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Production of chitinolytic enzymes from a novel species of *Aeromonas*

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A bacterial strain secreting potent chitinolytic activity was isolated from shrimp-pond water by enrichment culture using colloidal crab-shell chitin as the major carbon source. The isolated bacterium, designated as *Aeromonas* sp No. 16, exhibited a rod-like morphology with a polar flagellum. Under optimal culture conditions in 500-ml shaker flasks, it produced a chitinolytic activity of 1.4 U ml^{-1}. A slightly higher enzymatic activity of 1.5 U ml^{-1} was obtained when cultivation was carried out in a 5-liter jar fermentor using a medium containing crystalline chitin as the carbon source. The secretion of the enzyme(s) was stimulated by several organic nitrogenous supplements. Most carbon sources tested (glucose, maltose, N-acetylglucosamine, etc) enhanced cell growth, but they slightly inhibited enzyme secretion. Glucosamine (0.5% w/v) severely inhibited cell growth (16% of the control), but it did not significantly affect enzyme secretion. The production of chitinolytic enzymes was pH sensitive and was enhanced by increasing the concentration of colloidal chitin to 1.5%. The observed chitinolytic activity could be attributed to the presence of β-N-acetylglucosaminidase and chitinase. Chitinase was purified by ammonium sulfate fractionation and preparative gel electrophoresis to three major bands on SDS-PAGE. An in-gel enzymatic activity assay indicated that all three bands possessed chitinase activity. Analysis of the enzymatic products indicated that the purified enzyme(s) hydrolyzed colloidal chitin predominantly to N,N-diacetyl-chitobiose and, to a much lesser extent, the mono-, tri-, and tetramer of N-acetylglucosamine, suggesting that they are mainly endochitinases.

Keywords: chitin; chitinase; *Aeromonas* sp

Introduction

Chitin is an unbranched polysaccharide composed primarily of β-1,4 linked N-acetylglucosamine and, occasionally, glucosamine residues. It is the principal structural component of the exoskeletons of insects and such crustacea as shrimp and crab. The global distribution and abundance of chitin make it a vast, renewable source of both carbon and nitrogen. An estimation of the world-wide chitinous waste, suitable for use as the potential source of fermentation feedstock, is in excess of 150 kilotons per annum [3]. Hydrolysis of chitin is, however, relatively difficult due to its crystalline nature, which necessitates the use of strong mineral acids or bases [1,9].

In the past several years, there has been a steady increase in the demand of chitin and its hydrolytic derivatives for various industrial, clinical, and pharmaceutical applications. Some chitooligosaccharides and their derivatives have been shown to promote the growth of bifidobacteria, reduce the plasma concentration of cholesterol, accelerate wound healing, stimulate the immune system, display anti-tumor activity, and inhibit microbial growth [1,3,6,13,14]. These medical applications have added a great deal of value to chitooligosaccharides. Chitooligosaccharides are usually produced by the partial hydrolysis of chitin or chitosan with concentrated acids, followed by column chromatographic separation. The process is labor-intensive and not very cost-effective due to the low yield [13]. The potential of developing an enzymatic process for the production of chitooligosaccharides has attracted the attention of biotechnologists. To date, however, progress in the industrial-scale production of chitinolytic enzymes has been limited.

In Taiwan, solid waste from the seafood processing industry represents a rich source of chitin and its oligomer derivatives. Recovery of useful chitin-derivatives has been considered favorably as a potential solution to minimize pollution caused by the waste disposal of seafood processing companies. As part of our ongoing research on re-utilization of chitin waste, the present study is aimed to establish an easy, reliable, and inexpensive process for the production of chitinase (EC 3.2.1.14). Herein, we describe the isolation and identification of a novel chitin-degrading bacterium, *Aeromonas* sp No. 16, which secretes potent chitinase activity capable of degrading crystalline chitin, and report the optimization of conditions for enzyme production and the characterization of the purified enzymes.

Materials and methods

Chemicals

Chitin, chitosan, N-acetylglucosamine, and chitin oligosaccharides were purchased from Sigma Chemical Co (St Louis, MO, USA). Nutrient agar was obtained from Difco Laboratories (Detroit, MI, USA). All other chemicals were of reagent grade and were purchased from commercial suppliers.

Preparation of chitin substrates

One hundred grams of crab shell obtained from a local seafood processing plant were demineralized with 2.5 N HCl at room temperature for 10 min and deproteinized with 2 N NaOH at 100°C for 30 min. This yielded 17 g chitin

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with a purity of about 97.5%. Crystalline chitin was prepared by milling the chitin obtained and sieving it through a 60-mesh screen. The resulting products have a particle size of 0.25 mm or less. Colloidal chitin was prepared according to the method of Shimahara and Takiguchi [21] by using this chitin or practical-grade crab shell chitin purchased from Sigma Chemical Company. An aliquot of the preparation was dried to constant weight *in vacuo* to determine the concentration of chitin. The remainder was then diluted to the desired concentration and stored at 4°C.

Microorganism and cultivation

Bacterial strains were isolated from shrimp-pond water sampled from Northern Taiwan. One strain, which produced high chitinolytic activity, was identified as *Aeromonas* sp No. 16, according to Bergey's Manual [16], and was used in this study. Two reference strains, *Aeromonas hydrophila* and *A. sobria*, were obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. The bacteria were maintained at 4°C on nutrient agar medium supplemented with 0.03% colloidal chitin and subcultured once per month. For inoculum culture, a loopful of bacteria was inoculated into 50 ml of nutrient broth (Difco) in a 250-ml Erlenmeyer flask and incubated at 30°C for 9 h in a reciprocating shaker water bath set at 130 opm and a 3.85-cm stroke. For enzyme production, 2 ml of inoculum culture was added to 50 ml culture medium in a 500-ml shaker flask. The medium contained 3.0 g colloidal chitin, 0.5 g yeast extract, 1.0 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$ and 1.0 g KH_2PO_4 in 1 L distilled water, unless otherwise specified. The cultures were incubated at 30°C for 24 h in a rotary shaker set at 100 rpm and a 7-cm stroke. For large-scale experiments, 100 ml inoculum culture was added to a 2-L culture medium in a 5-L jar fermentor (Model LS-205, Hotech Instruments Corp, Taipei, Taiwan) and the cultivation was carried out at 30°C for 2 days under the conditions indicated. The pH of the medium was maintained between pH 7 and 8 by automatic addition of 28% ammonia water or 1 N HCl. Samples were taken at intervals for analysis.

Enzyme assays

Samples were centrifuged at 8000 × *g* for 5 min to remove cells and unhydrolyzed chitin. Total chitinase activity in the supernatant medium was assayed by measuring reducing sugars released from colloidal chitin. The reaction mixture contained 0.5 ml of 2.5% colloidal chitin, 1.4 ml of 50 mM phosphate buffer (pH 6.0), and 0.1 ml of a diluted supernatant sample. The reaction was allowed to proceed at 40°C for 10 min and terminated by boiling for 15 min. One unit (U) of chitinase is defined as the amount of enzyme required to produce the reducing equivalent of 1 μmol of N-acetylglucosamine per min under the assay conditions. For the determination of β-N-acetylglucosaminidase (EC 3.2.1.30; GlcNAcase) activity, the reaction mixture contained 0.1 ml of 10 mM *p*-nitrophenyl N-acetyl-β-D-glucosaminide, 0.8 ml of 50 mM phosphate buffer (pH 6.0), and 0.1 ml of a diluted supernatant sample. The reaction was carried out at 40°C for 5 min and terminated by addition of 2 ml of a 0.25 M calcium carbonate solution. The amount of *p*-nitrophenol released was determined colorimetrically by measuring the absorbance at 405 nm. One unit (U) of GlcNAcase is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per min under the assay conditions. All assays were carried out in duplicate. Either the enzyme or substrate was omitted from the control.

Gel electrophoresis and activity staining

Non-denaturing gel electrophoresis and SDS-PAGE were carried out using 7.5% gels. Proteins were stained with Coomassie brilliant blue R-250. Chitinase activity in a non-denaturing gel was detected by a modified method of Trudel and Asselin [26]. The resolving gel was incubated in 0.1 M phosphate buffer (pH 6.0) for 10 min, placed on a clean glass plate, and overlaid with agarose gel (1% (w/v), 0.75 mm) containing 0.01% (w/v) glycol chitin. The reaction was allowed to proceed at 37°C for 30–60 min. Following incubation, the agarose gel was dipped into a freshly prepared solution containing 0.01% (w/v) Calcofluor white M2R (Sigma) in 50 mM Tris-HCl (pH 8.9) for 5 min. The gel was then removed and rinsed in distilled water for about 1 h at room temperature to remove unadsorbed dye. Lytic zones were visualized under long-wave UV light and photographed. The PAGE gel was then stained with Coomassie brilliant blue R-250 to identify protein bands.

Analytical methods

Reducing sugars were measured colorimetrically using the ferri-ferrocyanide reagent, according to a modification of the method of Schales [7], and expressed as N-acetylglucosamine equivalent. The sugar profile of chitin hydrolysate was identified by ascending thin-layer chromatography using *n*-propanol : water : ammonia water (70 : 30 : 0.3 v/v) as the developing solvent. The dried chromatograms were impregnated with saturated $AgNO_3$ in acetone. Sugar spots were visualized by spraying the dried chromatogram with 0.5 N NaOH in ethanol solution followed by heating at 80°C for 20 min. Protein was determined by a protein assay kit (Sigma, procedure No. P 5656) using bovine serum albumin as the standard. Bacterial growth was measured by counting the number of colonies formed on nutrient agar plates and was expressed as colony-forming units (CFU) per milliliter. All results are the mean of at least two duplicate experiments.

Results

Isolation of chitinase-producing microorganisms

Pond water and soil samples obtained from shrimp farms in Northern Taiwan were used as the inoculum in an enrichment culture using a medium containing colloidal chitin as the major carbon source. Several days later, microorganisms showing a higher degree of chitin utilization than the sterile control were aseptically transferred to a fresh medium for further selection. After serial dilution with sterilized water, organisms in the culture were plated out on agar plates containing colloidal chitin. Colonies which exhibited a large zone of clearing were then isolated. About 100 microorganisms, mostly bacteria or actinomycetes, were obtained. Four of these isolates which secreted higher

Table 1 Biochemical characteristics of *Aeromonas* sp strain No. 16

Characteristics	Result
Nitrate reduction	+
Methyl red test	+
ONPG test	+
VP test	+
Arginine dihydrolase	+
Lysine decarboxylase	−
Ornithine decarboxylase	−
Tryptophan deaminase	−
Urease	−
Indole production	+
H_2S from cysteine	+
H_2S from thiosulfate	−
Casein hydrolysis	+
Esculin hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	+

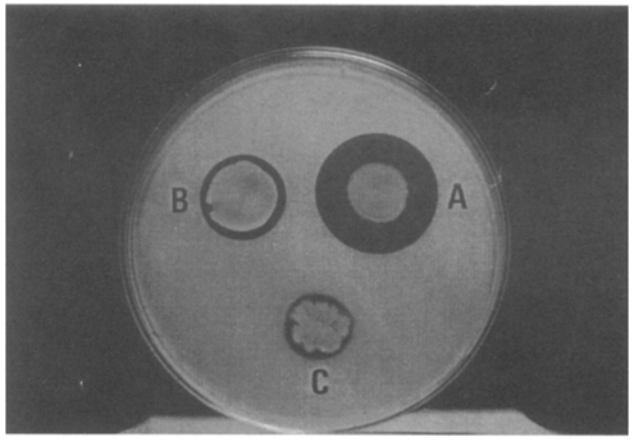

Figure 1 Zones of clearing of three *Aeromonas* strains grown on a chitin-containing nutrient agar plate. Bacterial strains were stabbed onto the colloidal chitin agar plate and incubated at 30°C for 24 h. The strains are: A, *Aeromonas* sp No. 16; B, *Aeromonas hydrophila* ATCC 7966; C, *Aeromonas sobria* ATCC 9071.

chitinolytic activity in culture broth were selected and found to be bacteria. Following purification by streaking, the selected isolates were subjected to screening in shaker flasks. Strain No. 16 was chosen for further study, since it formed the largest zone of clearing on agar plates and its chitinolytic activity appeared earliest during broth culture.

Taxonomic studies of strain No. 16

The morphology of the isolate was established by microscopic examination. After 24 h in shake culture in nutrient broth at 30°C, the cells were observed to be rods with round ends, 0.8–1.0 μm × 1.4–1.8 μm and are mobilized by a polar flagellum. Isolate No. 16 is Gram-negative, catalase-positive, and oxidase-positive and is capable of utilizing a variety of carbohydrates as the carbon source with the production of acid or acid and gas. When cultured on colloidal chitin agar plates, the colonies were circular, glistening, undulate, and white. Table 1 summarizes some additional characteristics of the isolate. Table 2 shows the results of the taxonomic identification obtained using three rapid identification kits. The biochemical characteristics of the isolate are consistent with those described for the genus *Aeromonas* in Bergey's Manual [16]. Several isolates of the *Aeromonas* species have been shown to produce constitutive as well as inducible chitinase [23]. Although the isolate resembles *Aeromonas hydrophila* and *A. sobria*, some differences were noticed, as shown in Figure 1 and Table 3.

Table 2 Taxonomic similarity of the isolated strain to the two *Aeromonas* species as determined by three commercial kits

Kit	Probability (%)	
	A. hydrophila	*A. sobria*
MICROBACT-MB24E MISC	99.99	<0.01
VITEK System	88	10
API 20NE	24.4	73.2

We therefore assigned the isolate to the genus *Aeromonas* and tentatively named it *Aeromonas* sp No. 16.

Culture conditions for chitinase production

The optimization of culture conditions for chitinase production by *Aeromonas* sp No. 16 was conducted by the single-dimensional search method. Only one parameter was varied at a time while others remained at constant levels. Parameters which were considered likely to affect chitinase production were then sequentially optimized. In general, cell growth and enzyme production were greatly influenced by nutrients in the culture medium. Environmental variables did not seem to influence chitinase synthesis directly, rather they affected enzyme production through their effects on cell growth. Since most microorganisms produce chitinase only when chitin or its derivatives are present [5], we first examined the effects of medium components on chitin induction. To compensate the relatively high carbon/ nitrogen (C/N) ratio of chitin (approx 8), compared with that of bacteria (4–5) [2], we included 0.05% yeast extract in the production medium for optimization. Table 4 shows the effects of various nitrogen sources (1.0%) on chitinase production. Among those tested, tryptone was most effective in stimulating the enzyme production. The optimal concentration of tryptone was later determined to be between 1.0 and 2.0% (w/v). With the exception of urea, better results have been obtained with the organic nitrogen sources than the two inorganic ones. The concentration of yeast extract supplement (from 0 to 2.0%) on chitinase production was

Table 3 Biochemical properties differentiating the three *Aeromonas* spp

Test	*A. hydrophila*	*A. sobria*	Strain No. 16
Esculin hydrolysis	+	−	+
L-Arabinose utilization	+	−	−
L-Histidine utilization	+	−	+
L-Arginine utilization	+	−	−
Salicin fermentation	+	−	+

Table 4 Effects of additional nitrogen sources on the production of chitinase by *Aeromonas* sp No. 16

Nitrogen source (1.0%, w/v)	Chitinase activity (U ml^{-1})
None	0.019
Tryptone	0.134
Soybean flour	0.107
Yeast extract	0.091
Casein	0.067
Peptone	0.058
Casamino acid	0.040
Sodium nitrate	0.022
Urea	0.012
Ammonium sulfate	0.008

Basal medium contained (per liter of distilled water): 3.0 g colloidal chitin, 0.5 g yeast extract, 1.0 g KH_2PO_4, and 0.3 g $MgSO_4 \cdot 7H_2O$. Cells were cultured at 30°C for 24 h at 100 rpm in 500-ml shaker flasks, each containing 50 ml of basal medium. The values presented are the means of at least two experiments.

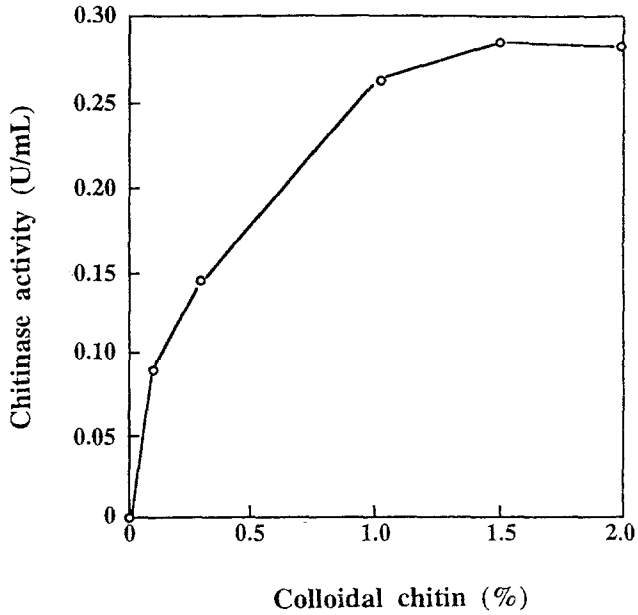

Figure 2 Effect of colloidal chitin concentration on the production of chitinase by *Aeromonas* sp No. 16. Cells were grown in 50 ml medium at 30°C for 24 h in 500-ml shaker flasks agitated at 100 rpm in a rotary shaker. The medium contained 10.0 g tryptone, 0.5 g yeast extract, 1.0 g KH_2PO_4, and 0.3 g $MgSO_4 \cdot 7H_2O$ in 1 L distilled water.

also investigated, and the best result was obtained with 1.5% (w/v). As chitin served as both an inducer and a carbon and/or nitrogen source for chitinase production, the effects of other carbon sources on chitinase production were examined to reveal their potential roles in regulation of chitinase production.

As shown in Table 5, all compounds tested, except for glucosamine, significantly enhanced cell growth but inhibited, slightly to moderately, chitinase production at 0.5%. When the productivity of the enzyme was compared on a per cell basis, glucosamine (0.5%) had an activity/cell mass five-fold higher than that of the control. This suggests that glucosamine has a pronounced stimulatory effect on chitinase production, despite its inhibitory effect on cell growth. Glucosamine was a good carbon source in the production of chitosanases from a different *Acinetobacter* sp [22]. Figure 2 shows the effect of colloidal chitin concentration on enzyme production. The secretion of chitinase into the culture medium increased almost linearly with increasing concentration of colloidal chitin up to 1.5%. No chitinase activity was detected when colloidal chitin was omitted from the medium, indicating that enzyme secretion was inducible by the substrate. Figure 3a shows a time course study of chitinase production by *Aeromonas* sp No. 16 in 500-ml shaker flasks containing 50 ml of the optimized production medium with colloidal chitin. After a lag of about 10 h, the growth of the cells entered the exponential phase which lasted for 2–3 h. The maximum cell count reached 7–8 $\times 10^9$ CFU ml^{-1} in about 24 h. The activity of chitinase in the supernatant medium became detectable soon after the onset of the stationary phase and increased rapidly, reaching a maximum of 1.4 U ml^{-1}, by 20 h. Recently, a similar profile of chitosanase production was also reported [22]. The activity remained almost unchanged thereafter for up to 2 days.

For large-scale cultivation, the use of colloidal chitin was not practical because the preparation of colloidal chitin was labor-intensive. Since crystalline chitin, crab shell powder, and HCl-treated crab shell powder are readily available and relatively inexpensive, we explored the possibility of substituting colloidal chitin with crystalline chitin. A typical result showing the production of chitinase in a 5-L jar fermentor, using a medium containing 1.5% crystalline chitin,

Table 5 Effects of additional carbon sources on chitinase production by *Aeromonas* sp No. 16

Carbon source (0.5%, w/v)	Cell growth ($\times 10^9$ CFU ml^{-1})	Chitinase activity (U ml^{-1})	Specific productivity
None	1.4	0.59	0.42
Glucose	2.7	0.53	0.20
N-Acetylglucosamine	1.7	0.59	0.35
Glucosamine	0.22	0.48	2.18
Maltose	2.8	0.43	0.15
Mannose	2.5	0.46	0.18
Galactose	2.2	0.42	0.19
Sucrose	3.0	0.51	0.17

Basal medium contained (per liter of distilled water): 10.0 g colloidal chitin, 20.0 g tryptone, 0.5 g yeast extract, 1.0 g KH_2PO_4, and 0.3 g $MgSO_4 \cdot 7H_2O$. Cultivation conditions were the same as described in Table 4. The data presented are the means of two independent experiments. Specific productivity was calculated by dividing the chitinase activity (U ml^{-1}) with the cell growth ($\times 10^9$ CFU ml^{-1}).

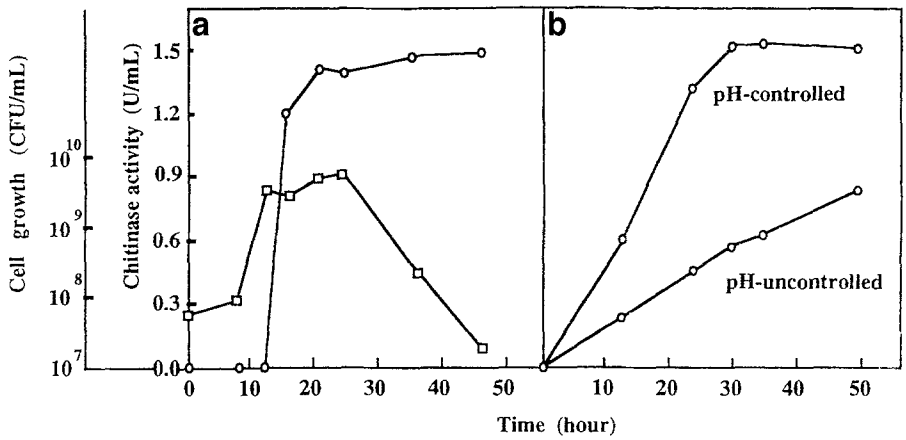

Figure 3 Time course of chitinase production by *Aeromonas* sp No. 16. Cells were grown, as described under Materials and Methods, at 30°C in optimized production medium in (a) 500-ml shaker flasks or (b) a 5-L jar fermentor with or without pH control. Media were sampled at different time intervals and the CFU (□) and chitinase activity (○) were determined.

is shown in Figure 3b. Despite a reduced growth rate of the cells in a crystalline chitin medium (data not shown), a slightly higher (1.5 U ml^{-1}) chitinase activity, as compared to that obtained from a colloidal chitin medium (Figure 3a), could be obtained in 30 h. It was also apparent that the maintenance of pH in the culture broth was critical for preserving enzymatic activity in the medium (Figure 3b). Similar results of pH sensitivity in chitinase production were also obtained by McCormack *et al* [9] and Reid and Ogrydziak [17].

Purification of chitinase produced by Aeromonas sp No. 16

In order to purify the enzyme(s), *Aeromonas* sp No. 16 was cultured for 20 h in a medium containing colloidal chitin. The supernatant medium was then brought to 80% saturation with ammonium sulfate and centrifuged at 10000 × *g* for 20 min. The pellet was re-dissolved in 2 ml phosphate buffer (20 mM, pH 6.0) and dialyzed overnight against the same buffer. The dialyzed enzyme (crude chitinase) was further purified by a second ammonium sulfate fractionation (30–60% saturation), followed by a preparative electrophoresis. Figure 4a and b shows the protein and chitinase activity profiles, respectively, from polyacrylamide gels containing both the crude and the purified enzymes. The purified enzyme preparation contained three major and several minor protein bands which all exhibited chitinase activity as determined by Calcofluor white M2R.

Identification of the enzymatic products

Figure 5 shows the hydrolytic products generated from various substrates using the crude and purified enzyme preparations. Colloidal chitin or crystalline chitin was predominantly hydrolyzed by the crude enzyme (culture filtrate) to N-acetylglucosamine and a trace amount of the di-, tri- and tetramer of N-acetylglucosamine. This indicates the presence of high levels of GlcNAcase activity in the culture broth (Figure 5a). The hydrolysis of chitosan (lane 4) is probably due to the presence of acetylated residues in the chitosan used. We have found that the chitosan used was about 15% N-acetylated, and similar results were obtained with chitinase from *Aeromonas hydrophila* [10]

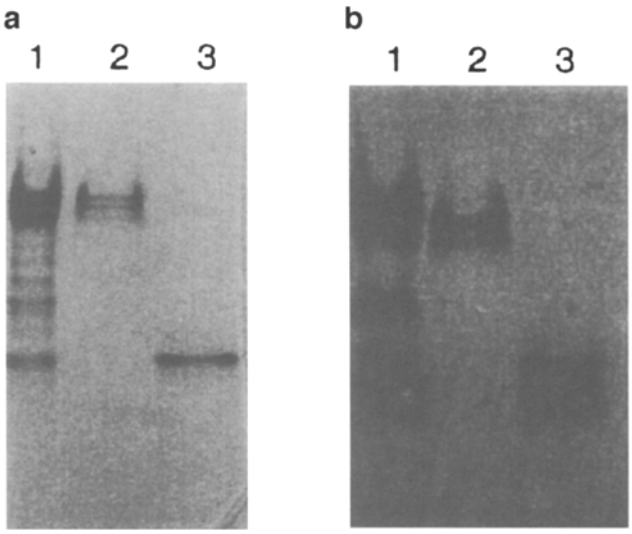

Figure 4 PAGE analysis of the crude and purified chitinase produced by *Aeromonas* sp No. 16. (a) Coomassie blue stain; and (b) chitinase activity stain. Lanes: 1, crude enzyme; 2 and 3, purified chitinases after preparative electrophoresis.

and *Bacillus circulans* [11]. In contrast, the purified enzyme preparation hydrolyzed colloidal chitin predominantly to N,N-diacetylchitobiose and trace amounts of the mono-, tri-, and tetrameric N-acetylglucosamine suggest that it contains mainly the endochitinase [12,19].

Discussion

Chitin not only serves as an important structural component of insects, crustaceans, and other lower organisms but also plays a crucial role in pathogen/plant interactions [12,15,19,29]. Its structure and function closely resemble those of cellulose, another important polysaccharide which constitutes the cell wall of plants. Although gene manipulation of chitinases has been intensively studied recently [4,24,27,28,33,34], the biochemical properties of those enzymes involved in the degradation of chitin are not well understood, much less their regulation such as induction by

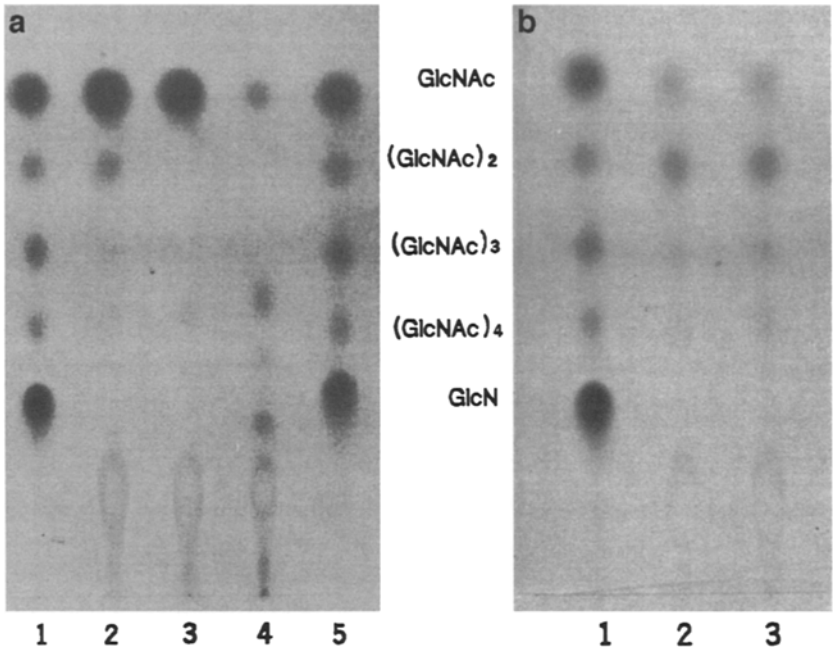

Figure 5 Thin-layer chromatograms of the enzymatic products generated by the crude (a) and purified (b) chitinase. Panel a, lanes 1 and 5, standards: glucosamine (GlcN), N-acetylglucosamine (GlcNAc) and its oligomers; lanes 2–4: products using colloidal chitin (2), crystalline chitin (3), or colloidal chitosan (4) as the substrate. Panel b, lane 1, standards; lanes 2 and 3, products using the two purified preparations of chitinases (Figure 4, lanes 2 and 3, respectively).

the substrate and inhibition by the intermediate metabolites [18,27]. By analogy with what has been learned about the degradation of cellulose by the cellulase complex, it is believed that degradation of chitin also requires the synergistic action of various chitinolytic enzymes, ie endochitinase, exochitinase, and GlcNAcase. The increasing demand of chitin derivatives for both industrial and medical applications in recent years has prompted the serious consideration of establishing a biocatalytic process for the hydrolysis of chitin. The feasibility of such a process relies greatly on the economy of securing the needed enzymes, the chitinase.

The isolated bacterium, *Aeromonas* sp No. 16, is a Gram-negative, catalase- and oxidase-positive, and flagellated bacterium which produces chitinase in a rather simple medium. All three substrates tested, including colloidal chitin, crystalline chitin, and chitosan, induced the enzyme secretion, though with different efficiencies (Table 6). The production of chitinase was significantly stimulated by organic nitrogen supplements (Table 4), which reflected the relatively high C/N ratio of chitin as both carbon and nitrogen sources for bacterial growth. With the exception of glucosamine, the addition of carbon source stimulated cell growth but slightly inhibited enzyme secretion (Table 5). The potent inhibitory effect of glucosamine on the growth of the isolate suggests that it may be an important regulator for certain enzyme(s) involved in chitin catabolism. Since glucosamine yielded the highest activity/cell mass ratio (Table 5), it remains to be seen whether addition of glucosamine to a near-confluent cell culture can further induce chitinase production in a large-scale culture. Experiments based on this strategy are currently under rigorous investigation. The secreted enzyme activity increased almost linearly with the increasing colloidal chitin substrate concentration up to 1.5%. At higher concentrations of the substrate, it was noticed that the culture time required to detect chitinase activity was prolonged (data not shown). This is, presumably, due to the adsorption of the secreted enzyme by the substrate, which might eventually be released upon hydrolysis of the substrate.

Table 6 Substrate specificity of the crude enzyme

Substrate	Specific rate of hydrolysis (μmol reducing sugar mg protein^{-1} min^{-1})
Colloidal chitin	1.168 (100)
Powdered chitin	0.958 (82)
Colloidal chitosan	0.325 (28)
Carboxymethyl cellulose	0

Chitinase activities were determined in 0.05 M phosphate buffer (pH 6.0) containing 0.5% (w/v) of various substrates at 40°C for 60 min. The numbers in parentheses indicate percent relative activities.

Under optimal conditions, the isolated strain produced a specific chitinase activity of about 1.4–1.5 U ml^{-1} which is considerably higher than those reported previously [8,9,17,20,25,29,31,32]. When colloidal chitin was used as the substrate, the predominant product generated by the crude enzyme was N-acetylglucosamine (Figure 5a), suggesting the presence of GlcNAcase in the culture medium. The majority of this GlcNAcase could be readily separated from chitinase activity by the two-step ammonium sulfate fractionation, as shown in Figure 5b. By this procedure, about 94% of the GlcNAcase activity could be removed, while 80% of the chitinase activity was recovered. This resulted in a 14-fold increase in the ratio of chitinase to

GlcNAcase (from 1.3 to 17.9) in the purified preparation. In-gel activity assays indicated that the chitinase produced by *Aeromonas* sp No. 16 existed as a multiple-enzyme complex containing at least three major polypeptides. The presence of multiple isoforms of chitinase observed here is consistent with previous reports [5,29,30,35]. At least six chitinases have been identified in the spent culture media of *Aeromonas* sp [30] and *Bacillus circulans* [35], respectively.

Enzymatic degradation of crystalline chitin is thought to start with the random hydrolysis of the internal glucosidic bonds by endochitinase, which generates non-reducing chain ends for the subsequent action of exochitinase. The formation of N,N-diacetylchitobiose as the major product by the purified enzyme preparation (Figure 5) suggests that a significant amount of the enzyme acts as endochitinase. The ability of the isolated bacterium to utilize crystalline chitin substrate is especially noteworthy. Crystalline chitin is very resistant to degradation because of its structure and water insolubility. Although relatively inexpensive and readily available, chitin acquired from the bioconversion processing of the seafood industry, which is demineralized and deproteinated, remains highly crystalline. The degradation of crystalline chitin and chitosan by *Aeromonas* sp No. 16 greatly increases its potential for the future development of a biocatalytic process for the production of useful chitin derivatives as well as chitinolytic enzymes.

Acknowledgements

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