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# Purification and Characteristics of Chitosanase from *Bacillus* sp. HW-002

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Chitosanase from *Bacillus* sp. HW-002 was purified with CM-cellulose column chromatography, and HPLC with DEAE-TSK gel and YMC-pack Diol 120. The purified enzyme appeared as a single band on SDS-polyacrylamide gel. The molecular weight of the enzyme was estimated to be about 46 kDa on SDS-polyacrylamide gel, and was estimated to be about 23 kDa by GFC. The optimal pH of chitosanolytic activity was about pH 5.5-6.0, and the purified enzyme was most stable at pH 5.0. The optimal temperature of chitosanolytic activity was 65°C and the enzyme was stable at 45°C for 1 h. Chitosan was the most favorable substrate among various β-glucan. UVmax of the purified enzyme was 195 nm and was not noted around 280 nm. The main product of enzyme reaction with chitosan was chitobiose.

Recently, chitosan derivatives and its partially degraded oligosaccharides have been applied to medical, food and agricultural usage, resulting in an increased demand for chitosanase (14). Low molecular weight chitooligosaccharides have received attention because of their interesting biological properties (6), including their inhibitory effects on the growth of fungi and bacteria, host associated antitumor activity, activation of immune response, and their ability to elicit phytoalexin production in higher plants. These biological properties are defferently shown accordig to sugar units. Therefore, production of selectively depolymerized chitooligosaccharide is required. Enzymatic hydrolysis has advantage for the production of regular sugar unit chitooligosaccharide due to enzymatic specificity.

Chitin is the β-1,4 linked polymer of *N*-acetyl-D-glucosamine, and chitosan is generally a partially *N*-acetylated compound. Chitosan can be hydrolyzed to chitooligosaccharides by enzymes called chitosanases. Chitosanases are classified by substrate specificity. Chitosanases which hydrolyzed only chitosan have been isolated from *Bacillus* sp. PI-7S (15), *Bacillus* sp. R-4 (17) and *Pseudomonas* sp. H-14 (20). Chitosanases with carboxy methyl cellulase (CMCase) activity have already been isolated from *Mycobacter* sp. (1) and *Streptomyces griseus* (11), and chitosanases from *Streptomyces griseus* (10), *Aeromonas hydrophila* (7) and *Pycnoporus cinnabarinus* (9) hydrolyzed both chitosan and the *N*-acetyl-β-D-glucosaminidic bonds in partially *N*-acetylated chitosan. Chitosanases from *Bacillus circulans* WL-2 (8), *Enterobacter* sp. G-1 (19) and *Acinetobacter* sp. CHB 101 (16) hydrolyzed both chitin and chitosan. Most of microbial chitosanases produced chitooligosaccharides of various sugar units as a result of non-specific endo-type cleavage reactions (4, 6, 10, 15), but chitobiose and chitotriose were produced by chitosanases from *Pseudomonas* sp. H-14 (20) and *Enterobacter* sp. G-1 (19).

We isolated bacteria from soil sample, which produced chitosanase highly. A previously published paper reported on the isolation, taxonomical studies and cultural conditions of chitosanase-producing bacteria (6). In this paper, we present the methods of purification and the characteristics of chitosanase from *Bacillus*. sp. HW-002.

## MATERIALS AND METHODS

### Production of Chitosanase

A chitosanase production medium was composed of 0.5% sucrose, 0.75% yeast extract and 0.75% peptone at pH 6.7. Working conditions were 2 l working volume, 300 rpm agitation, 1.5 vvm air supply in a 3 l jar fermenter with 0.01% silicon oil added as Antifoam. Maximum chitosanase productivity of 2,620 U/l was obtained at a final pH of 7.4 after 32 h.

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**Enzyme and Protein Assay**

Chitosanase activity was assayed by measuring the amount of reducing sugars released from the enzyme reaction using the dinitrosalicylic acid (DNS) method (2). The substrate was 0.3% chitosan dissolved in 50 mM sodium acetate butter (pH 5.6). 10 μl enzyme solution was added to a 490 μl substrate solution, and the reaction mixture was incubated in a water bath at 40°C for 10 min. The reaction was terminated by addition of 500 μl 1 N KOH, and then the mixture was centrifuged at 5,000× g for 5 min. The supernatant (0.5 ml) was transferred to a 5 ml tube and added to 0.5 ml DNS solution. The mixture was boiled for 15 min, and then cooling with tap water. The optical density was measured by spectrophotometer (Shimadzu UV-240) at 540 nm. One unit (U) of activity was defined as the amount of enzyme which liberated 1 μmole of reducing sugar per min and glucosamine was used as the standard.

Protein concentrations were estimated with a protein assay kit (Bio-Rad), using bovine serum albumin as the standard.

**Purification of the Enzyme**

After removal of the bacterial cells from the culture broths by centrifugation at 10,000× g for 10 min, the chitosanase was concentrated from the supernatants by ethanol precipitation. The enzyme was precipitated with 50% saturation of ethanol. After standing at -10°C for 2 h, the precipitate was collected by centrifugation at 10,000× g for 10 min. The precipitate was dissolved in an appropriate volume of 50 mM sodium acetate buffer (pH 5.6), followed by dialysis against the same buffer for 10 h at 4°C. The dialysate was centrifuged to remove the insoluble materials and the supernatant was used as the crude concentrates of the chitosanase. The crude enzyme was loaded on a CM-cellulose column (2.5×25 cm) which was equilibrated with 50 mM sodium acetate buffer at pH 5.6, and was washed with 100 ml sodium acetate buffer, pH 5.6. The charged enzyme was released with a linear gradient of NaCl from 0 to 0.3 M NaCl in the same buffer at a flow rate of 5 ml/20 min. Eluates were collected with a 5 ml/tube, and the active fraction was concentrated by freeze drying. The enzyme was dialyzed against a 30 mM Tris-HCl buffer (pH 6.8) and the dialysate was further purified by High Performance Liquid Chromatography (HPLC) using the TOSO HPLC system. A DEAE-TSK gel column (7.5×75 mm) was used for anion exchange chromatography of the crude enzyme. After washing with 30 mM Tris-HCl buffer (pH 6.8) for 5 min, the charged chitosanase was eluted with a linear gradient of NaCl from 0 to 0.3 M NaCl in the same buffer at a flow rate of 1.0 ml/min for 40 min. The active fraction was lyophilized and was dissolved in 10 mM Tris-HCl buffer (pH 6.8) containing 0.5 M NaCl. A YMC-pack Diol 120 column (8×500 mm) was used for gel filtration and the enzyme was eluted with 30 mM Tris-HCl buffer (pH 6.8) containing 0.5 M NaCl at a flow rate of 1 ml/min. The eluates were detected by ultraviolet (UV) detector at 280 nm during the HPLC.

**Characterization of Chitosanase**

The purity and molecular weight of chitosanase were determined by sodium dodesyl sulfate(SDS)-polyacrylamide gel electrophoresis(PAGE) (3) and gel filtration chromatography (GFC) using HPLC. The molecular weight standard was established using an electrophoresis calibration kit (Pharmacia) and standard proteins of GFC grade (Sigma). The effects of pH on the activity and the stability of the enzyme was investigated in a buffer from pH 3.0 to 10.0. For investigation of the pH stability, the purified enzyme was treated with buffer of various pH at 30°C for 1 h. The buffers were adjusted from pH 2.0 to 9.0 using 50 mM McIlvain buffer. The effects of temperatures between 30°C and 70°C were investigated and heat stability was examined in 50 mM sodium acetate buffer (pH 6.0) at 45°C and 55°C for 1 h. Substrate specificity was investigated with soluble chitosan, colloidal chitosan, insoluble chitosan, colloidal chitin, insoluble chitin and carboxy methyl cellulose. Purified chitosanase which was dissolved in distilled water was investigated with ultraviolet spectrum from 190 nm to 300 nm. The reaction product of chitosanase was analyzed by GFC with two linked YMC-pack Diol 60 (8×1000 mm), using a refractive index (RI) detector, and the eluant was a 1 M NaCl solution. 100 ml chitosan (1%) was hydrolyzed by 30 units of chitosanase with slow shaking (60 rpm) at 40°C for 5 h. The reaction mixture was precipitated with 70% saturation of methanol and centrifuged at 10,000× g for 5 min. The supernatant was concentrated by vacuum evaporator at 37°C, the reaction product dissolved in distilled water and passed through an IRA-420 (Sigma) anion exchange column (2×20 cm). The eluate was concentrated by vacuum evaporator and analyzed by HPLC.

## RESULTS AND DISCUSSION

**Purification of Chitosanase**

After cultivation of *Bacillus.* sp. HW-002 in a jar fermenter, approximately 27,200 units/l chitosanase were detected as extracellular enzyme in the supernatant. Enzyme in the 200 ml cell free supernatant was precipitated with 200 ml ethanol and 78% chitosanase activity was recovered. The precipitate was dissolved in 10 ml acetate buffer (50 mM, pH 5.6) and dialyzed against a 50 mM acetate buffer at pH 5.6. The enzyme solution was centrifuged to remove insoluble materials at 10,000× g for 10 min and 67% enzyme activity was recovered at this step. The total supernatant was loaded onto a CM-cellulose column and was eluted by a linear gradient of

NaCl from 0 to 0.3 M NaCl. An active fraction of chitosanase was presented in the tube number 31-40 as shown in Fig. 1. The active fraction was concentrated to 2 ml by lyophilizer, dialyzed in a cellulose tube (cut off 8 kDa) against a 30 mM Tris-HCl buffer at pH 6.8 and 41% chitolytic activity was recovered at this step. Anion exchange chromatography was performed by HPLC with a DEAE-TSK gel column (5×75 mm). The crude enzyme was charged, washed with buffer for 5 min and eluted with a linear gradient of NaCl from 0 to 0.3 M NaCl for 40 min. The elution time of the chitosanase was 28.50 min as shown in Fig. 2. The active fraction

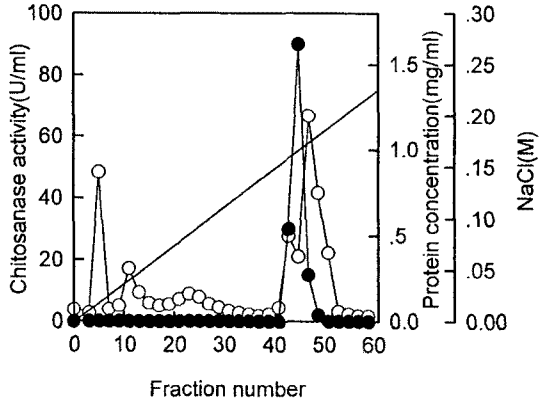


**Fig. 1.** Chromatogram of CM-cellulose column chromatography.
The column (2.5×25 cm) was equilibrated with 50 mM sodium acetate buffer, pH 5.6. The enzyme was eluted with a linear gradient of 0-0.3 M NaCl and 5 ml fractions were. collected. Protein concentration was estimated by protein assay kit (Bio-Rad). Chitosanase activity (●), protein concentration (○).

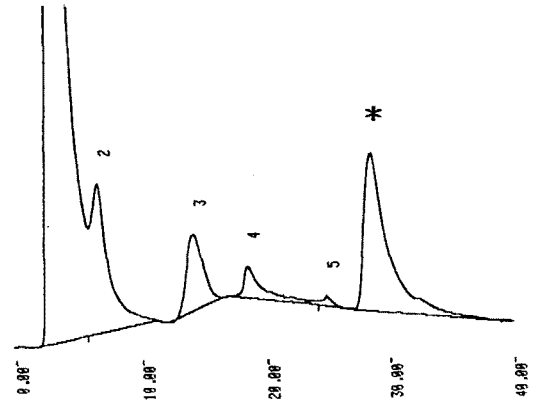


**Fig. 2.** Chromatogram of the enzyme HPLC with DEAE-TSK gel column.
The column (7.5×75 mm) was equilibrated with 30 mM Tris-HCl buffer, pH 6.8. The enzyme was injected and was washed with same buffer for 5 min. The enzyme was eluted with a linear gradient of 0-0.3 M NaCl at the flow rate of 1 ml/min. The protein peak was detected by UV-detector at 280 nm and each protein peak were collected. Chitosanase activity peak (*).

was concentrated and GFC was performed by HPLC. The enzyme was eluted at 15.60 min in GFC (Fig. 3). This enzyme has an affinity for the YMC-pack Diol 120 column in a low salt solution, thus elution time was delayed but adequate elution time was gained by 0.5 M NaCl solution. Chitosanase was purified 251-fold, and 1,145 units with a 21% recovery yield were gained.

**Characteristics of Chitosanase**

Chitosanase from *Bacillus* sp. HW-002 was purified by a series of purifying steps: 50% saturation with ethanol, CM-cellulose, DEAE-TSK gel and YMC-pack Diol 120.

Only one chitosanolytic activity was detected from the culture of *Bacillus* sp. HW-002 on the chitosanase production medium. The purified chitosanase appeared as a single band on SDS-PAGE (Fig. 5). The molecular weight of the enzyme was estimated to be about 46 kilodaltons (kDa) by SDS-PAGE, and 23 kDa by GFC (Fig. 4). The molecular weight of chitosanase from *Bacillus* sp. HW-002 differed greatly from chitosanases from *B. megaterium* P1 (12), which had molecular weights of 43, 39.5 and 22 kDa by SDS-PAGE. Chitosanases of *B. circulans* MH-K1 (18) was 32 kDa by GFC and *Bacillus* sp. R-4 (17) was 31 kDa by SDS-PAGE. These molecular weights were not similar to that of enzyme from *Bacillus* sp. HW-002. The elution time of purified enzyme (25 min) was delayed by low salt eluant (30 mM Tris-HCl), in comparision with molecular weight by SDS-PAGE, but the enzyme was rapidly eluted (15.6 min) by a high salt eluant (30 mM Tris-HCl containing 0.5 M NaCl), in comparision with a low salt eluant. The results were similar to those for *B. megaterium* P1 (12), for which the molecular weights of three chitosanases appeared as 43, 39.5 and 22 kDa by SDS-PAGE, whereas low molecular weights for all three enzymes were estimated to be about 23, 13 and 5.5 kDa by gel filtration. Also, the molecular weight of chitosanase from *Bacillus* sp. PI-7S (15) was

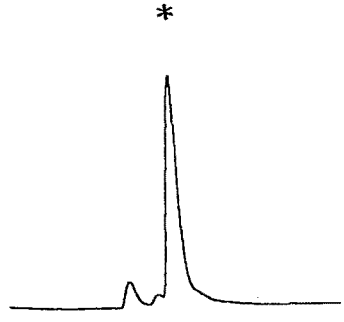


**Fig. 3.** Chromatogram of the enzyme by HPLC with YMC-pack Diol-120.
The gel filtration was performed with 30 mM. Tris-HCl buffer, pH 6.8, at a flow rate of 1 ml/min. Protein was detected by UV-detector at 280 nm and the enzyme was eluted in 15.50 min. Chitosanase activity peak (*).

estimated to be about 43 kDa by SDS-PAGE but was estimated to be about 25 kDa by TSK gel G-3,000 SW GFC. The retardation of the enzyme on GFC is suggestive of non-globular protein conformation or of interaction with the gel filtration matrix.

The optimal pH of chitosanolytic activity was approximately pH 5.5-6.0, and chitosanolytic activity was most stable at pH 5.0 as shown in Fig. 6. The optimal pH of enzyme activity from *Bacillus* sp. HW-002 was similar to chitosanase from *Bacillus* sp. R-4 (17), but the pH stability of chitosanase from *Bacillus* sp. R-4 was approximately pH 4.0-9.0.

The optimal temperature of chitosanolytic activity was

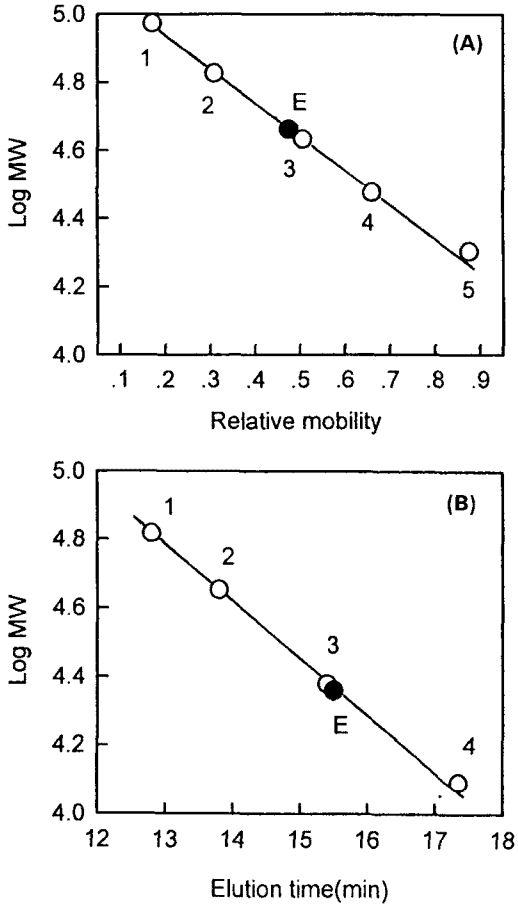


**Fig. 4.** The estimation of molecular weight of the purified chitosanase.

(A) SDS-PAGE was performed as described in Fig. 5. The moleuclar weight of enzyme was estimated to be about 46 kDa. 1, Phosphorylase b (97.4 kDa); 2, Bovine serum albumin (66 kDa); 3, Ovalbumin (45 kDa); 4, Carbonic anhydrase (30 kDa); 5, Soy trypsin inhibitor (20.1 kDa); E, Chitosanase (46 kDa). (B) GFC was performed with 30 mM Tris-HCl buffer (pH 6.8) containing 0.5 M NaCl, at a flow rate of 1 ml/min. The moleuclar weight of enzyme was estimated to be about 23 kDa. 1, Bovine serum albumin (66 kDa); 2, Egg albumin (45 kDa); 3, Trypsin (24 kDa); 4, Cytochrome c (12.4 kDa); E, Chitosanase (23 kDa).

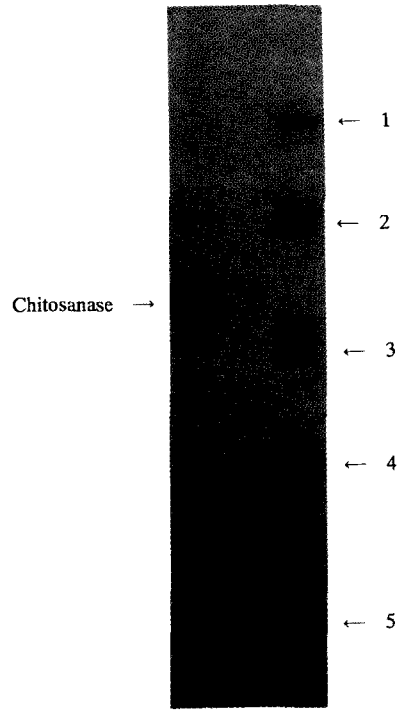


**Fig. 5.** SDS-polyacrylamide gel electrophoretic pattern of the purified chitosanase.

For the estimation of purity and molecular weight was used 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue R-250. 1, Phosphorylase b (97.4 kDa); 2, Bovine serum albumin (66 kDa); 3, Ovalbumin (45 kDa); 4, Carbonic anhydrase (30 kDa); 5, Soy trypsin inhibitor (20.1 kDa).

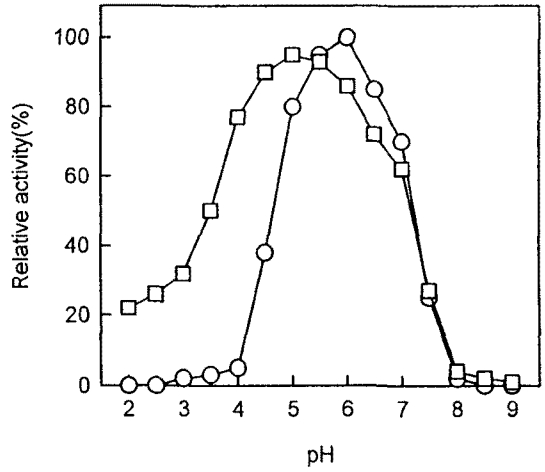


**Fig. 6.** Effect of pH on activity and stability of the purified chitosanase.

Optimal pH on activity of purified chitosanase (○): The purified enzyme was incubated with chitosan in various pH values buffer at 40°C for 10 min. The buffers were adjusted from pH 2.0 to pH 9.0 by 50 mM McIlvain buffer. The pH stability of chitosanase (□): Purified enzyme was incubated in various pH values buffer at 30°C for 1 h, and the remaining activities were measured.

at 65°C (Fig. 7). The chitosanolytic activity was stable at 45°C for 1 h, and decreased to approximately 20% of its original activity when the enzyme was treated at 50°C for 1 h, as shown in Fig. 8.

The substrate specificity of the purified enzyme was analyzed with 0.3% soluble chitosan, colloidal chitosan, insoluble chitosan, colloidal chitin, insoluble chitin, and CMC as substrates. When the enzyme activity with soluble chitosan was benchmarked at 100%, the relative enzyme activities with colloidal chitosan and colloidal chitin were 31% and 5%, respectively. However, the enzyme did not degrade insoluble chitosan, insoluble chitin or CMC (Table 1). This result was not similar to those of chitosanases from *B. circulans* WL-2 (8), *Enterobacter* sp. G-1 (19) and *Acinetobacter* sp. CHB 101 (16), which hydrolyzed chitin and chitosan.

The enzyme exhibited maximal absorption(UVmax) at 195 nm and had not UVmax at 280 nm as shown in Fig. 9. Thus the chitosanase has either very little or no aromatic amino acid content. This finding differs greatly from the result of the chitosanases from *Streptomyces* sp. (13) and *Bacillus sp.* R-4 (17) which exhibited UVmax

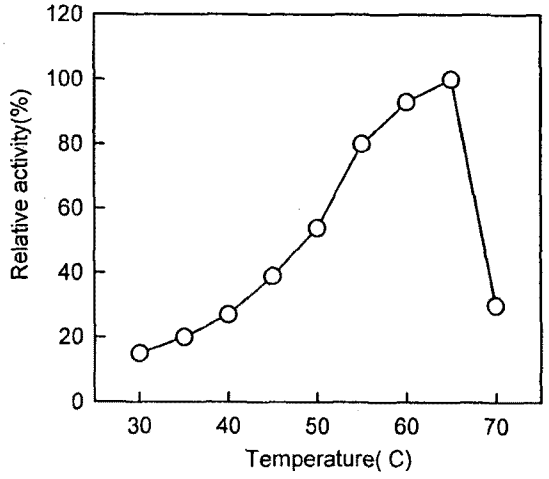


**Fig. 7.** Effect of temperature on activity of the purified chitosanase.
The purified enzyme was incubated with chitosan in 50 mM sodium acetate buffer (pH 5.6) at each temperature for 10 min, and the released reducing sugar was examined.

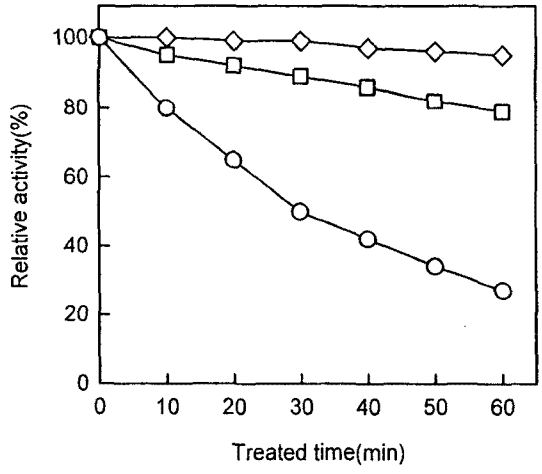


**Fig. 8.** Effect of temperature on stability of the purified chitosanase.
After the enzyme was treated in 50 mM sodium acetate buffer (pH 5.6) at 45, 50 and 55°C for 10-60 min, remaing activities were measured. 45°C (◇), 50°C (□), 55°C (○).

**Table 1.** Substrate specificity of the chitosanase against different kinds of β-1,4-glucans.

| Substrate | Relative actitity (%) |
|---|---|
| Soluble chitosan | 100 |
| Colloidal chitosan | 31 |
| Colloidal chitin | 5 |
| Insoluble chitosan | 0 |
| Insoluble chitin | 0 |
| Carboxy methyl cellulose | 0 |

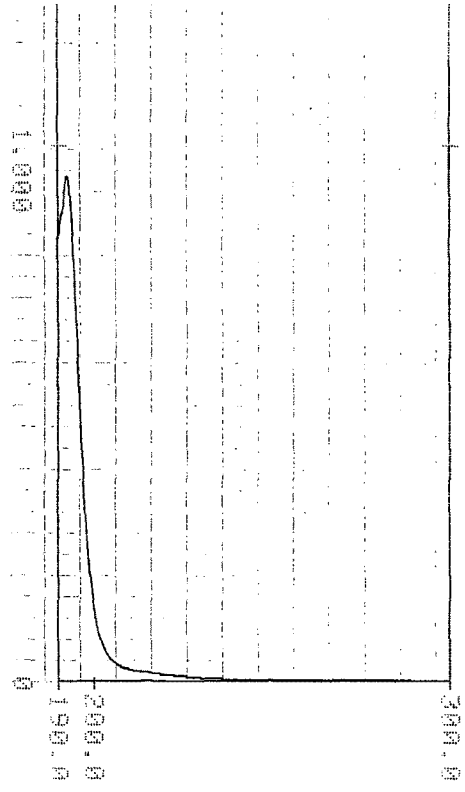

**Fig. 9.** UV-spectrum of the purified chitosanase.
After gel filtration chromatography the active fraction was dialyzed against distilled water at 4°C for 10 h and spectrum of the enzyme was investigated from 190 nm to 300 nm by UV-spectrophotometer.

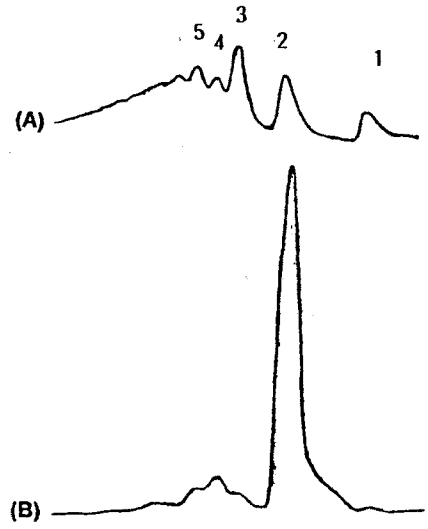


**Fig. 10.** Chromatogram of the end product of enzyme reaction by HPLC with two linked YMC-pack Diol 60.
After 100 ml chitosan (1%) was hydrolyzed by 30 units purified chitosanase at 40°C for 5 h, the reaction mixture was precipitated with 70% saturation of methanol and centrifuged at 10,000×g. The supernatant was concentrated by vacuum evaporator and was dissolved in distilled water. The dissolved compound was passed through IRA-420 anion exchange column (2×20 cm) and concentrated by vacuum evaporator. The treated end product was analyzed by gel filtration chromatography with 1.0 M NaCl solution, at a flow rate of 0.5 ml/min and was detected by RI detector. The elution time of main peak (B) was 72 min. (A) Standard of chitooligosaccharide: 1, *N*-acetylglucosamine; 2, Chitobiose; 3, Chitotriose; 4, Chitotetraose; 5, Chitopentaose. (B) End product of the chitosanase reaction.

at 278 nm.

When the chitosanase was highly concentrated or throughly dialyzed against deionized water, the enzyme became insoluble and was partially precipitated. Addition of NaCl, however, was found to be effective for the solubilization of chitosanase. The chitosanase was completely soluble at higher concentrations of NaCl greater than 0.2 M. This characteristic was very similar to that of chitosanase from *Bacillus* sp. R-4 (17).

The enzyme reaction mixture was analyzed by HPLC with two linked YMC-pack Diol 60. The main end product of the enzyme reaction with chitosan as a substrate was chitobiose but very small amounts of chitotriose, chitotetraose, and chitopentaose were also produced, as shown in Fig. 10. This result is interesting because the chitosanases from *Bacillus* sp. PI-7S (15), *Bacillus* sp. No. 7M (5), *B. circulans* MH-K1 (18) and *Streptomyces griseus* (10) produced variously sized chitooligosarccharides by endo-splitting, but chitosanase from *Bacillus* sp. HW-002 produced almost all chitobiose. Therefore, further study will be required for clarifying the cleavage pattern.

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