzyme was defined as the amount that liberated 1 μmol equivalent D-glucosamine in 1 min at 37°C.

Kinetic studies were carried out at 22°C with 10 ml of the same reaction mixture (pH 5.6) but in the presence of 10 μg of bovine serum albumin per 1.5-ml reaction. Proteins were measured by using the BCA protein assay reagent obtained from Pierce Chemical Co.

Chitosan, chitin, carboxymethylcellulose, D-glucosamine, *N*-acetyl-D-glucosamine, chitobiose, chitotriose, and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. The chitosan used was 81% deacetylated, and the degree of substitution of carboxymethyl cellulose (CMC) was between 65 and 85%. Avicel (microcrystalline cellulose) is from Fluka. Chitosan solutions were prepared as described by Fenton and Eveleigh (5).

Chitosanase was purified from an induced culture of *B. megaterium* P1. A 5-liter culture was first grown to the

* Corresponding author.

stationary phase in Trypticase soy broth. The cells were pelleted, suspended in 1 liter of inducing medium, and incubated for 48 h with vigorous agitation. Bacteria were eliminated by centrifugation; then phenylmethylsulfonyl fluoride and EDTA at final concentrations of 1 and 10 mM, respectively, were added to the crude extract. Initial purification steps consisted of ammonium sulfate fractionation. The pellet obtained from the ammonium sulfate cut corresponding to 50 to 90% saturation was suspended in approximately 30 ml of concentrated 10× buffer A (1× buffer A is 10 mM KH_2PO_4, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride [pH 7.3]) and thoroughly dialyzed against 1× buffer A. Proteins were diluted to 0.5 mg/ml and applied to a cation exchanger (FPLC Mono-S; Pharmacia Fine Chemicals) equilibrated with buffer A and eluted with a 0 to 0.5 M KCl gradient. Chitosan-degrading activity was detected in a fraction eluting between 0.05 and 0.08 M KCl. This fraction was concentrated by using Sephadex G-25 (10) and applied on a gel filtration column (FPLC Superose-12; Pharmacia) equilibrated with 100 mM phosphate buffer–50 mM KCl (pH 6.8). The active fraction corresponded to pure enzyme A and could be stored at −20°C in 50% glycerol without loss of activity for at least 6 months.

The fraction unretained by the cation exchanger possessed residual chitosan-hydrolyzing activity, which was further purified by a second application on the cation exchanger (Mono-S) previously equilibrated in buffer B (20 mM sodium acetate [pH 4.5]). Two active fractions were eluted by using a 0 to 0.5 M NaCl gradient; fraction 1 eluted between 0.10 and 0.13 M NaCl, and fraction 2 eluted between 0.18 and 0.21 M NaCl. The two fractions were concentrated and applied separately to the gel filtration column, yielding chitosanases B and C, respectively. Both chitosanase fractions could be stored in 50% glycerol at −20°C.

The end products of the enzymatic hydrolysis of chitosan were analyzed by descending chromatography on Whatman 3MM paper. The mobile phase used was butanol-pyridine-water (60:40:30) (9), and sugars were developed by a modified Elson-Morgan procedure (2) as follows. Reagent A consisted of 5 g of KOH dissolved in 20 ml of H_2O, to which 80 ml of 95% ethanol was added; reagent B consisted of 1 ml of acetylacetone in 99 ml of 95% ethanol; and reagent C was 5 g of *para*-dimethylaminobenzaldehyde dissolved in 5 ml of concentrated HCl, to which was added 95 ml of 95% ethanol. The chromatogram was first dipped into a mixture of reagents A and B (1:10, vol/vol), dried for 20 min, and heated for 3 min at 120°C. The paper was then dipped in reagent C and dried by heating at 70°C for 5 to 10 min. Extensive digestions of chitosans were carried out for 90 h under standard conditions with a 10-fold excess of chitosanase A and a second addition of enzyme after 24 h.

RESULTS AND DISCUSSION

The chitosan-degrading population among the bacterial flora from various samples represents a significant portion of total heterotroph bacteria. The highest frequency of chitosan-hydrolyzing organisms was estimated at 5% of the total bacterial population and was found in a sample taken near the edge of a pond. Significantly lower frequencies were found from soil samples taken near tree roots (0.7%) or under dead leaves (0.2%). These findings correlate well with the relative frequencies reported by Davis and Eveleigh (4), who found that chitosan-hydrolytic colonies represented 7.4 and 1.5% of the total heterotroph population in a sample taken from a salt marsh and from a forest sample, respectively. Muddy samples on the basis of these data would appear to represent a significant source of chitosan-degrading bacteria. Nine colonies selected by their capacity to produce a clearing halo on selective agar plates were further purified. Six of these colonies were identified as *B. megaterium* strains (P1 through P6), whereas the remaining three were typed as *Bacillus lentus* strains (P7 through P9). The rather low diversity among the bacterial strains capable of degrading chitosan suggests that the chitosan-degrading microorganisms represent a fairly homogeneous population.

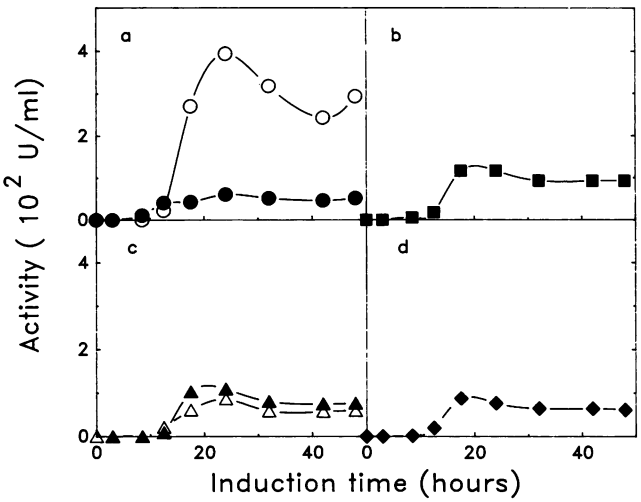

FIG. 1. Induction of chitosanase activity in *B. megaterium* P1 by various substrates. A stationary culture (450 ml) was centrifuged, and the cells were suspended in 100 ml of inducing medium containing appropriate salts and either (a) chitosan flakes at 0.2% (●) and 1% (○) (wt/vol) concentration, (b) 1% (wt/vol) crystalline chitin (■), (c) yeast extract (50 mg) and glucosamine hydrochloride at 0.2 mM (▲) and 2.0 mM (△), or (d) 1% (wt/vol) Avicel (◆).

Induction studies of the purified strains revealed *B. megaterium* P1 as a particularly potent source of chitosanase activity. Chitosanase activity was secreted into the medium and was best induced by chitosan flakes at a 1% concentration. Similar concentrations of chitin or Avicel resulted in a substantially lower yet basal expression of chitosanase activity. D-Glucosamine also showed the same basal inducing capacity at both 2.0 and 0.2 mM (Fig. 1). The highest yield in chitosanase activity (up to 1 U/ml) was obtained by first amplifying cells in rich medium and then concentrating these cells in a selective medium containing chitosan. Simultaneous growth and induction resulted in only 5% of the previous enzymatic activity being secreted and required 8 days rather than 48 h for maximal induction of enzymatic activity.

Purification of chitosanase A is summarized in Table 1. Chitosanase A initial activity was increased 450- to 500-fold by using a combination of ammonium sulfate fractionation, cation-exchange chromatography, and gel filtration, giving an overall yield of 35%. The final specific activity ranged from 150 to 310 U/mg, depending upon the particular chitosan preparation used for assaying activity. It should be noted that the chitosanase assay reflects total enzymatic activity; individual chitosanase activities A, B, and C cannot be distinguished in the crude extract. However, given the extremely low specific activities of chitosanases B and C relative to chitosanase A together with similar overall yields (~1 mg/liter of induced culture), the purification scheme in Table 1 is truly representative of chitosanase A. On the basis

TABLE 1. Purification procedure for chitosanase A[a]

Purification step	Total protein (mg)	Total U	Sp act (U/mg of protein)	% Yield	Purification factor
Crude extract	1,505	469	0.3	100	
50 to 90% $(NH_4)_2SO_4$ fraction	93.3	322	3.5	69	11
Mono-S cation exchanger (0.06 to 0.08 M KCl)	1.70	278	163.8	59	528
Superose gel filtration	1.05	163	154.8	35	499

[a] Activity was assayed at 37°C for 30 min.

of migration on sodium dodecyl sulfate gels, the three fractions appeared homogeneous and corresponded to relative molecular masses of 43 (chitosanase A), 39.5 (chitosanase B), and 22 (chitosanase C) kilodaltons (kDa) (Fig. 2). The relatively low specific activities of chitosanases B and C have permitted only limited characterization.

Estimation of the apparent molecular weights based on elution volume on gel filtration yielded substantially lower molecular masses for all three enzymes: 23, 13, and 5.5 kDa for chitosanases A, B, and C, respectively (data not shown). Retardation of proteins on gel filtration columns is suggestive of a nonglobular protein conformation or of interaction with the agarose gel filtration matrix.

The substrate specificities of chitosanases A, B, and C were compared (Table 2). Of the three, chitosanase A possessed the greatest activity and specificity for chitosan, whereas its activity toward CMC represented only 0.4% of the activity displayed toward chitosan. Chitosanases with CMCase activity have already been isolated from *Myxobacter* and *Streptomyces griseus* strains (6, 8), but in both cases chitosanase and CMCase activities were of comparable rates. Enzyme B showed similar activity toward both chitin and chitosan, although its specific activity toward chitosan was relatively low when compared with that of enzyme A. Enzyme C displayed even lower but detectable activity toward chitosan and hydrolyzed all substrates tested at comparable rates. Chitosanases B and C from *B. megaterium* P1 represent the first example of a chitinase and chitosanase activity associated with the same enzyme. The possibility that the purified enzymes possess lysozymelike activity could be eliminated, since hydrolysis by chitosanase A, B or C of the lysozyme substrate, a *M. lysodeikticus* suspension, was negligible.

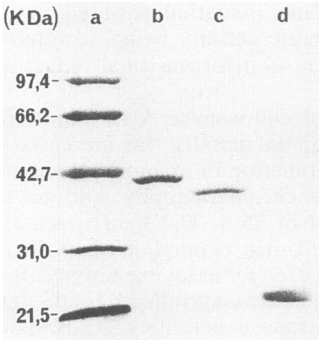

FIG. 2. Migration on sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis of chitosanase activities isolated from *B. megaterium* P1. Lanes: a, molecular mass markers (Bio-Rad Laboratories); b, chitosanase A; c, chitosanase B; d, chitosanase C. A 3-μg sample of protein was applied in each lane.

TABLE 2. Substrate specificity of the chitosanases isolated from *B. megaterium* P1[a]

Substrate	Activity (μmol/min per mg of enzyme)		
	Enzyme A	Enzyme B	Enzyme C
Chitosan (81% deacetylated)	148.2	1.64	0.20
Chitin	0.09	0.92	0.24
CMC (65 to 85% substituted)	0.64	0.37	0.21
Avicel	0.01	0.28	0.15

[a] The reaction temperature was 22°C (enzyme A) or 37°C (enzymes B and C).

To investigate the mode of action of these three chitosanases, end products of chitosan hydrolysis were analyzed by paper chromatography. Extensive digestion by the chitosanase A, B or C released the same oligomeric products, predominantly dimers and trimers of D-glucosamine, whereas no monomeric residues were detected (Fig. 3). In this regard, chitosanase A is similar to the other bacterial chitosanases studied, which all yield oligomeric end products. All chitosanases examined to date appear to depolymerize chitosan by endoglycolytic cleavage (5, 6, 8, 9, 14), and it would seem reasonable to assume that at least chitosanase A also hydrolyzes chitosan in the same manner.

Kinetic studies were only undertaken for chitosanase A for reasons previously stated. The reciprocal plot of the

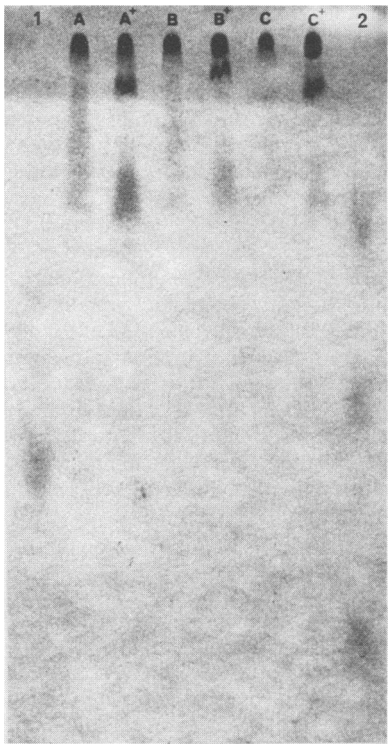

FIG. 3. Paper chromatography of chitosan hydrolysis end products. Lanes: 1, D-glucosamine standard (20 μg); 2, *N*-acetyl-D-glucosamine, chitobiose, and chitotriose standards (20 μg each); A, digestion with chitosanase A for 24 h; A^+, digestion shown in lane A but continued after a second addition of enzyme, for a total incubation time of 90 h; B and B^+, as in A and A^+ but with chitosanase B; C and C^+, as in A and A^+ but with chitosanase C.

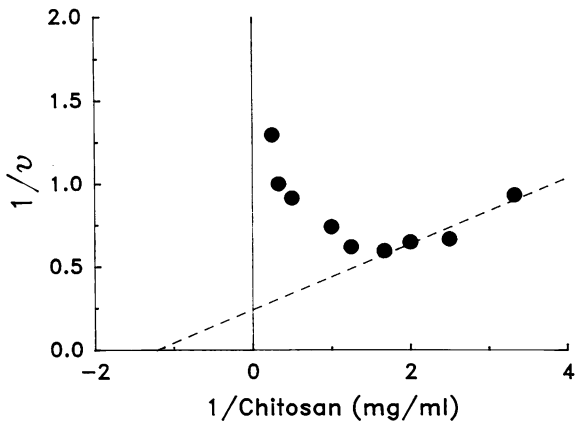

FIG. 4. Double-reciprocal plot of chitosanase A initial velocity as a function of chitosan substrate concentration. Estimation of K_m and V_{max} utilized the velocities measured for the lowest four chitosan concentrations (dotted line). Kinetic studies were carried out at 22°C at pH 5.6 under conditions corresponding to linear variation of activity with enzyme concentration.

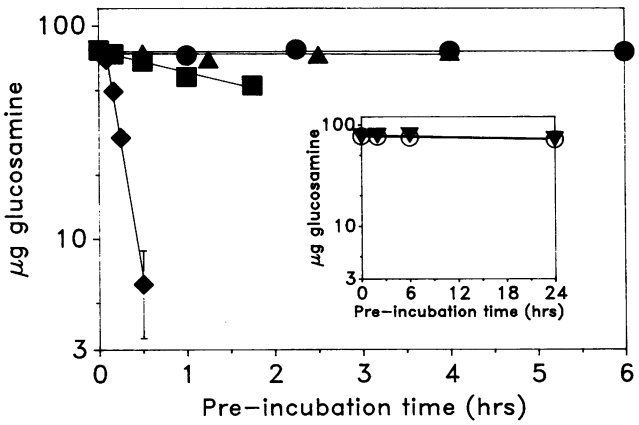

FIG. 6. Thermostability of chitosanase A activity. The enzyme (0.1 μg) was preincubated in 100 μl of buffer containing 10 μl of bovine serum albumin at 4°C (○), 21°C (▼), 35°C (●), 45°C (▲), 55°C (■), and 60°C (◆) for various times. Substrate and remaining constituents of the 1.5-ml reaction mixture were then added, and digestion was allowed to proceed for 1 h at 20°C.

initial velocity data (Fig. 4) deviates from linearity at high substrate concentration, which is characteristic of a substrate-inhibited enzyme. On the basis of extrapolations from velocities obtained at low chitosan concentrations, values of 286.3 U/mg and 0.82 mg/ml were determined for V_{max} and K_m, respectively, with small variations observed among the different chitosan preparations used. However, these values for the kinetic parameters should be interpreted with caution.

Careful analysis of the primary velocity data suggests that at least part of the substrate inhibition observed may be apparent. As can be seen from the initial velocity curves (Fig. 5), a lag occurred before the linear phase of the reaction; this lag period increased with substrate concentration (Fig. 5, inset). Not taking into account this lag period could lead to underestimation of the true reaction rates. Furthermore, the assay is limited by the fact that it does not quantitate all of the reducing ends produced, since the longer polymers are precipitated by the stop solution. The Nelson-Somogyi assay could also create misleading interpretations, since the various products released by the enzyme need not possess identical molar absorbancy.

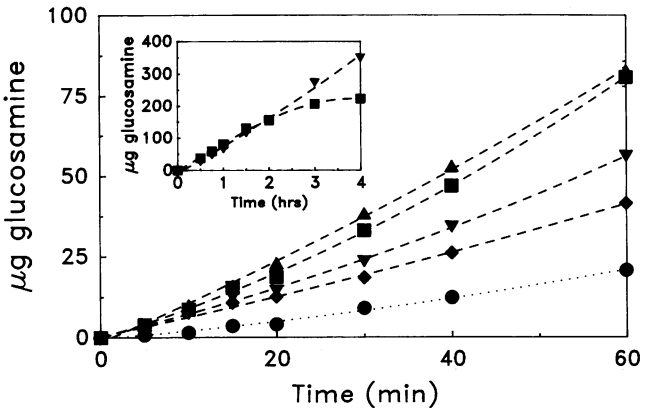

FIG. 5. Representative data of initial reaction rates for chitosanase A. Samples (1.0 ml) were removed from the reaction mixture at precise time intervals, the reaction was immediately stopped by the addition of NaOH, and reducing sugars were measured. Chitosan concentrations shown here are 0.2 (●), 0.4 (▲), 0.8 (■), 2.0 (▼), and 4.0 (◆) mg/ml. Enzyme concentrations are 20.5 mU/ml except for the 0.2 mg/ml chitosan concentration, where 6.8 mU of enzyme per ml was used. The insert shows longer reaction times at the 0.8- and 2.0-mg/ml concentrations.

The effect of pH and temperature on chitosanase A was investigated. The enzyme activity was uniform between pH 4.5 to 6.5 but showed a sharp drop at pH 7.0. It is noteworthy that the chitosan substrate precipitated at this same pH.

The optimum temperature for substrate hydrolysis by chitosanase A was approximately 50°C. Enzymatic thermal stability studies indicated that the enzyme was relatively labile and lost all activity at 50°C (data not shown). However, the addition of bovine serum albumin (0.1 mg/ml) in the preincubation buffer increased the thermal stability of chitosanase A. Under these conditions, only temperatures higher than 55°C produced a noticeable decrease in activity (Fig. 6). The apparent discrepancy between the optimum temperature and the lability of chitosanase A at 50°C may reflect that the optimum temperature is related to the chemical reaction catalyzed, whereas thermal stability refers to lability of the protein structure.

Comparisons were made with chitosanases from microorganisms previously described in the literature (5, 6, 8, 9, 13, 14). Two broad chitosanase families have been defined on the basis of substrate specificity (7): chitosanases that are specific for chitosan, such as those isolated from *Penicillium islandicum* (5), *Streptomyces* strain sp. 6 (9), *Bacillus circulans* (4), and *Bacillus* sp. strain R4 (13); and chitosanases that show substantial activity toward both chitosan and CMC, such as those isolated from *Streptomyces griseus* (8) and *Myxobacter* sp. (6). Chitosanase A possesses only relatively low CMCase activity, similarly to that of the *Bacillus* sp. strain 7-M chitosanase, and as such cannot be clearly classified in either family and may represent a distinct family of chitosanase. However classification relying solely upon detection of a CMCase activity, which is dependent upon the sensitivity of the assay used, should be treated with caution.

Chitosanase from *Bacillus* sp. strain 7-M, a bacterium extracted from soil by Uchida and Ohtakara (14), shares several of the characteristics of the *B. megaterium* P1 enzyme. The molecular sizes of both enzymes are similar:

estimates from sodium dodecyl sulfate gels were 43 kDa for the *B. megaterium* chitosanase and 41 kDa for the *Bacillus* sp. strain 7-M enzyme, whereas estimates on the basis of gel filtration were both lower, corresponding to 24 and 30 kDa, respectively. *B. megaterium* P1 and *Bacillus* sp. strain 7-M chitosanases each yield oligomeric species as end products of chitosan hydrolysis. Both chitosanases appear to possess similar optimum temperatures; however, thermal stability of the *Bacillus* sp. strain 7-M chitosanase was assayed over a 15-min period at 37°C, whereas the *B. megaterium* enzyme was stable for at least 1 h at this temperature. Although the V_{max}s of both chitosanases are of the same order of magnitude, the K_m of the *B. megaterium* P1 chitosanase is substantially lower, suggesting tighter substrate binding. Catalytic efficiency as measured by the ratio V_{max}/K_m is some 30 times higher than that for the *Bacillus* sp. strain 7-M enzyme.

The two chitosanases also differ significantly on the basis of their pH activity profiles. The maximum activity for the *B. megaterium* P1 chitosanase corresponds to a broad region ranging from pH 4.5 to 6.5, whereas the *Bacillus* sp. strain 7-M enzyme possesses a well defined peak at pH 6.0. These differences could indicate either completely different enzymes or evolutionary divergence of a chitosanase gene common to the *Bacillus* species.

A single type of hydrolyzing activity has been isolated in each chitosan-degrading bacterium investigated to date. This is in striking contrast with the bacterial cellulose degradation systems, which secrete at least three different activities, depolymerizing their substrate by a combination of exo- and endoglycolytic modes as well as by a cellobiase (3). All *B. megaterium* P1 enzymes that are capable of depolymerizing a chitosan of high degree of polymerization (d.p.) have been isolated, and no exoglycolytic-type activity is present. Nonetheless, a "chitosanbiase" activity capable of hydrolyzing only low-d.p. chitosans may have remained undetected by virtue of the large d.p. of the substrate used in the assay as well as by the fact that only activities secreted in the medium were analyzed.

The possibility that D-glucosamine is not the end product of the *Bacillus* chitosan hydrolysis should be considered, since D-glucosamine does not induce chitosanase secretion above the basal level even though expression of bacterial hydrolytic systems is frequently induced in the presence of low concentrations of their end products. How *B. megaterium* P1 utilizes the chitosan oligomers remains to be investigated.

ACKNOWLEDGMENTS

This work was supported by Les Actions Structurantes du Québec and Les Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.

We thank D. Beaudry for helpful discussions and C. Jutras for assistance and preparation of this manuscript.

LITERATURE CITED

1. **Allan, G. G., J. R. Fox, and N. Kong.** 1978. *In* R. A. A. Muzzarelli and E. R. Parsier (ed.), Proceedings of the first international conference on chitin and chitosan, p. 64–78. MIT Press, Cambridge, Mass.
2. **Chaplin, M. P.** 1986. Monosaccharides, p. 15. *In* M. F. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis. IRL Press, Oxford.
3. **Coughlan, M. P.** 1985. The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnol. Genet. Eng. Rev. **3**:39–109.
4. **David, B., and D. E. Eveleigh.** 1984. Chitosanases: occurrence, production and immobilization, p. 161–179. *In* J. P. Zikalis (ed.), Chitin, chitosan and related enzymes. Academic Press, Inc., Orlando, Fla.
5. **Fenton, D. M., and D. E. Eveleigh.** 1981. Purification and mode of action of a chitosanase from *Penicillium islandicum*. J. Gen. Microbiol. **126**:151–165.
6. **Hedges, A., and R. S. Wolfe.** 1974. Extracellular enzyme from *Myxobacter* AL-1 that exhibits both β-1,4 glucanase and chitosanase activities. J. Bacteriol. **120**:844–853.
7. **Kreger, D. R.** 1954. Observations on cell walls of yeasts and some other fungi by X-ray diffraction and solubility tests. Biochim. Biophys. Acta **13**:1.
8. **Ohtakara, A., H. Ogata, Y. Taketomi, and M. Mitsutomi.** 1984. Purification and characterization of chitosanase from *Streptomyces Griseus*, p. 147–160. *In* J. P. Zikakis (ed.), Chitin, chitosan and related enzymes. Academic Press, Inc., Orlando, Fla.
9. **Price, J. S., and R. Stork.** 1975. Production, purification, and characterization of an extracellular chitosanase from *Streptomyces*. J. Bacteriol. **124**:1574–1585.
10. **Saul, A., and M. Don.** 1984. A rapid method of concentrating proteins in small volumes with high recovery using Sephadex G-25. Anal. Biochem. **138**:451–453.
11. **Seng, J.-M.** 1988. Chitine, chitosane et dérivés: de nouvelles perspectives pour l'industrie. Biofutur **71**:40–44.
12. **Spiro, R. G.** 1966. Analysis of sugars found in glycoproteins. Methods Enzymol. **8**:7–9.
13. **Tominaga, Y., and Y. Stujisake.** 1975. Purification and some enzymatic properties of the chitosanase from *Bacillus R-4* which lyses *Rhizopus* Cell Walls. Biochim. Biophys. Acta **410**:145–155.
14. **Uchida, Y., and A. Ohtakara.** 1988. Chitosanase from *Bacillus* species. Methods Enzymol. **161**:501–505.