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Isolation and Characterization of Chitosanase-Producing Microorganism, *Aureobacterium* sp. YL, from Crab Shells

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Abstract A bacterial strain producing extracellular chitosanase was isolated from crab shells and identified as a member of the genus *Aureobacterium*. The production of chitosanase was proportionally related to the microbial growth, induced by the presence of chitosan, and repressed by glucose at 0.5% (w/v) concentration or higher. The optimal culture conditions for the production of chitosanase were 30°C and pH 7.0. Among the nitrogen sources tested, incubation with 0.25% (w/v) concentrations of tryptone and casitone showed the best production of chitosanase. The chitosanase of *Aureobacterium* sp. YL produced chitobiose as a major product and glucosamine, chitotriose, chitotetraose, and chitopentaose as minor products from chitosan.

Key words: *Aureobacterium* sp. YL, chitosanase, chitosan, chitooligosaccharide

Chitosan, poly-(β→1-4)-D-glucosamine, occurs in nature only in the cell walls of fungi belonging to the order *Mucorales* [2]. This polymer has a unique polyamine characteristic which makes it water-soluble in acidic pH condition, and is positively charged and easily modifiable chemically. Chitosan has many diverse applications such as an antifungal compound [7], a flocculating agent [14], and a hydrating agent (Nanba, T. and M. Izumi. 1987. Japanese patent, Kokai Tokkyo Koho JP, 62,273,905 [87.273,905] Cl. A61K7/00). In addition, low molecular weight chitosan oligomers have been found to reduce blood cholesterol levels [10], accelerate wound healing [13], stimulate the immune system [24], and act as an antitumoral agent [24].

Chitosan oligomers have been prepared by the chemical depolymerization method in which concentrated hydrochloric acid is used. An alternative method is the use of chitosanolytic enzymes. The chitosanolytic activities have been found in many microorganisms [4, 5, 8, 12, 15, 17, 18, 19, 21, 22, 25, 28, 30].

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To obtain a novel chitosanase-producing microorganism which can be used in the production of chitosan oligomers, various types of microorganisms from crab shells obtained from a Korean local market were screened for their ability to form a clear halo on a chitosanase detection agar plate. Several strains showed chitosanolytic activities on the detection agar plates, and one strain that formed the largest halo was selected. This report describes the identification and characteristics of the bacterial strain and the chitosan-hydrolysis pattern of the chitosanase secreted by this bacteria.

Materials and Methods

Reagents

Chitin was prepared from crab shells obtained at the Noryangjin fishery market, Korea, using the method of Kim and Cho [11]. Chitosan was prepared from chitin according to the method of No and Meyers [16]. The degree of N-acetylation (D. A.) in the chitosan was determined using the method of Baxter *et al.* [3]. Chitosan with the D. A. of 1.3–3.3% was used as a substrate. Other chemical reagents such as inorganic salts were of analytical grade.

Screening of Chitosan Hydrolyzing Bacterial Strains

The samples of crab shells obtained from Noryangjin fishery market, Korea, were spreaded onto a chitosanase detection medium (3 g of chitosan, 1 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 0.5 g of K_2HPO_4, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.001 g of $ZnCl_2$, 0.01 g of $CaCl_2 \cdot 2H_2O$, and 0.005 g of $MnCl_2$ in 1 l, pH 7.0) solidified with 2% agar. After incubation for 7 days at 30°C, a single colony showing a prominent chitosanolytic activity was selected and identified by examining its morphological, cultural, and biochemical characteristics, according to *Bergey's Manual of Determinative Bacteriology* [9] and *Bergey's Manual of Systematic Bacteriology* [23]. The morphological characteristics were studied using a scanning electron microscope (JSM-35CF, Jeol, Japan).

Culture Conditions

The isolated strain was inoculated into 30 ml of a seed-culture medium (1 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 0.5 g of K_2HPO_4, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.001 g of $ZnCl_2$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.005 g of $MnCl_2$, 2.5 g of tryptone, and 2.5 g of casitone in 1 l, pH 7.0) and incubated at 30°C for 30 h on a shaking incubator (VS-8480SR, Vision Scientific Co., Korea) at 200 rpm. The resulting cultures were inoculated (5%) into an appropriate volume of a main culture medium, which was identical with the seed-culture medium except for the addition of chitosan (0.3%), and incubated under the same conditions for an additional 15 h.

Preparation of Chitosanase Enzyme

The culture supernatant was collected by centrifugation (Micro 17R, Hanil Industrial Co., Korea) at 12,000 rpm for 10 min. Crude enzyme proteins were obtained by 80% ammonium sulfate precipitation and dissolved in an appropriate volume of 100 mM sodium acetate buffer (pH 5.0).

Enzyme Assay

The substrate solution for chitosanase activity assay contained 5 mg of chitosan in 1 ml of 100 mM sodium acetate buffer (pH 5.0). Then, 0.8 ml of the enzyme solution was mixed with 0.2 ml of the substrate solution and incubated with shaking (KMC-1205SW1, Vision Scientific Co., Korea) at 30°C for 30 min, followed by boiling for 10 min. The amount of D-glucosamine released was determined using the method of Rondle-Morgan and Morgan [20]. One unit of enzyme activity was defined as the chitosanolytic activity that released 1 μmol of D-glucosamine per min at 30°C.

Localization of Chitosanase in the Chitosanolytic Strain

The isolate was grown in 50 ml of a main culture medium according to the culture conditions mentioned above. Half of the culture broth (25 ml) was centrifuged (SS-34 rotor, Sorvall® RC-28S, Du Pont, Newtown, U.S.A.) at 15,000 rpm for 10 min. The supernatant was taken as an extracellular fraction. The rest of the culture broth was sonicated for 1 min and cooled for 3 min on ice bath. The samples were sonicated 40 times at 1.1 A in a sonicator (Kyu Sonic, Kyungil Co., Korea) and then centrifuged. The resulting supernatant was used as an extracellular and cytoplasmic fraction. The chitosanase activity was assayed as mentioned above.

Analysis of the Enzymatic Reaction Products

The products of the enzymatic hydrolysis of chitosan were analyzed by thin layer chromatography on Silica Gel 60 F_{254} plastic sheets (Merck, Darmstadt, Germany) using the n-propanol/30% ammonia (2:1, v/v) solvent system. Amino sugar was visualized using a ninhydrin reagent [21]. HPLC was carried out with a carbohydrate analysis column (3.9× 300 mm, Waters Co., Milford, U.S.A.). Elution was carried out with water/acetonitrile (28:72, v/v) at a flow rate of 1.0 ml/min. The elution pattern was monitored by measuring the absorbance at 210 nm due to N-acetyl groups. N-Acetylation of chitooligosaccharides was performed by the method of Amano *et al.* [1]

RESULTS AND DISCUSSION

Isolation and Identification of Bacterial Strain Hydrolyzing Chitosan

About 16 chitosanolytic microorganisms were isolated from crab shells from Noryangjin fishery market, Korea. The isolates formed clear halos around their colonies in the chitosanase detection agar plates. Among them, the bacterial strain forming the largest clear halo was selected for further studies. The chitosanolytic activity of the strain on the chitosanase detection agar plate is shown in Fig. 1. The strain was subjected to a taxonomic analysis according to *Bergey's Manual of Systematic Bacteriology* [23]. The morphological, cultural, and biochemical characteristics of this strain are summarized in Table 1. The cell morphology, confirmed as being rod-shape by scanning electron microscopy, is shown in Fig. 2. The strain existed singly and the cell size was ~1.1×0.3 μm. Even in 3 day-old cultures, there were no marked coccus-shape cells observed, which suggested that the strain did not have a rod-coccus growth cycle. On the basis of these results, the strain was assigned to a member of the genus *Aureobacterium*.

The characteristics of the strain was compared with *Aureobacterium liquefaciens* [23, 27] as shown in Table 1.

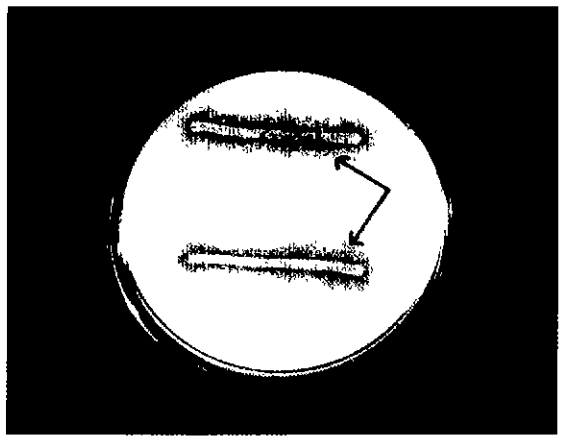

Fig. 1. Chitosanolytic activity of *Aureobacterium* sp. YL on the chitosanase detection agar plate.
The production of chitosanase was detected by a clear halo around the microorganism The arrows point to a clear halo in a chitosanase detection agar plate incubated at 30°C for 2 days.

Table 1. The morphological, cultural, and biochemical characteristics of *Aureobacterium* sp. YL, compared with *Aureobacterium liquefaciens* [23, 27] (+: positive, -: negative).

Characteristics	*Aureobacterium* sp. YL	*Aureobacterium liquefaciens*
Color of colonies	yellow	yellow
Shape	rod	rod
Motility	-	-
Endospore formation	-	-
Gram staining	+	+
Acid-fast staining	-	-
Oxygen requirement	strictly aerobic	strictly aerobic
Oxidation-fermentation test	oxidative	oxidative
Growth in the presence of 2.0% NaCl	+	+
Hydrolysis of:		
gelatin	-	+
starch	+	-
casein	+	+
Nitrate reduction	-	+
Voges-Proskauer test	-	-
Methyl red test	+	+
Urease production	-	+
Oxidase test	-	-
Indole test	-	-
Catalase test	+	+
H_2S formation	-	+
Acid production from carbohydrates	glucose	glucose, galactose
Assimilation of:		
acetate	+	+
citrate	-	-
succinate	+	+
lactate	+	+ (weak)
propionate	+	+
hippurate	-	+
Growth at 37°C	+	-

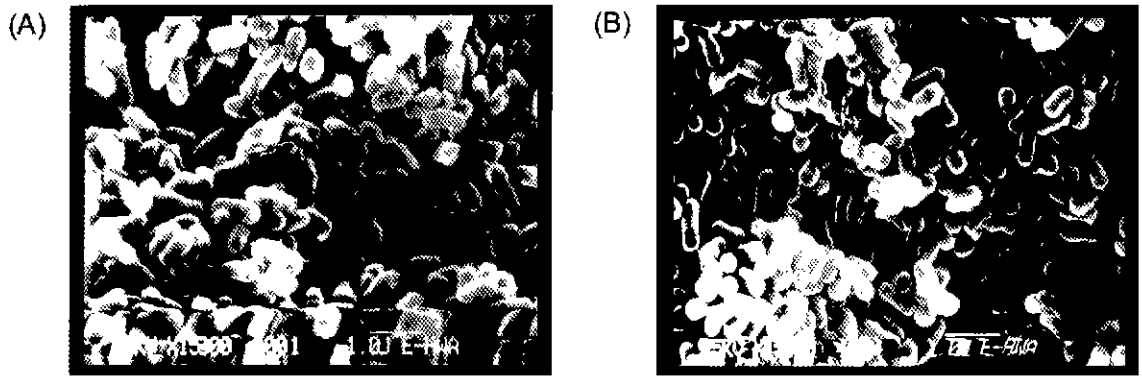

Fig. 2. Scanning electron microscopy (SEM) micrograph (×15,000) of *Aureobacterium* sp. YL after 1 day (A) and 3 days (B) of incubation.
This strain was rod-shaped and existed singly. The cell size was ~1.1 × 0.3 μm. In older cultures (3 days), there was no marked rod-coccus growth cycle and endospores were not formed. The size bar indicates 1 μm.

Although the two species were very similar in morphological and biochemical characteristics, there were some differences. *Aureobacterium* sp. YL hydrolyzed starch, whereas *Aureobacterium liquefaciens* hydrolyzed gelatin. *Aureobacterium* sp. YL grew at 37°C and produced acid from only glucose. In addition, this strain was negative for tests of nitrate reduction, urease production, H_2S formation, and assimilation of hippurate. On the other

hand. *Aureobacterium liquefaciens* was positive. Therefore, the isolated strain was named as *Aureobacterium* sp. YL.

Chitosanolytic enzymes have been found in many microorganisms such as *Bacillus* sp. [18, 25, 28], *Nocardia orientalis* [15, 21], *Streptomyces* sp. [4, 19], *Nocardioides* sp. [17], *Myxobacter* sp. [8], *Pseudomonas* sp. [30], *Acinetobacter* sp. [22], *Penicillium* sp. [5], and *Aspergillus* sp. [12]. However, from our literature search, we could not find any reports on chitosanolytic activities of the genus *Aureobacterium*. Thus, our finding might be the first report for the chitosan-hydrolyzing activities of the genus *Aureobacterium*.

Localization of Chitosanase in the Chitosanolytic Strain

The chitosanase activities in both extracellular and cytoplasmic fractions (14.37±0.33 mU/ml) were similar to that of the extracellular fraction alone (13.91±0.31 mU/ml). This result indicates that most of the chitosanase produced by *Aureobacterium* sp. YL was secreted into the culture medium. As in the cases of chitosanases of other microorganisms [4, 5, 8. 17, 18, 19, 21. 22, 30], this chitosanase was an extracellular enzyme. However, chitosanase activity per 1 ml of a culture medium was relatively low in *Aureobacterium* sp. YL (15- 30 mU/ml), compared with *Bacillus megaterium* P1 (1.000 mU/ml) [18] and *Pseudomonas* H-14 (650 mU/ml) [28]. This low productivity could be manipulated through mutation using a mutagen such as UV so as to enhance production of chitosanolytic enzyme.

Effects of Chitosan and Cell Growth on the Production of Chitosanase

The cell growth pattern in the main culture medium was similar to the one in the seed-culture medium as shown in Fig. 3. The cultures reached a stationary phase after approximately 17 h of incubation. However, chitosanase was only produced in the main culture medium. This result suggests that the chitosanase of *Aureobacterium* sp. YL is inducible by the presence of chitosan in the main culture medium, because the main culture medium was prepared by the addition of chitosan to the seed-culture medium. The chitosanase activity increased in proportion to the microbial growth and reached a peak after 14 h of incubation, which corresponded to the late logarithmic phase of growth.

Effect of Glucose Concentration on the Production of Chitosanase

As shown in Fig. 4, chitosanase was produced best when glucose was not present in the culture medium. The more glucose added to the medium, the less chitosanase was produced. Furthermore, chitosanase production was not induced in the medium containing more than 0.5% glucose. The results suggest that glucose might catabolically repress the chitosanase production in the strain.

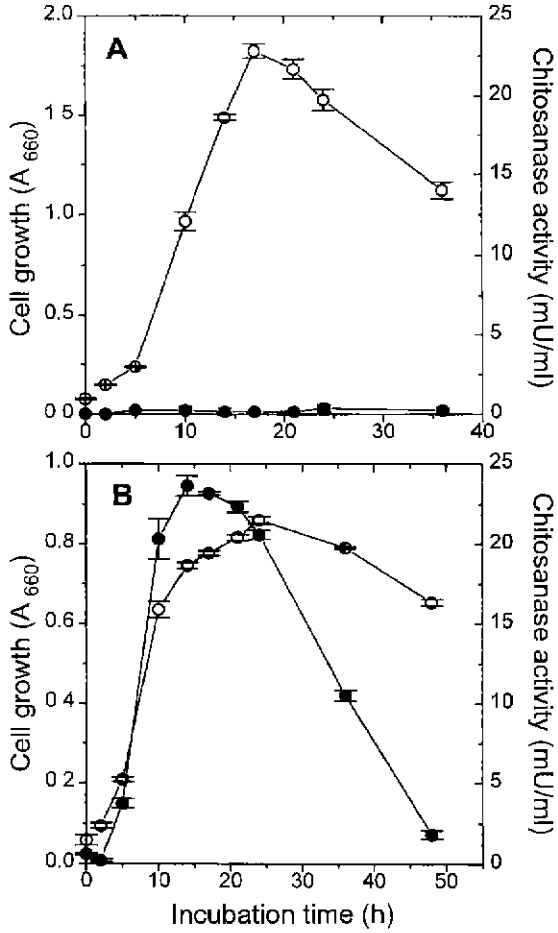

Fig. 3. Effects of chitosan and cell growth on the production of chitosanase.

Seed cultures (30°C, 200 rpm, 30 h) of *Aureobacterium* sp. YL were inoculated (5%) into the seed-culture medium (A) and the main culture medium (B) and incubated at 30°C on a rotary shaker (200 rpm) respectively. At various time points, the culture broths (1 ml) were sampled. Cell growth was determined by absorbance at 660 nm and the chitosanase activities were measured as mentioned in MATERIALS AND METHODS. The data are the mean values of duplicate experimental results. Error bar indicates the range of standard deviation of two data points. ○, cell growth profile; ●, chitosanase activity profile

Effects of Nitrogen Sources on the Production of Chitosanase

The best nitrogen source for chitosanase production is shown in Fig. 5. Chitosanase was produced better in a medium containing casitone and tryptone. Thus, tryptone and casitone were added to the culture medium for chitosanase production.

Effects of Temperature and pH on the Production of Chitosanase

The optimal temperature and pH for chitosanase production are shown in Fig. 6 and Fig. 7, respectively. The optimal condition was 30°C and pH 7.0.

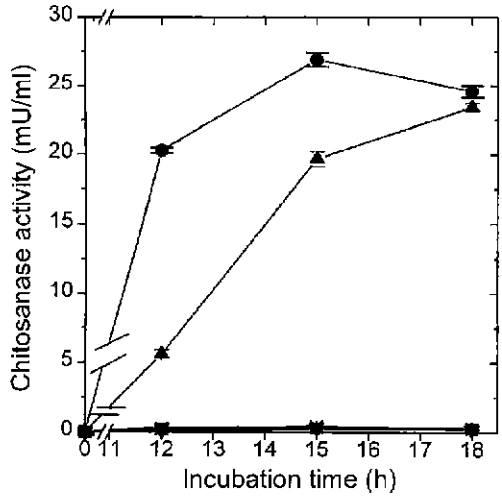

Fig. 4. Effect of glucose concentration on the production of chitosanase.

Seed cultures (30°C, 200 rpm, 30 h) of *Aureobacterium* sp. YL were inoculated (5%) into the main culture medium supplemented with various concentrations of glucose and incubated at 30°C on a rotary shaker (200 rpm). At various time points, the culture broths (1 ml) were sampled and the chitosanase activities were measured as mentioned in MATERIALS AND METHODS The data are the mean values of duplicate experimental results. Error bar indicates the range of standard deviation of two data points. ●, 0%; ▲, 0.1%; ▼, 0.5%; ◆, 1.0%; +, 2.0%; ×, 3.0%

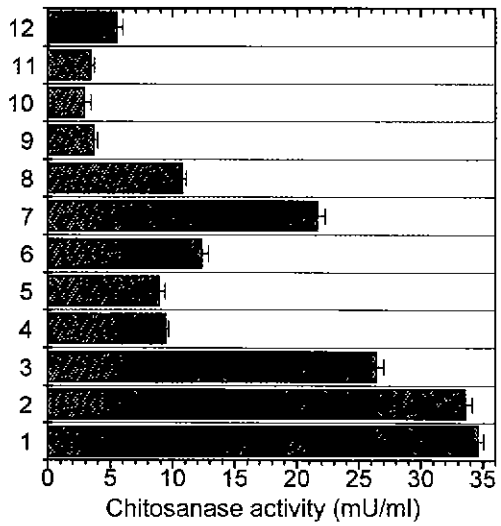

Fig. 5. Effects of nitrogen sources on the production of chitosanase.

Seed cultures (30°C, 200 rpm, 30 h) of *Aureobacterium* sp. YL were inoculated (5%) into the chitosanase detection medium supplemented with various nitrogen sources to a final concentration of 0.5%, and incubated at 30°C for 15 h with shaking (200 rpm). The culture broths (1 ml) were sampled and the chitosanase activities were assayed as mentioned in MATERIALS AND METHODS. The data are the mean values of duplicate experimental results. Error bar indicates the range of standard deviation of two data points. 1: Casitone + Tryptone 2 Casitone 3 Tryptone; 4· Peptone; 5. Yeast extract, 6. Casamino acid; 7: Brain heart infusion; 8: NZ amine A: 9: Proline; 10: $NaNO_3$; 11: NH_4Cl; 12: No addition.

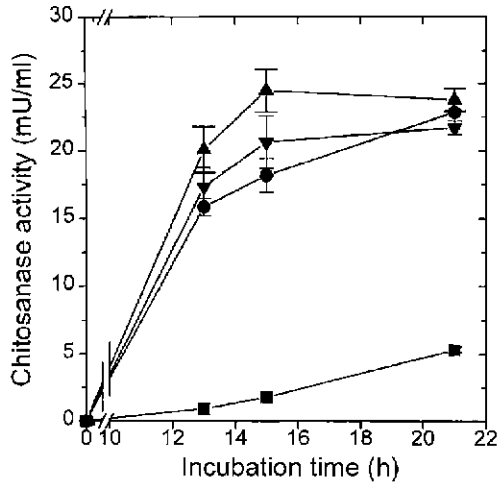

Fig. 6. Effect of temperature on the production of chitosanase.

Seed cultures (30°C, 200 rpm, 30 h) of *Aureobacterium* sp. YL were inoculated (5%) into the main culture medium adjusted to pH 7.0 and incubated at various temperatures on a rotary shaker (200 rpm). At various time points, the culture broths (1 ml) were sampled and the chitosanase activities were measured as mentioned in MATERIALS AND METHODS The data are the mean values of duplicate experimental results. Error bar indicates the range of standard deviation of two data points. ■, 20°C; ●, 25°C, ▲, 30°C, ▼, 35°C.

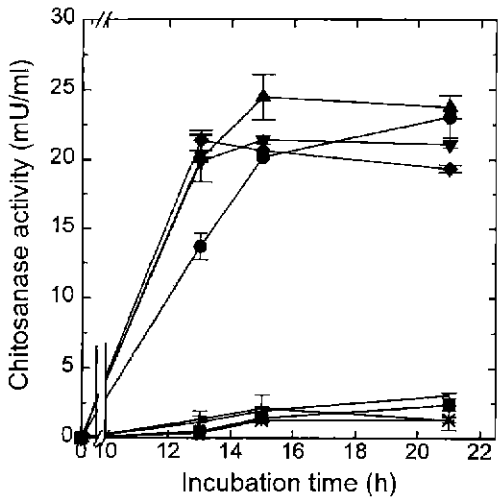

Fig. 7. Effect of pH on the production of chitosanase.

Seed cultures (30°C, 200 rpm, 30 h) of *Aureobacterium* sp. YL were inoculated (5%) into the main culture medium adjusted to various pHs and incubated at 30°C on a rotary shaker (200 rpm) The culture broths (1 ml) were sampled at various time points and the chitosanase activities were measured as mentioned in the text. The data are the mean values of duplicate experimental results Error bar indicates the range of standard deviation of two data points. -, pH 5.0; ■, pH 6.0; ●, pH 6.5, ▲, pH 7 0; ▼, pH 7.5; ◆, pH 8.0; +, pH 9.0; ×, pH 10 0.

Analysis of Enzymatic Reaction Products

The result of thin-layer chromatography analysis is shown in Fig. 8A. This chitosanase hydrolyzed chitosan to chitobiose and chitotriose. Even after a prolonged incubation, the product patterns were similar. However, HPLC analysis after N-

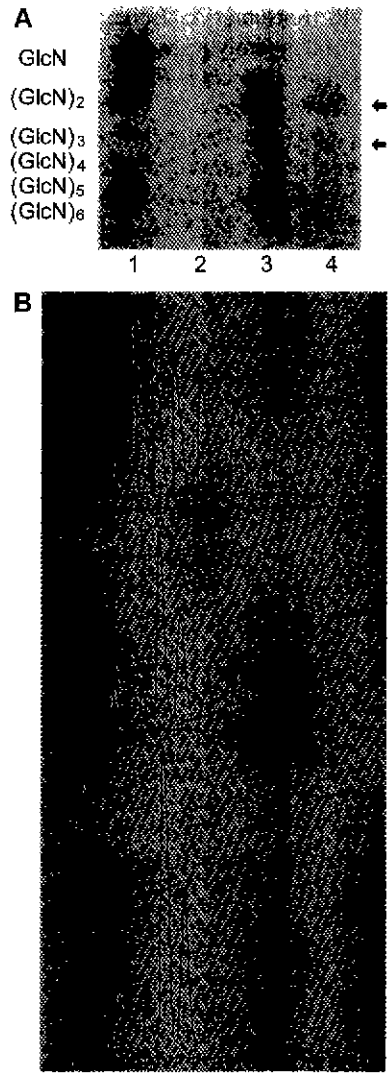

Fig. 8. Hydrolysates of chitosan by the chitosanase of *Aureobacterium* sp. YL.

A substrate (Chitosan 0.1 mg/ml in a 100 mM sodium acetate buffer, pH 5.0) was incubated with 50 mU of the crude enzyme solutions in 1 ml of a 100 mM sodium acetate buffer (pH 5.0) at 30°C for 30 min or 24 h. After incubation, the reaction mixtures were heated for 10 min in boiling water, filtered through a centricon 30 (Amicon Co., Danvers, U.S.A.), lyophilized. (A) Thin layer chromatogram. The powder was dissolved in 50 μl of water. Ten μl amounts of the products were spotted on Silica Gel 60 F_{254} plastic sheets (Merck, Darmstadt, Germany), developed in the solvent system: n-propanol/30% ammonia (2:1, v/v), and visualized using ninhydrin reagent. Lane 1, standards: glucosamine (GlcN) and glucosamine oligomers from dimers $(GlcN)_2$ to hexamers $(GlcN)_6$; Lane 2, substrate solution (chitosan) only of 24 h incubation; Lane 3, enzymatic hydrolysates of 24 h incubation; Lane 4, enzymatic hydrolysates of 30 min incubation. (B) HPLC chromatogram. The lyophilized powder was N-acetylated by the method of Amano *et al.* [1] and analyzed by HPLC with a carbohydrate analysis column (3.9×300 mm, Waters Co.) using a solvent system of 72% acetonitrile and 28% water. Panel 1, standards: N-acetylglucosamine (GlcNAc) and N-acetylglucosamine oligomers from dimers $(GlcNAc)_2$ to hexamers $(GlcNAc)_6$. Peak 1: GlcNAc, Peak 2: $(GlcNAc)_2$, Peak 3: $(GlcNAc)_3$, Peak 4: $(GlcNAc)_4$, Peak 5: $(GlcNAc)_5$, Peak 6 $(GlcNAc)_6$; Panel 2, N-acetylated hydrolysates of 30 min incubation; Panel 3, N-acetylated hydrolysates of 24 h incubation. Peak a corresponds to GlcNAc. Peak b corresponds to $(GlcNAc)_2$, Peak c corresponds to $(GlcNAc)_3$, Peak d corresponds to $(GlcNAc)_4$, and Peak e corresponds to $(GlcNAc)_5$.

acetylation of released products showed that hydrolysates contained saccharides from glucosamine monomer to pentamer (Fig. 8B). If all chitooligosaccharides were converted to N-acetylchitooligosaccharides in this acetylation reaction, it can be inferred that this chitosanase produced a chitooligosaccharides mixture from chitosan at the molar ratio of GlcN:$(GlcN)_2$:$(GlcN)_3$:$(GlcN)_4$:$(GlcN)_5$=1:42.8:17.2:4.1:4.5 after 24 h of incubation.

From the results, we concluded that this chitosanase produced chitobiose as the major product, and glucosamine, chitotriose, chitotetraose, and chitopentaose as the minor products.

Most of the chitosanases reported from other microorganisms [4, 6, 17, 18, 21, 22, 26, 30] produced chitobiose and chitotriose. Exo-β-D-glucosaminidase from *Nocardia orientalis* [15] produced only D-glucosamine as a final product. In contrast, the chitosanase secreted from *Aureobacterium* sp. YL produced chitotetraose and chitopentaose, which might be a useful enzymatic characteristic for the production of chitooligosaccharides.

Acknowledgments

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