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Effect of Chitin Sources on Production of Chitinase and Chitosanase by *Streptomyces griseus* HUT 6037

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Abstract The advantage of using *Streptomyces griseus* HUT 6037 in the production of chitinase or chitosanase is that the organism is capable of hydrolyzing amorphous or crystalline chitin and chitosan according to the type of the substrate used. We investigated the effects of the enzyme induction time and chitin sources, CM-chitosan and deacetylated chitosan (degree of deacetylation 75-99%), on production of chitosanase. We found that this strain accumulated chitosanase when cells were grown in the culture medium containing chitosanaceous substrates instead of chitinaceous substrates. The highest chitosanase activity was obtained at 4 days of cultivation with 99% deacetylated chitosan. Soluble chitosan (53% deacetylated chitosan) was found to induce chitinase as well as chitosanase. The specific activities of chitinase and chitosanase were 0.91 and 1.33 U/mg protein at 3 and 5 days, respectively. From the study of the enzymatic digestibility of various degrees of deacetylated chitosan, it was found that $(GlcN)_3$, $(GlcN)_4$ and $(GlcN)_5$ were produced during the enzymatic hydrolysis reaction. The results of this study suggested that the sugar composition of $(GlcN)_3$ was homogeneous and those of $(GlcN)_4$ and $(GlcN)_5$ were heterogeneous.

Keywords: chitooligosaccharide, chitosanase, deacetylated chitosan, CM-chitosan, *Streptomyces griseus*

INTRODUCTION

Chitin, chitosan and their oligosaccharides, which are very effective in the treatment of ulcerative colitis and other gastrointestinal inflammation disorders, can not be absorbed or digested directly in the gastrointestinal tract but absorbed only in the hydrolyzed oligosaccharide forms, such as cellulose [1-3]. The production of these oligosaccharides by chemical process causes environmental problems, and the chemical production of these oligosaccharides in a large scale has been difficult. Therefore, studies of oligosaccharide production by microorganisms have been performed recently. Chitinase (poly(1,4-(*N*-acetyl-β-D-glucosaminide)) glycanohydrolase, [E.C. 3.2.1.14]) produces low-molecular-weight, soluble multimers of *N*-acetyl-β-D-glucosamine (GlcNAc) and the dimer *N,N'*-diacetyl chitobiose, while chitobiase (β-D-*N*-acetyl-glucosaminidase, [E.C. 3.2.1.30]) hydrolyzes the dimer (chitobiose) to produce a monomer (GlcNAc). Combined use of chitinase and chitobiase is needed for splitting chitin to GlcNAc [2,4-7]. Chitinase and chitosanase are produced by a wide range of organisms including bacteria, insects, viruses, plants and animals and play important physiological and ecological roles [8]. Microorganisms such as *Serratia marcescens* [9-12], *Trichoderma harzianum*[13], *Streptomyces* sp. [14,15] and *Myrothecium verrucaria* [16] produce extracellular chitinase and chitobiase. Chitosanase (chitosan *N*-acetylglucosaminohydrolase [E.C. 3.2.1.132]) hydrolyzes β-1,4-linkages between GlcNAc and D-glucosamine (GlcN) residues in chitosan by an endowise manner but not chitin. This enzyme has been obtained from microbial sources such as *Rhizopus rhizopodiformis*, *Bacillus* sp., *Bacillus* R-4, *Myxobacter* and *Streptomyces griseus* [17,18]. *Streptomyces griseus*, the most active strain, can hydrolyze colloidal chitosan, soluble chitosan, glycol chitosan, carboxymethyl chitosan (CM-chitosan) and carboxymethyl cellulose (CM-cellulose) producing glucosamine oligosaccharide $(GlcN)_n$ (n=1-6) [19].

One advantage of using the organism *S. griseus* HUT 6037 is that it produces both chitinase and chitosanase depending on the substrate. These enzymes are capable of hydrolyzing amorphous or crystalline chitin and chitosan. In this study, we investigated the optimization of the induction protocol and the effect of chitin sources (powdered chitin, colloidal chitin) for the production of chitinase and the effect of CM-chitosan and deacetylated chitosan (degree of deacetylation 75-99%) on the production of chitosanase. In addition, we investigated the composition of chitooligosaccharides produced from deacetylated chitosan by *S. griseus* HUT 6037 chitosanase during the hydrolysis reaction. The optimum reaction time for the selective production of large amounts of chitooligosaccharides was also investigated.

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MATERIALS AND METHODS

Microorganism

S. griseus HUT 6037 was kindly provided by Prof. Masaru Mitsutomi (Saga University, Department of Applied Biotechnology, Fukuoka, Japan). The strain was maintained on agar slant containing 1.0% mannitol, 0.2% peptone, 0.1% meat extract, 0.1% yeast extract and 0.05% $MgSO_4 \cdot 7H_2O$. This strain was transferred to 50 mL of a growth-stage medium at pH 7.0, consisting of 1.0% mannitol, 0.2% peptone, 0.1% meat extract, and 0.1% yeast extract, and grown at 30°C for 24, 36 and 48 hrs to determine the optimal enzyme induction time. One mL of the growth-stage medium was inoculated into a 250 mL Erlenmeyer flask containing a production-stage medium pH 7.0, consisting of 0.05% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4$. Each of 0.2% (w/w) of powdered chitin, colloidal chitin, soluble chitosan and deacetylated chitosan was added into a production-stage medium as a substrate, and cultivated at 30°C on a rotary shaker.

Powdered Chitin

Purified chitin was purchased from Sigma Chemical Co. This chitin was pretreated with ball-mill (particle size of chitin was less than 250 μm), since ball-milled chitin of a nominal diameter of 180-250 μm yielded the highest level of chitinase activity [20].

Colloidal Chitin

Various forms of swollen chitin were also prepared according to the method of Berger and Reynolds [21]. Crab shells were ball-milled for 3 h and then soaked in 2% (w/v) $KMnO_4$ solution at room temperature for 20 h. The remaining powder was then washed with a 1% (w/v) oxalic acid solution to reduce the residual $KMnO_4$ and MnO_2 content and to remove oxidized protein matter.

Swollen colloidal chitin was prepared by washing the foregoing chitin with acetone to form a chitin paste, and then the paste was slowly added to 7 to 9 volumes of concentrated HCl cooled in an ice bath to *ca.* 4°C to arrest hydrolysis. The syrupy liquid was filtered in a glass column through a sintered glass plate filled with glass wool. The chitin containing filtrate was dropped into a vigorously stirred aqueous 50% ethanol solution to precipitate the chitin in a highly dispersed state. The colloidal residue was centrifuged at 8,000 rpm for 20 min and resuspended in water three to five times, sedimented by gravity and washed several times with 100 mM potassium phosphate buffer containing 1 mM $CaCl_2$ (pH 6.0) to remove excess acid and alcohol. Finally, the swollen chitin solution was dialyzed against 100 mM potassium phosphate buffer until a pH of 5 to 6 was maintained.

Soluble Chitosan

The powdered chitin (180-210 μm) obtained after ball-milling was alkaline-hydrolyzed with 47% (w/w) sodium hydroxide solution at 25°C for 56 h. The sample was neutralized by successive washing with deionized water.

Deacetylated Chitosan

The powdered chitin (100 g) pretreated with ball mill was treated with 870 mL of 47% (w/w) sodium hydroxide solution at 110-120°C for 1-3 h under nitrogen atmosphere. The chitosan product obtained by alkali treatment was washed with water at about 80°C to neutrality and washed with water repeatedly for two or more times to obtain the chitosan product which were deacetylated 85-95%. To obtain 99% deacetylated chitosan, 95% deacetylated chitosan was dissolved into 2% acetic acid then the solution was poured into a large amount of 1 N sodium hydroxide solution before deacetylation [22]. Measurements of the degree of deacetylation were made by the modified method employed for deacetylated chitosan by Sannan *et al*. [23] and infrared spectroscopic method [24,25]

Carboxymethyl Chitosan [26]

A 95% deacetylated chitosan was added to 2-propanol (50 mL), containing mono-chloroacetic acid (6 g), and the mixture was stirred for 1 h. The material isolated upon filtration was added to water (200 mL), and the pH was adjusted to neutrality with concentrated HCl; the mixture was stirred for 1 h. The resulting viscous solution was passed through glass wool, and then added slowly to 1 liter of acetone. The sodium salt of carboxymethyl chitosan obtained was washed with absolute ethyl alcohol and dried.

Chitosanase Activity Assay

Chitosanase activity was measured with glycol chitosan as a substrate [27]. The reaction mixture containing 0.5 mL of 2% glycol chitosan in 0.1 M phosphate buffer at pH 7.5 and 0.5 mL of enzyme solution was incubated at 37°C for 10 min, at which time the reaction was stopped by boiling for 4 min. This mixture was cooled in an ice bath after adding 1 mL of acetyl acetone and 1 mL of distilled water and boiling for 20 min. Five mililiter of ethanol and Ehrlich's reagent (*p*-dimethylaminobenzaldehyde: DMAB) was added and incubated at 65-70°C for 10 min. It was centrifuged at 8,000 rpm for 5 min to remove the substrates, and the absorbance was measured at 540 nm using an ELISA plate reader (Anthos ht III, Anthos Labtec. Instruments, Austria). Aminosugar liberated was determined by the method of Rondle and Morgan [28], with glucosamine as a standard. One unit of chitosanase activity was defined as

the amount of the enzyme that liberated 1 μmol of aminosugar per hour at 37°C.

Chitinase Activity Assay

Chitinase activity was measured by the colorimetric method of Ressig *et al.* [29] with some modification to allow for multi-sample analysis using an ELISA plate reader. The reaction mixture contained 0.8 mL of 1.25% (w/v) suspension of colloidal chitin, 0.1 mL of 100 mM potassium phosphate buffer (pH 6.0), and 1.0 mM calcium chloride. The reaction mixture was mixed with 0.1 mL of enzyme (supernatant or cell lysate) solution diluted 5 to 200 folds in the same phosphate buffer and incubated at 37°C for 1 h, at which time the reaction was stopped by boiling for 5 min. The chitin blank and samples were then centrifuged at 5,000 rpm for 5 min, and 0.275 mL of each supernatant was collected and assayed for GlcNAc concentration.

GlcNAc concentration was determined by mixing 0.055 mL of 0.8 M potassium tetraborate (pH 9.9) with the 0.275 mL supernatant sample and boiling this mixture for 3 min. The mixture was then cooled in an ice bath. 0.1 mL of 0.112 M DMAB in analytical-grade glacial acetic acid, which contained 12.5% (w/v) 10 N HCl, was then added, and immediately after mixing, 100 mL of the mixture was deposited in an Elisa plate maintained at 37°C. GlcNAc standard and blank solutions were also added to the plate, and after precise 20 min of reaction time, the plates were cooled to 4°C for 3 min and the absorbance of each well was measured at 545 nm in an ELISA plate reader. One unit of activity was defined as the amount of enzyme able to liberate 1 mg of GlcNAc per hour.

Chitobiase Activity Assay

Chitobiase activity was determined by measuring the amount of *p*-nitrophenol released when an aliquot of the enzyme solution was incubated with an aqueous solution of *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine (pNP-GlcNAc). Fifty mililiter of the 5 mM pNP-GlcNAc solution with 20 mL of the enzyme solution was appropriately diluted in Tris (hydroxymethyl-aminomethane)-maleate buffer at pH 7.0. After 10 min of the incubation period at 37°C, the reaction was stopped by the addition of 100 mL of 0.25 M Na_2CO_3. The liberated *p*-nitrophenol was measured at 405 nm in an ELISA plate reader. One unit of chitobiase activity was equal to the amount of enzyme necessary to liberate 1 mmol of *p*-nitrophenol per minute.

Determination of Reducing Sugar and Total Protein

The amount of total reducing sugar was determined by the method of Imoto and Yagishita [30]. Total protein concentrations were determined by the Bio-Rad protein assay, which follows the colorimetric procedure of Bradford [31]. In this study, the assay method was modified to accommodate the multi-sample format of an ELISA plate reader. All samples and standards were assayed in triplicate at 595 nm.

Analysis of N-acetylchitooligosaccharides by High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a pump, a UV/VIS detector, a RI detector, a sample injector, and a data module integrator (Waters, USA). Sugars were separated on a μBondapak NH_2 column (3.9 × 300 mm) using 70% acetonitrile-water mixture as the mobile phase, at a flow rate of 1.5 mL per min. *N*-Acetylchitooligosaccharides were detected by monitoring the UV absorbance.

Analysis of Chitooligosaccharides by HPLC

The HPLC system consisted of a pump, a UV/VIS detector, an RI detector (Japan Spectroscopic Co. Ltd., Tokyo, Japan), a sample injector (Model 7125, Rheodyne Inc., CA, USA), and a D-2500 Chromato-Integrator (Hitachi Ltd., Tokyo, Japan). Sugars were separated on a Radial-PAK μBondapak NH_2 column (8.0 × 100 mm, Millipore Co., Milford, MA, USA) using 70% acetonitrile-water mixture as the mobile phase, at a flow rate of 2.0 mL per min. Chitooligosaccharides were detected by monitoring the refractive index.

Hydrolysis of Deacetylated Chitosan by Chitosanase

A reaction mixture consisting of 100 mL of 0.6% substrates (degree of deacetylation; 53-99%) in 0.1 M acetate buffer (pH 5.5), 5 mL of 2% NaN_3, and 10 mL of chitosanase (5.0 units) was incubated for 48 h at 37°C. After the addition of 4 mL of chitosanase (2.0 units), the mixture was further incubated for 48 h at 37°C. The reaction was stopped by boiling for 5 min.

Hydrolysis of Chitooligosaccharides by Chitosanase

A reaction mixture containing 50 μL (200 μg) of chitooligosaccharides, 25 μL of 0.02 M phosphate buffer (pH 5.5) and 25 μL of enzyme solution [0.0025 units for $(GlcN)_4$, 0.0125 units for $(GlcN)_3$ and GlcN-GlcNAc-GlcN-GlcN, 0.125 units for the other hetero-chitooligosaccharides] was incubated at 30°C for 10 min. The reaction was stopped by boiling for 5 min and the products formed from each substrate were analyzed by HPLC.

Determination of the Numbers of Sugar Residues of Oligosaccharides

The numbers of sugar residues of *N*-acetylchitooligosaccharides and chitooligosaccharides were determined by HPLC using UV detector and RI detector, respectively. The number of sugar residues of chitooligosaccharides was analyzed by thin-layer chromatography (TLC) using silica gel 60 (aluminum sheet, Merck, USA) and a solvent system consisting of isopropanol:pyridine:acetic acid:water (10:6:6:9). Chromatography was car-

ried out at room temperature. After drying, the plate was sprayed with 0.2% ninhydrin in ethanol and amino sugars were visualized by heating at 100°C.

RESULTS AND DISCUSSION

Effect of Induction Time and Substrates on Chitinase Activity

S. griseus HUT 6037 produced chitinase when powdered or colloidal chitin was used as a sole carbon source in batch fermentation. This strain was grown in a seed culture medium for 24, 36 and 48 h to determine the optimal induction time of producing chitinolytic enzyme of highest activity. We found that chitinase activity was different depending on the induction time. Total chitinase activity in the culture broth using powdered chitin was generally higher than that of using colloidal chitin. The highest chitinase activity (8.6 U/mL) was observed at 5 days of cultivation with powdered chitin at the induction time of 36 h. However the specific activity of chitinase was higher when colloidal chitin was used as a carbon source (Fig. 1). The maximum specific activity of chitinase was observed at 5 days of cultivation when the induction time was 48 h with colloidal chitin as a carbon source. The specific activity of chitinase obtained using powdered chitin (8.3 U/mg protein) was lower than that obtained using colloidal chitin (12.6 U/mg protein). The optimal induction time and carbon source for maximizing chitinase activity were found to be 48 h cultivation in the seed culture medium and colloidal chitin, respectively.

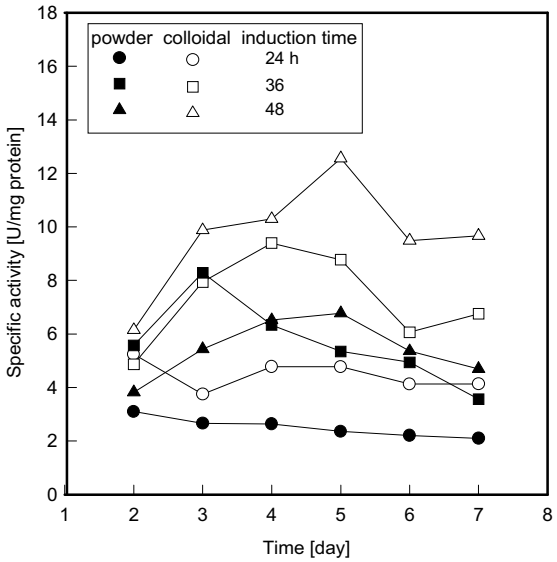

Fig. 1. Specific activity of chitinase during batch fermentation of *S. griseus* HUT 6037 using powdered chitin and colloidal chitin at different induction time.

Production of Chitosanase with Deacetylated Chitosan and CM-chitosan

A preliminary experiment showed that *S. griseus* HUT 6037 produced chitinase when chitinaceous sources were used as a carbon source. To investigate the characteristics of chitinase/chitobiase and chitosanase production according to carbon sources, we used deacetylated chitosan which has various degrees of deacetylation as well as CM-chitosan as a chitosan derivative. It was found that this strain accumulated chitosanase in the culture medium when a chitosanaceous substrate instead of chitinaceous substrate was used as a carbon source.

As shown in Fig. 2, total chitosanase activity in the culture broth reached a maximum value at 4 days of cultivation regardless of the types of chitosan. The highest chitosanase activity was obtained when CM-chitosan was used as a carbon source. The results shown in Fig. 2 also indicate that the activity of chitosanase increased with increasing the degree of the deacetylation of chitosan. Chitosanase activity using 99% deacetylated chitosan was 1.3 times higher than that of 75% deacetylated chitosan. The specific activity of chitosanase depending on CM-chitosan and degree of deacetylated chitosan were presented in Fig. 3. When CM-chitosan was used as a carbon source, total chitosanase activity in the culture broth was the highest (Fig. 2), but the specific activity of chitosanase was the lowest.

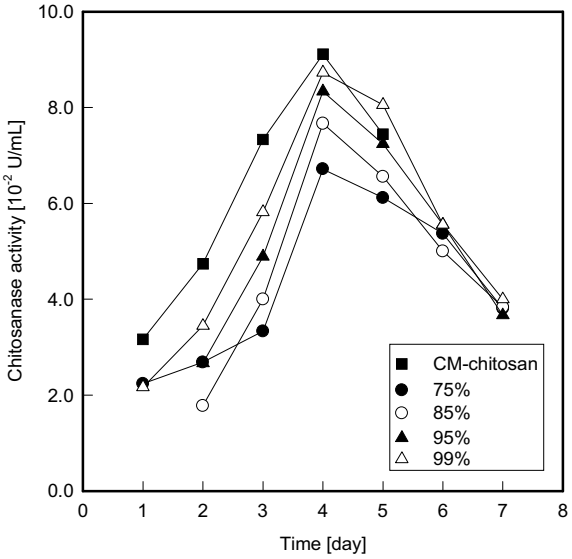

Fig. 2. Chitosanase activity in the culture medium during batch fermentation of *S. griseus* HUT 6037 using chitosan deacetylated at various degrees and CM-chitosan induction time was 48 h.

S. griseus HUT 6037 could produce both chitinase and chitosanase. Chitinase activity (8.6 U/mL) in this work was very low compared with that of *Serratia marcescens* QM B1466 (23.6 U/mL) reported in the previous work [20]. In this regard, this strain may be more suitable for the production of chitosanase rather than

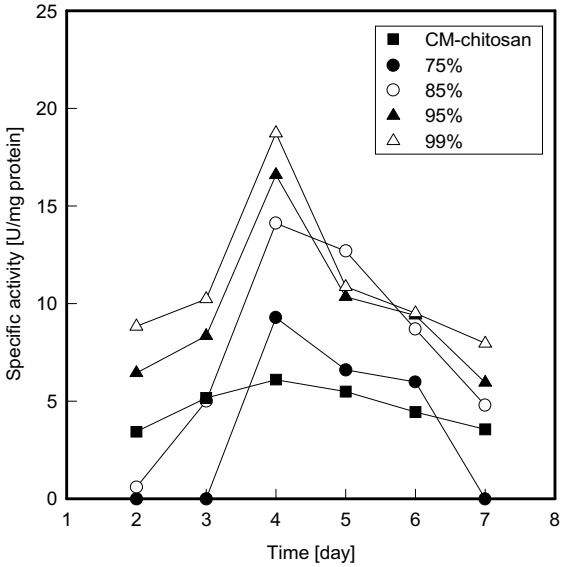

Fig. 3. Specific activity of chitosanase during batch fermentation of *S. griseus* HUT 6037 using chitosan deacetylated at various degrees and CM-chitosan. Induction time was 48 h.

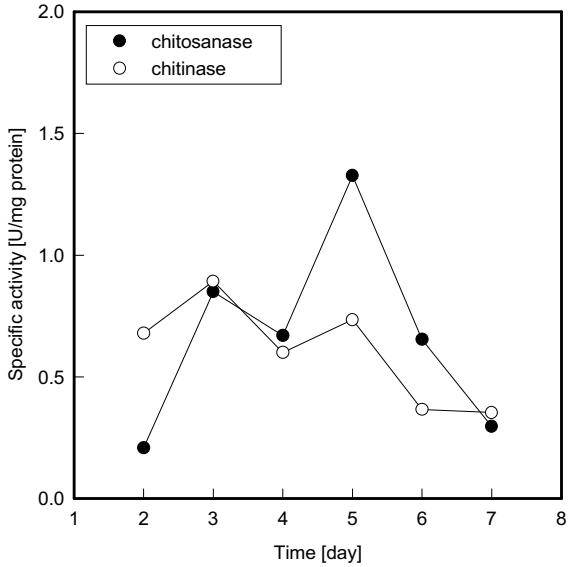

Fig. 4. Specific activity of chitinase and chitosanase during batch fermentation of *S. griseus* HUT 6037 using soluble chitosan. Induction time was 48 h.

chitinase. We also measured chitobiase activities; however, this enzyme activity was rarely detected.

Production of Chitinase/Chitobiase and Chitosanase with Soluble Chitosan

Generally, soluble chitosan was defined as partially (45-55%) deacetylated chitosan. Therefore, we attempted to produce both chitinase and chitosanase with *S. griseus* HUT 6037 using 53% deacetylated chitosan as a carbon source. The activity of enzyme using soluble chitosan differs from that obtained using 75-99% deacetylated chitosan. Maximum activities of chitinase and chitosanase were 0.04 U/mL and 0.92 U/mL, respectively at 5 days of cultivation with soluble chitosan while the maximum activity of chitosanase was observed at 4 days of cultivation using 75-99% deacetylated chitosan. Chitobiase activity was rarely detected in this cultivation. These results indicate that the enzymes produced during cultivation cleave both the *N*-acetyl-β-D-glucosaminidic and the β-glucosaminidic linkages in soluble chitosan molecules. Fig. 4 shows change of specific activities of chitinase and chitosanase during cultivation the specific activity of chitosanase was maximum at 5 days of cultivation. The specific activity of chitinase was changed similarly to that of chitosanase. The specific activity and the optimum cultivation period for production of chitinase and chitosanase were 0.91 and 1.33 U/mg protein at 3 and 5 days of cultivation, respectively.

Identification of Sugar Residues in Chitooligosaccharides

HPLC chromatograms of reaction mixture after enzymatic hydrolysis one of various chitosans were shown in Fig. 5. $(GlcN)_3$, $(GlcN)_4$ and $(GlcN)_5$ were detected in each sample using an RI detector, although the concentration of these chitooligosaccharides was very low. When the reaction products were analyzed with a UV detector, on the other hand, $(GlcNAc)_3$ and oligosaccharides above the hexamer of *N*-acetylchitooligosaccha- rides were detected in each sample. However the oligosaccharide above the hexamer of *N*-acetylchitooligosac-charides was presented as a trace and $(GlcNAc)_6$ was detected only when soluble chitosan was used as a substrate. It was found that the peak of $(GlcNAc)_3$ was reduced with the increase in the deacetylation degree of chitosan. On the other hand, $(GlcNAc)_4$ and $(GlcNAc)_5$ were not detected. It was suggested that the sugar composition of $(GlcN)_3$ was homogeneous but those of $(GlcN)_4$ and $(GlcN)_5$ were heterogeneous which may be composed with GlcNAc-GlcN-GlcNAc-GlcN or $(GlcNAc)_2$-$(GlcN)_2$ and $(GlcNAc)_3$-$(GlcN)_2$ or $(GlcNAc)_4$-GlcN.

In this study, we found that *S. griseus* HUT 6037 could secrete chitinase with a chitinaceous substrate, chitosanase with a chitosanaceous substrate, and both chitinase and chitosanase when soluble chitosan was used as a carbon source. The optimal induction protocol and carbon source for the production of chitinase by this strain were 48 hrs and colloidal chitin at 5 days of cultivation, respectively. The specific activity of chitinase when using a colloidal chitin was about 2 times higher than that when using a powdered chitin. We also found that specific activity of chitosanase depends on the degree of deacetylation of modified chitosan. As a result, 99% deacetylated chitosan was most suitable in the production of chitosanase with the highest specific activity. On the other hand, 95% deacetylated chitosan

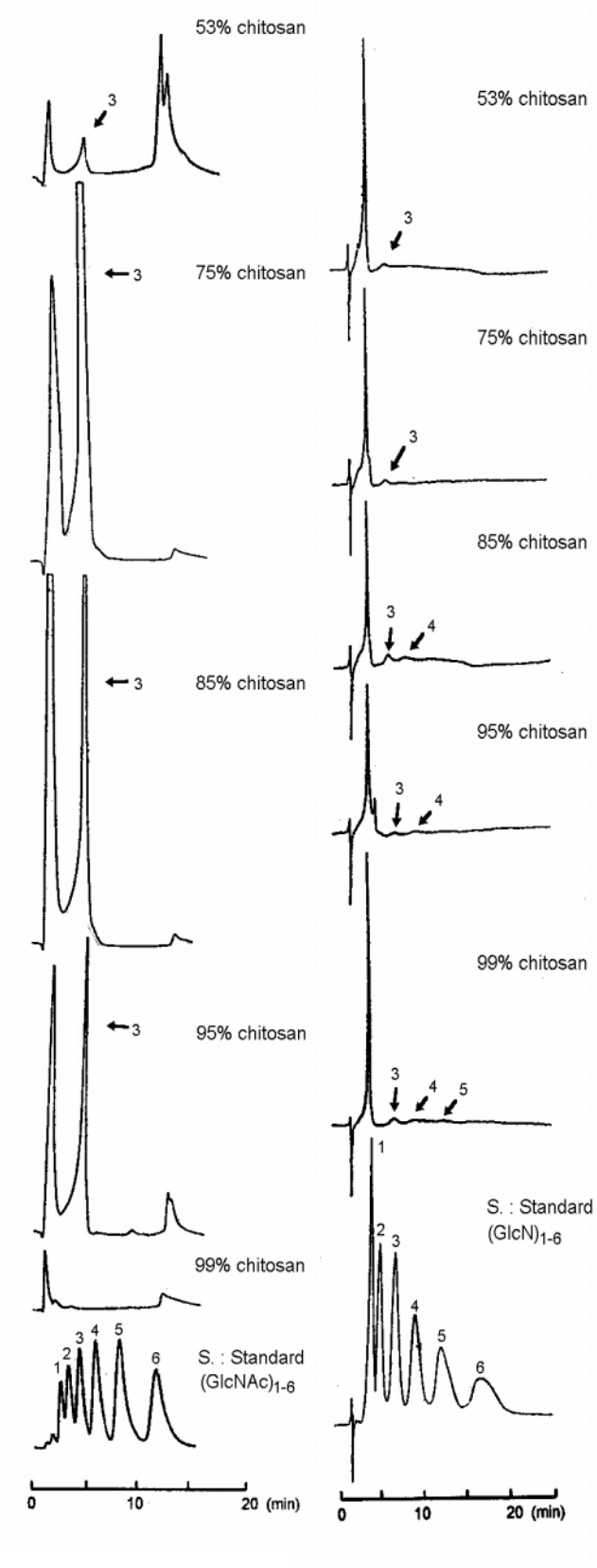

Fig. 5. HPLC chromatograms of chitooligosaccharides produced by enzymatic hydrolysis of various deacetylated chitosans (53-99%) analyzed with UV and RI detectors.

was most suitable for the production of chitoooligosaccharides ($(GlcN)_3$, $(GlcN)_4$ and $(GlcN)_5$). Since chitooligosaccharides attracted much attention in the biomedical, pharmacological, food industrial, and biotechnological fields, the results presented in this study may be useful for further studies on chitooligosaccharides.

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