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Production of Two Chitosanases from a Chitosan-Assimilating Bacterium, *Acinetobacter* sp. Strain CHB101

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A bacterial strain capable of utilizing chitosan as a sole carbon source was isolated from soil and was identified as a member of the genus *Acinetobacter*. This strain, designated CHB101, produced extracellular chitosan-degrading enzymes in the absence of chitosan. The chitosan-degrading activity in the culture fluid increased when cultures reached the early stationary phase, although the level of activity was low in the exponential growth phase. Two chitosanases, chitosanases I and II, which had molecular weights of 37,000 and 30,000, respectively, were purified from the culture fluid. Chitosanase I exhibited substrate specificity for chitosan that had a low degree of acetylation (10 to 30%), while chitosanase II degraded colloidal chitin and glycol chitin, as well as chitosan that had a degree of acetylation of 30%. Rapid decreases in the viscosities of chitosan solutions suggested that both chitosanases catalyzed an endo type of cleavage reaction; however, chitosan oligomers (molecules smaller than pentamers) were not produced after a prolonged reaction.

Chitosan, a deacetylated derivative of chitin (a linear polysaccharide consisting of β-1,4-linked *N*-acetylglucosamine residues), occurs only in the cell walls of fungi belonging to the order Mucorales in nature (3). However, chitosan molecules that have different degrees of acetylation (D.A.) can be readily obtained by chemically deacetylating chitin that is extracted from an abundant source, shrimp and crab shells. Low-molecular-weight chitosan oligomers have received attention because of their interesting biological properties, including their inhibitory effects on the growth of fungi and bacteria (2, 8) and their ability to elicit phytoalexin production in higher plants (6, 11). Chitosanolytic enzymes have been found in a variety of microorganisms, including bacteria (4, 7, 15–17, 19, 23–25) and fungi (1, 5, 21, 22), in part because these enzymes potentially could be used in the preparation of chitosan oligomers. Thus, an enzymatic procedure would replace the chemical depolymerization method in which concentrated hydrochloric acid is used. Most of microbial chitosanases that have been purified and characterized catalyze an endo type of cleavage reaction in which the relative velocity is highly dependent on the D.A. of chitosan and requires the addition of chitosan to the medium. To obtain a novel chitosanase which could be used for large-scale production of chitosan oligomers, we screened various types of microorganisms that are able to use chitosan as a sole carbon source. We found one strain which produced extracellular chitosanolytic enzymes in cultures that did not contain chitin- or chitosan-related compounds. In this paper, we describe the physiological properties of this bacterial strain and the purification and characterization of two chitosanases produced by it.

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MATERIALS AND METHODS

Culture conditions. The medium used throughout this study was M9 synthetic medium (18). To determine the productivity of chitosanolytic enzymes, glucose was replaced with various carbon sources. When chitosan was used as the carbon source (at a final concentration of 0.25%), it was autoclaved separately from other constituents after it was dissolved in 0.1 N HCl and the pH of the solution was adjusted to 5.6 with NaOH. Cells were grown at 30°C with shaking, and culture fluid was obtained after the cells were sedimented by centrifugation. The culture supernatant was used for a chitosanase assay after overnight dialysis against 25 mM sodium acetate buffer (pH 5.6) (buffer B).

Screening of bacterial strains by using chitosan as a sole carbon source. The samples used in experiments to screen for chitosan-assimilating bacterial strains were obtained from soils from around Ueda City, Nagano Prefecture, Japan. Microbial cells were washed out of the samples with deionized water, and appropriate amounts of the resulting cell suspensions were inoculated into 500-ml Erlenmeyer flasks containing 50 ml of M9 medium supplemented with 0.25% chitosan with a D.A. of 30% as the sole carbon source (M9-chitosan medium). After the samples were incubated at 25°C for 7 days on a rotary shaker (150 rpm), a 2-ml portion of each preparation was transferred into 50 ml of fresh M9-chitosan medium, and the resulting cultures were incubated under the same conditions for an additional 7 days. Subsequently, the samples were spread onto M9-chitosan medium solidified with 1.5% agar. Clear zones (halos) were observed around some microbial colonies after incubation for several days at 30°C. Pure cultures were obtained by successively isolating single colonies and growing them on M9-chitosan agar.

Chitosanase assay. Unless indicated otherwise, chitosan with a D.A. of 30% was used as the substrate in a standard chitosanase assay, and the amounts of reducing sugars liberated during hydrolysis of chitosan were determined by the method of Imoto and Yagishita (9). Each 2-ml reaction mixture contained 1 ml of 0.5% chitosan in 50 mM sodium acetate buffer (pH 5.6) and 1 ml of enzyme solution. After incubation at 37°C for 15 min, the reaction was terminated by immersing the test tube in boiling water for 3 min; 1 U of activity was defined as the amount of enzyme which liberated 1 μmol of reducing sugar per min. Glucosamine was used as the standard in this assay. A viscometric chitosanase assay was performed by using the method of Ohtakara (13). The reaction mixtures (7 ml) contained 0.1% chitosan with a D.A. of 30% in 30 mM sodium acetate buffer (pH 5.6) and various amounts of purified chitosanase. The reactions were performed in an Ostwald viscosimeter (Shibata model 1) kept at 37°C, and the flow time of each mixture was determined at appropriate intervals.

Purification of chitosanases. All purification procedures were performed at 4°C. To the culture supernatant obtained from 500 ml of a culture containing 0.2% D-glucosamine as the sole carbon source, enough ammonium sulfate was added to achieve 80% saturation. The resulting precipitate was collected by centrifugation and dissolved in 20 ml of 20 mM sodium acetate buffer (pH 4.6) (buffer A). The sample was dialyzed against buffer A and loaded onto a CM-Sepharose CL-6B (Pharmacia) column (2.2 by 25 cm) equilibrated with buffer A. Washing the column with a linear gradient of buffer A containing 0 to 1 M KCl resulted in elution of adsorbed proteins with chitosanase activity in two separate peaks. The active fractions were pooled separately, and each pool was placed on

a Sephadex G-100 column (2.2 by 51 cm) equilibrated with buffer B. The active enzyme was eluted with buffer B, and the resulting preparation was considered the purified enzyme.

Analytical methods. Protein contents were determined with a protein assay kit (Bio-Rad), using bovine serum albumin as the standard. The purity of the enzyme was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (12). The products of enzymatic hydrolysis were analyzed by thin-layer chromatography by the method of Sakai et al. (17). Each reaction mixture (50 μl) contained 2.5 μmol of sodium acetate buffer (pH 5.6), substrate (0.5 μmol for chitooligomers and 125 μg for chitosan with a D.A. of 0%), and 3.0 mU of purified chitosanase I or 3.0 mU of purified chitosanase II. In the case of crude proteins obtained from the culture fluid, we used an amount corresponding to 2 μg of proteins. After incubation at 37°C overnight, the reaction was terminated by immersing the reaction tube in boiling water for 3 min. The reaction products were analyzed by thin-layer chromatography with Merck HPTLC Silica Gel 60 (aluminum sheets). Amino sugars were detected by the ninhydrin reaction.

Chemicals. Chitosan 70H (D.A., 30%), glycol chitosan (degree of polymerization, ≥400), chitobiose, chitotriose, chitotetraose, and chitopentaose were obtained from Wako Junyaku Co., Ltd. Chitosan 10B (D.A., 0%), chitosan 9B (D.A., 10%), and chitosan 8B (D.A., 20%) were purchased from Funakoshi Co., Ltd. Glycol chitin was obtained from Seikagaku Kogyo Co., Ltd. Colloidal chitin was prepared by the method of Shimahara and Takiguchi (20), using commercially available chitin powder from shrimp shells (Nakarai Tesque, Inc.).

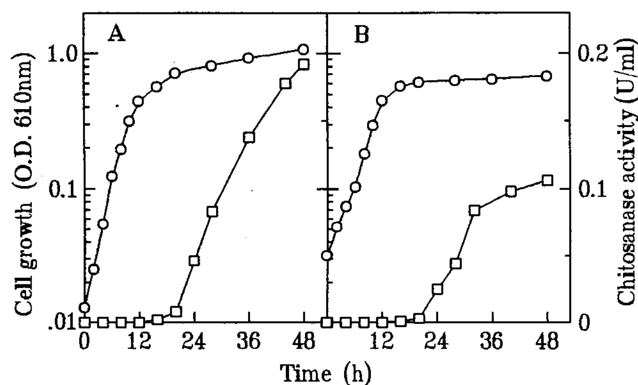

FIG. 1. Cell growth and extracellular chitosanolytic activity of *Acinetobacter* sp. strain CHB101. Cells were grown in M9-glucose medium (A) and in M9-chitosan medium (B) at 30°C with shaking. At appropriate intervals, cell density was measured by determining the A_{610}, and the chitosanolytic activity in the culture fluid was determined by using chitosan with a D.A. of 30% as the substrate. Symbols: ○, cell growth (optical density at 610 nm [O.D.610nm]); □, chitosanase activity.

RESULTS

Isolation of the chitosan-assimilating bacterium *Acinetobacter* sp. strain CHB101. During our screening experiments, we observed various kinds of microbial colonies that formed halos on M9-chitosan agar. Several bacterial cultures in which the colonies formed large, clear halos were purified and then tested for extracellular chitosanolytic activity when they were grown in M9-glucose and M9-chitosan liquid media. All of these isolates exhibited significant levels of chitosanase activity in M9-chitosan medium; however, only one of the isolates (isolate 5) produced chitosanase in M9-glucose medium (data not shown). These preliminary results suggested that the chitosanolytic enzyme(s) of isolate 5 was produced constitutively or was not susceptible to catabolite (glucose) repression. Isolate 5 was chosen for further study because of its apparent suitability for large-scale production of chitosanase.

Bacterial isolate 5 was subjected to a taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology* (10) and was identified as a member of the genus *Acinetobacter*. This taxonomic identification was based mainly on the following criteria: the organism was gram negative, aerobic, oxidase negative, catalase positive, nonmotile, and rod shaped. At the present time, a single species, *Acinetobacter calcoaceticus*, has been proposed for all strains of the genus *Acinetobacter*, although future studies may provide a basis for establishing other species (10). Isolate 5 was similar to *A. calcoaceticus* type strain ATCC 23055 in almost all traits tested, but differed in its ability to utilize chitosan and its inability to utilize acetate as a sole carbon source. Therefore, isolate 5 was tentatively designated *Acinetobacter* sp. strain CHB101.

Chitosanase production by *Acinetobacter* sp. strain CHB101. Strain CHB101 was investigated for extracellular chitosanase productivity when it was grown in M9-glucose medium and M9-chitosan medium. This strain grew well in M9-chitosan medium with a generation time of 280 min, although the growth rate in this medium was lower than the growth rate in M9-glucose medium (generation time, 110 min) (Fig. 1). In both cultures, the level of chitosanase activity in the culture fluid was 0.10 to 0.20 U/ml after 2 days of incubation. Interestingly, the level of activity remained low (less than 0.01 U/ml) while the cells were growing exponentially and began to increase when the cultures reached the stationary phase (Fig. 1). This indicated that the production and/or secretion of chitosanase may depend on the growth phase of a batch culture. The possibility that a preexisting intracellular chitosanase leaked out during cell lysis was ruled out by the observation that the number of viable cells in a culture (ca. 1×10^9 cells per ml) did not decrease during the stationary phase (after up to 4 days of incubation). Furthermore, significant levels of chitosanase activity were not detected in cell extracts obtained from exponentially growing cultures (data not shown).

Degradation activity for other polysaccharides was assayed by using both stationary-phase cultures grown in M9-glucose medium and stationary-phase cultures grown in M9-chitosan medium. Both types of cultures exhibited significant levels of degradation activity for chitosan with a D.A. of 30%, chitosan with a D.A. of 0%, and glycol chitin but did not exhibit any degradation activity for glycol chitosan or carboxymethyl cellulose (data not shown). The extracellular proteins in M9-glucose medium cultures were fractionated by using CM-Sepharose CL-6B column chromatography to identify the protein(s) with chitosanolytic activity. When chitosan with a D.A. of 30% was used as the substrate, the activity eluted in two separate peaks. The peak which eluted at a high ionic strength (peak II) was active against both chitosan with a D.A. of 30% and glycol chitin. The same elution pattern was observed with chitosan cultures grown in M9 medium (data not shown). Tests to determine the effects of various carbon sources on production of extracellular chitosanase revealed that the two peaks of chitosanase activity were also observed in stationary-phase cultures when we used M9 medium containing glucosamine, *N*-acetylglucosamine, or glycerol as the carbon source (data not shown). However, the strain did not grow at all when either colloidal chitin or glycol chitin was used as the carbon source.

Purification and characterization of two chitosanases from *Acinetobacter* sp. strain CHB101. Two chitosanases were purified from stationary-phase cultures grown in M9-glucosamine medium in order to examine their roles in chitosan degradation (Table 1). Glucosamine was used as the carbon source for growth because the resulting culture fluid exhibited the highest specific activity observed with the carbon sources tested (data not shown). Like the elution pattern described above, chitosanase activity eluted in two separate peaks during CM-Sepharose chromatography. The chitosanolytic proteins that eluted in peaks I and II were designated chitosanases I and II, respectively. Both of these chitosanases were purified by successive Sephadex G-100 gel chromatography to homogeneity, as

TABLE 1. Purification of chitosanases I and II from *Acinetobacter* sp. strain CHB101 culture fluid

Step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	% Recovery
Culture supernatant	453	101	—[a]	—	100
0–80% $(NH_4)_2SO_4$ gradient	27	107	2.06	52	106
Chitosanase I					
CM-Sepharose	11	24	0.22	109	24
Sephadex G-100	21	17	0.05	334	17
Chitosanase II					
CM-Sepharose	11	28	0.07	406	28
Sephadex G-100	21	16	0.02	800	16

[a] —, Protein contents could not be determined because of a disturbance caused by unknown constituents in the sample.

judged by SDS-polyacrylamide gel electrophoresis. The molecular masses of chitosanases I and II were estimated to be 37 and 30 kDa, respectively. Both of these chitosanases eluted from Sephadex G-100 columns in the positions predicted for proteins with their molecular weights, as judged by SDS-polyacrylamide gel electrophoresis, indicating that they were monomeric. Kinetic properties were determined by using purified chitosanases I and II.

(i) Effects of pH and temperature on activity. Both chitosanases exhibited the same level of activity at a broad range of pH values (pH 5 to 9) when glycol chitin was used as the substrate. Chitosanases I and II exhibited maximum activity at 65 and 50°C, respectively, when they were assayed under standard conditions by using chitosan with a D.A. of 30% in 50 mM sodium acetate buffer (pH 5.6).

(ii) Substrate specificity. The activities of the two chitosanases with various substrates are summarized in Table 2. Chitosanase I degraded chitosan with a D.A. of 10 to 30% effectively and exhibited low activity with chitosan having a D.A. of 0%, colloidal chitin, and glycol chitin. On the other hand, chitosanase II preferentially degraded glycol chitin and exhibited an insignificant level of activity with chitosan with a D.A. of 0 to 10%. Neither chitosanase hydrolyzed glycol chitosan or carboxymethyl cellulose.

(iii) Reduction in the viscosity of a chitosan solution. Both chitosanase I and chitosanase II reduced the viscosity of a solution of chitosan with a D.A. of 30% extensively at an early stage of the reaction (Fig. 2). The rates of reduction were similar for chitosanases I and II when the same amount of enzyme was used, as judged by the values obtained in the standard reducing sugar assay.

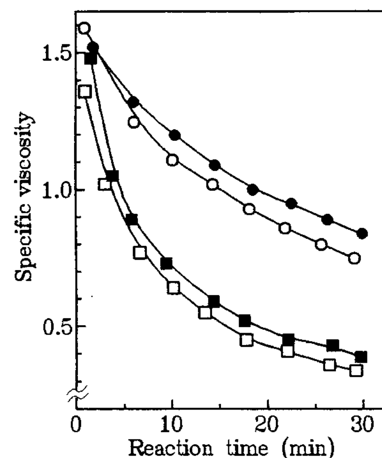

FIG. 2. Reductions in the viscosity of chitosan solutions obtained with chitosanases I and II. Reductions in viscosity were determined with an Ostwald viscosimeter. Specific viscosity was defined as follows: [(flow time of reaction mixture)/(flow time of distilled water)] − 1. Different amounts of enzymes were used. Symbols: ○, 4 mU of chitosanase I; □, 16 mU of chitosanase I; ●, 4 mU of chitosanase II; ■, 16 mU of chitosanase II.

(iv) Analysis of the reaction products. The reaction products were analyzed by thin-layer chromatography after a prolonged reaction in which chitosan with a D.A. of 0% or chitooligosaccharides were used as the substrates. Neither chitosanase produced glucosamine oligomers smaller than the pentamer, which was the longest oligomer that could be separated and detected under the chromatography conditions used (Fig. 3, lanes 1 and 2). Furthermore, chitobiose, chitotriose, chitotetraose, and chitopentaose were not degraded by either enzyme after prolonged reactions (Fig. 3, lanes 3 and 4). The reaction products were analyzed in a similar way by using crude proteins obtained from the culture fluid by ammonium sulfate precipitation (Table 1). Chitosan with a D.A. of 0% was degraded to produce a mixture of chitobiose and chitotriose (Fig. 3, lane 6), and chitopentaose was degraded to chitobiose and chitotriose (Fig. 3, lane 5).

TABLE 2. Substrate specificities of chitosanases I and II

Substrate[a]	Relative activity (%)[b]	
	Chitosanase I	Chitosanase II
Chitosan (D.A., 30%)	86	45
Chitosan (D.A., 20%)	100	35
Chitosan (D.A., 10%)	85	17
Chitosan (D.A., 0%)	20	10
Glycol chitin	22	100
Colloidal chitin	15	30
Glycol chitosan	7	2
Carboxymethyl cellulose	2	2

[a] The experiments were performed by using the standard assay conditions described in the text. Each reaction mixture contained substrate at a concentration of 0.25%.

[b] The amount of reducing sugar liberated after a 15-min reaction relative to the largest amount for each chitosanase (280 and 190 nmol for chitosanases I and II, respectively).

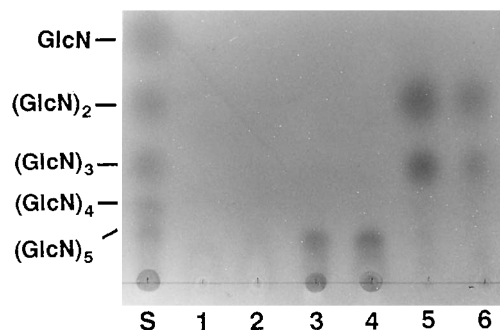

FIG. 3. Analysis of enzymatic hydrolysates by thin-layer chromatography. The substrates used were chitosan (lanes 1, 2, and 6) and chitopentaose (lanes 3 through 5). The enzymes used were purified chitosanase I (lanes 1 and 3), purified chitosanase II (lanes 2 and 4), and crude proteins (lanes 5 and 6). Lane S contained a mixture of chitooligomers ranging from glucosamine (GlcN) to chitopentaose [$(GlcN)_5$].

DISCUSSION

The bacterial strain used in this study, which was determined to be a member of the genus *Acinetobacter*, was isolated from soil and was able to use chitosan with a D.A. of 30% as a sole carbon source. This strain, tentatively designated *Acinetobacter* sp. strain CHB101, produced two extracellular chitosanases in M9 synthetic medium containing glucose, chitosan with a D.A. of 30%, glycerol, glucosamine, or *N*-acetylglucosamine as a source of carbon. The previously described chitosanase-producing bacterial strains are members of the following genera: *Bacillus* (15, 19, 23), *Pseudomonas* (25), *Myxobacter* (7), *Enterobacter* (24), *Streptomyces* (4, 16), and *Nocardia* (17). To our knowledge, this is the first report of a chitosanase-producing bacterium belonging to the genus *Acinetobacter*.

Strain CHB101 grew well in M9 synthetic medium containing chitosan with a D.A. of 30% as the sole carbon source (Fig. 1). Our data suggest that exogenously added chitosan is rapidly degraded to low-molecular-weight saccharides that can support the growth of bacterial cells. However, our observation that the amounts of extracellular chitosanases increased when cultures reached the stationary phase seems to contradict this observation (Fig. 1). Plausible explanations for this are that a small amount of extracellular chitosanase during the exponential growth phase may be sufficient to produce a critical level of saccharides which support cell growth and that there may be other chitosanolytic enzymes which were not detected by the standard assay (determination of reducing sugar). The two chitosanases which we purified (chitosanases I and II) seem to catalyze an endo type of cleavage reaction, as judged from the rapid reductions in the viscosity of chitosan solutions (Fig. 2). The final products from chitosan after a prolonged reaction were chitooligosaccharides (oligosaccharides larger than the pentamer) which seemed not to be taken up directly by the bacterial cells (Fig. 3). An analysis of the degradation products in which we used crude proteins in the culture fluid clearly revealed that there was an unknown enzyme(s) which was capable of producing chitobiose and chitotriose from chitopentaose as well as chitosan (Fig. 3). We did not detect this enzyme in a standard assay in which chitosan was used as the substrate, possibly because the enzyme did not degrade high-molecular-weight chitosan at a significant rate. This enzyme apparently degrades chitosan oligomers produced by chitosanases I and II into smaller units (chitobiose and chitotriose) which are easily taken up and metabolized by strain CHB101 cells. We are now trying to purify the enzyme which catalyzes the cleavage of chitopentaose into chitobiose and chitotriose. A kinetic study of this enzyme should clarify the mechanism of chitosan degradation and utilization in this bacterium.

Acinetobacter sp. strain CHB101 produced two extracellular chitosanases (chitosanases I and II) whose substrate specificities differed with respect to the D.A. of polysaccharides consisting of glucosamine and *N*-acetylglucosamine (Table 2). Most of the chitosanolytic bacterial strains described previously produce a single chitosanase which degrades chitosan molecules that have D.A. values within a narrow range but does not degrade chitin. Because strain CHB101 produces two different types of chitosanases, we presumed that this organism could degrade chitinous polymers with a broad range of D.A. values (from 0 to 100%), although it could not grow at all in M9 medium containing colloidal chitin as a sole carbon source. It might be possible to call chitosanase II a chitinase, because the enzyme preferentially degrades glycol chitin. We are now trying to molecularly clone the chitosanase genes from strain CHB101. The fact that the extracellular levels of chitosanases I and II began to increase during the stationary phase of growth suggested that production of the enzymes depends on the growth phase in batch cultures (Fig. 1). If this is the case, production of chitosanases may be regulated at the level of gene transcription, although we cannot rule out the possibility that a preexisting inactive form of chitosanase is processed to an active form and secreted during the stationary phase. If cloned chitosanase genes were obtained, the regulation of chitosanase production and secretion could be clarified in detail.

Strain CHB101 produced chitosanases in the absence of the substrate chitosan. Moreover, we found that almost the same level of chitosanase was produced in a complex medium composed of peptone and yeast extract as in M9 synthetic medium (data not shown). This trait is highly desirable for large-scale production of chitosanase for industrial use. It should be noted that enzymatic cleavage of chitosan with purified chitosanase I or II produces chitosan oligomers that have potential use as antibacterial or antifungal reagents (2, 8) and as elicitors (6, 11). Chitosanases I and II could be used to efficiently produce chitosan oligomers which are difficult to obtain by acid hydrolysis.

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