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# Identification of Chitinolytic System in *Allium fistulosum*

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**Abstract** □ Chitinase was partially purified from *Allium fistulosum* L. (green onion). Protein fraction precipitated from ammonium sulfate was passed through CM-Sepharose and Sephacryl HR-200. The specific activity of the chitinase was 6.4 units/mg and total recovery was 6.3%. The analysis of the products from the digestion of N-acetychitohexaose indicated that chitinase was endo in action, with oligerms from N-acetylchitobiose to chitotetraose. N-Acetylglucosaminidase from the same species hydrolyzed oligomers obtained from chitinase reaction to lower oligosaccharides. These data demonstrated that chitinolytic system exists in green onion.

**keywords** □ *Allium fistulosum*, chitin, chitinolytic system, N-acetylglucosaminidase, chitinase

Chitin, the second most plentiful natural polymer, is composed of 1,4 linked N-acetyl-D-glucosamine (Review,[1]). This is mainly produced by a variety of marine animals, insects, and fungi. Recent biomedical application of chitooligosacharides has increased interest in finding new kinds of chitinase[2,3].

Chitinase (EC 3.2.1.14) is an enzyme that typically hydrolyzes chitin. Either chitobiose or chitotriose is formed, depending on the mode of action[4]. Chitinase is widely distributed in nature and is important in ecological system. Endochitinase is, in general, found in many species of higher plants[5-7] and hydrolyzes insoluble chitin to give soluble N-acetylchitooligosaccharides, while exochitinase from mixrobial sources cleaves the non-reducing terminus of chitin and yields mainly N,N′-diacetylchitobiose[8-10]. Another exo-type enzyme, N-acetylucosaminidase (EC 3.2.1.30) cleaves terminal N-acetylglucosamine (GlcNAc) residues from N-acetyl-D-glucosaminides, including oligosaccharides[11]. Both types of enzyme are required for the complete hydrolysis of chitin[12,13]. Most of chitinase are observed in microorganism, but the enzyme is produced by a variety of plant and animal species as well[14,15]. The presence of chitinase in plant was initially described by Powing and Irzykiewicz[16]. They proposed that plant chitinase played a role as a defense mexhanism against pathogen and this idea has been supported from many other workers[7,17,18].

This paper is to find the inexpensive source of chitinase out of edible plants for the utilization of chitin. Since we observed that green onion has chitinolytic system including chitinase and N-acetylglucosamidase (GlcNAcase), we characterized some of their properties.

## EXPERIMENTAL AND METHODS

Green onion was purchased in the local market. Practical chitin (from crab), chitosan (from crab), glycolchitosan, N,N′-diacetyl chitobiose, N,N′,N″-triacetyl chitotriose, *p*-nitrophenyl-N-acetylglucosaminide (pNPGlcNAc), glucosidase (almond), lysozyme, *Micrococcus lysodektius* and *p*-dimethylaminobenzaldehyde (DMAB) were purchased from Sigma Chemical Co. Acrylamide, bisacrylamide, and sodium dodecylsulfate (SDS) were also from Sigma Chemical Co. CM-Sepharose, Sephadex G-100 and Sephacryl HR-200 were purchased from Pharmacia. Bio-Gel P-4 (−400 mesh) was from Bio-Rad. Acetonitrile and water for high-performance liquid chromatography were of HPLC grade. Other chemicals were all of reagent grade.

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Ultrafiltration was done with Amicon kit using PM 10 membrane (Danvers, MA, USA).

***Purification of chitin***

It was followed essentially by the literature procedure with a slight modification[19]. In brief, ten grams of practical grade crab chitin flakes were suspended in 1 *l* of 2N HCl and stored for 2 days at room temperature with occasional stirring. After 2 days, demineralized chitin flakes were obtained through filtration and washed with water until neutral. Then, they were refluxed with 1N NaOH solution for 36 hours with stirring. Crude chitin chips were collected and washed with water until neutral. The chips were added to 500 m*l* of 95% ethanol, and air dried.

***Preparation of colloidal chitin***

Colloidal chitin was prepared using the purified chitin according to Shimahara *et al.*[19]. Glycol chitin was prepared by N-acetylation with acetic anhydride[20].

***Preparation of N-acetylchitooligosaccharides***

N-Acetylchitooligosaccharides were prepared from crab chitin (purified from the above method) by the partial hydrolysis and were separated on a Bio-Gel P-4 column[21]. In brief, ten grams of chitin were suspended in 140 m*l* of hydrochloric acid at 0℃ and incubated in 40℃ bath for 2 hrs with stirring. The hydrolyzate was neutralized with 200g of lead carbonate and was filtered. The filtrate and washings were freeze-dried. An 2.5 m*l* aliquot of the concentrate was loaded on a Bio-Gel P-4 column (2.5×90 cm) and was eluted with water at 0.2 m*l*/min. Fractions (3.8 m*l*) were collected and their absorbances were read at 210 nm. The fractions corresponding to each peak were pooled and freeze-dried.

***N-Acetylglucosaminidase assay***

This was essentially performed according to literature procedure[13]. The reaction mixture, consisting of 50 μ*l* of 4 mM *p*-NPGlcNAc, 100 μl of sodium acetate buffer, pH 5.0, and 50 μ*l* of enzyme solution, was incubated for 10 min at 37℃. The reaction was stopped by adding 1 m*l* of 1 M KOH to 100 μ*l* of reaction mixture.

***Chitinase assay***

Endochitinase activity was assayed according to Boller *et al.*[5]. In a routine assay, the reactiion mixture contained 100 μ*l* of colloidal chitin (10 mg/m*l*) or glycol chitin (10 mg/m*l*), 100 μ*l* of enzyme solution, and 300 μ*l* of acetate buffer (pH 5.0). Incubation was carried out at 37℃ for 2 hours and stopped by heating for 3 min. Then, two hundred microliters of reaction mixture was taken out and incubated by adding 40 μ*l* of glucosidase from almond (2U) or 0.2U of N-acetylglucosaminidase partially purified from green onion for 1 hour. The reaction was stopped by boiling for 3 min with the addition of 100 μ*l* of potassium borate buffer (pH 9.0). The reducing power was measured according to Reissing *et al.*[22]. The tubes were incubated for 20 min at 37℃ by mixing 3 m*l* of DMAB. The absorbance was measured at 580 nm. Exochitinase activity was assayed by the same procedure except for glucosidase addition.

One unit of chitinase is defined as the amount that catalyzes the liberation of 1 μmole of N-acetylglucosamine.

***Purification of N-acetylglucosaminidase and chitinase***

***Step 1 : Extraction and precipitation:*** The green onion, 2 kg, was homogenized in 0.05 M citrate buffer (3 m*l*/g). The homogenate was shaken at 4℃ for 1 hour and filtered through nylon. Then, it was centrifuged at 12,000 rpm for 20 min. The protein was precipitated to 80% with $(NH_4)_2SO_4$ and stayed overnight at 4℃. After centrifugation, the precipitate was dissolved in 100 m*l* of water and dialyzed against 3 *l* of 50 mM citrate buffer (pH 4.0).

***Step 2 : Chromatography on CM-Sepharose:*** The enzyme solution containing 196 mg of protein from step 1 was applied on CM-Sepharose column (2.5 cm×22 cm), which had been equilibrated with 0.05 N citrate buffer (pH 4.0). The column was washed until the absorbance at 280 nm was below 0.05 and eluted with a linear pH gradient from pH 4.0 to 6.0 in the same buffer. Eluate was collected in 5 m*l* fractions and they were assayed for both GlcNAcase and chitinase. The main fractions were pooled and concentrated with Amicon ultrafiltration unit using PM 10 membrane.

***Step 3 : Chromatography on Sephacryl HR-200:*** The enzyme solution (3 m*l*) was applied on a Sephacryl column (1.5×120 cm) previously equilibrated with 0.05 M citrate buffer (pH 5.0), and eluted with the same buffer, at a flow rate of 15 m*l*/hr.

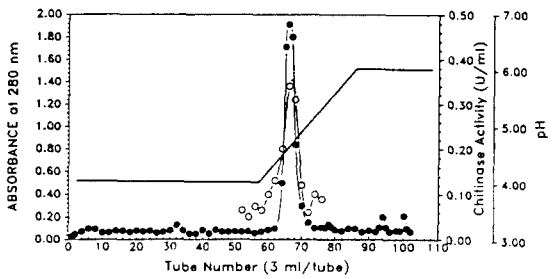


**Fig. 1. Purification of GlcNAcase and chitinase by CM-Sepharose.**

Ammonium sulfate precipitate containing GlcNAcase and chitinase activities was applied on a 2.5×30 cm CM-Sepharose column and the column was washed with sodium citrate buffer (pH 4.0). Chitinolytic activity (64-68, ○—○) was released from the column with 250 ml, pH 4.0 to pH 6.0 gradient in sodium citrate (——). Fractions were collected and protein was measured at 280 nm (●—●).

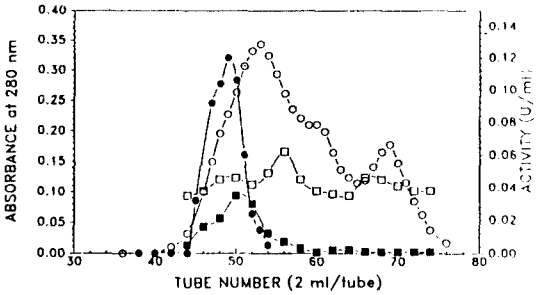


**Fig. 2. Purification of GlcNAcase and chitinase using by Sephacryl HR 200.**

The active fraction (3 ml) obtained from CM-Sepharose was applied on Sephacryl HR-200 column (1.5 cm×110 cm). Fractions were collected (2 ml/tube), and protein was measured at 280 nm (○—○). GlcNAcase activity was measured at 420 nm (●—●). Chitinase activity was assayed with glucosidase (□—□) or without glucosidase (■—■). Each tube was collected and divided into four fractions, I (45-49), II (50-53), III (54-58) and IV (67-72).

***Protein Assay***

Protein concentration was measured spectrophotometrically at 280 nm or by the method of Bradford using BSA as a standard[23].

***Polyacrylamide Gel electrophoresis (PAGE)***

The molecular weights of GlcNAcase and chitinase were determined by SDS-PAGE on 10% gels according to the method of Lammeli[24].

***Enzymatic degradation of collodal chitin and N-acetylchitohexaose with chitinase (fraction IV)***

N-acetylchitohexaose (0.16 μmole) was dissolved in 10 μl of citrate buffer (pH 5.0) and treated with 0.2 U of chitinase overnight. Then 0.1 U of GlcNAcase was added to reactiion mixture and incubated for 4 hours. At the same time, 100 μl of colloidal chitin (10 mg/ml) was incubated with 0.1 U of chitinase overnight. The reaction was stopped by centrifugation.

***High performance liquid chromatography (HPLC) of N-acetylchitooligosaccharides***

HPLC was performed to analyze the reaction mixture of colloidal chitin and chitinolytic enzyme from green onion. The system was equipped with a SP 8860 ternary HPLC pump, fixed-volume loop Rheodyne (Cocti, CA) $7125 injector and Spectra 100 variable wavelength detector. The data was processed using SP4270 integrator. The reaction mixture was injected on a Sepheri-5 amino column of dimension 4.6 mm×22 cm from Spectra Physics and eluted with acetonitrile (75%) and water (25%) at 1.0 ml/min.

## RESULTS

***Chitinolytic activity in green onion***

Ammonium sulfate fraction from green onion showed the reducing power of N-acetylglucosamine with *p*-dimetylaminobenzaldehyde. Chitinase in the system was specific for colloidal chitin and glycol chitin. A fraction eluted at pH gradient (4.0-6.0) using CM-Sepharose showed chitinolytic activity (Fig. 1). Both activities could not be separated. This was separated using Sephacryl HR-200 and divided into four fractions (Fig. 2). A fraction (I) showing GlcNAcase was eluted after void region (80 ml) and collected (Fig. 2). After concentration, this was reapplied on the same column, and the fraction showing the activity was collected. This fraction was used as a source of GlcNAcase. Fractions of II, III and IV in Fig. 2 showed chitinase activities acting on glycol chitin (Fig. 3). The open bars represent only chitinase activity and the filled bars indicate that N-acetylchitooligosaccharides produced in the reaction mixture of glycol chitin with each fractions

**Table I. Purification of chitinase**

| Step | Total volume (ml) | Total[1] protein (mg) | Total[2] activity (units) | Specific activity (units/mg) | Yield[3] (%) |
|---|---|---|---|---|---|
| Supernatant | 2,750 | 1157 | 440 | 0.38 | 100 |
| $(NH_4)_2SO_4$ | 100 | 82 | 96 | 1.2 | 21 |
| CM-Sepharose | 99 | 24 | 43 | 1.7 | 9.8 |
| Sephacryl HR | 26 | 4.4 | 28 | 6.4 | 6.3 |

[1]Based on Bio-Rad microassay
[2]One unit is defined as the amount that catalyzes the liberation of 1 μmole of N-acetylglucosamine in one hour.
[3]Based on total activity.

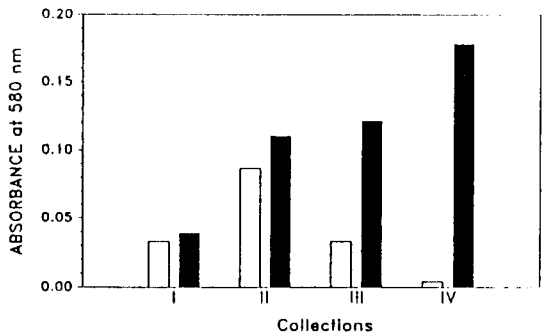


**Fig. 3. Chitinase activities of four fractions obtained from Sephacryl HR-200.**
The open bars indicate chitinase activity in the absence of GlcNAcase. The filled bars indicate chitinase activity in the presence of GlcNAcase.

were hydrolyzed by GlcNAcase from green onion. While II and III showed both GlcNAcase and chitinase activities, IV showed only chitinase activity. Since chitooligosaccharides greater than chitobiose do not show reducing power and GlcNAcase cannot hydrolyze glycol chitin, it can be explained that chitinase in the fractions of I, II, and III first cleaved glycol chitin to glycol chitooligosaccharides and then GlcNAcase coexisted in each fractions converted oligosaccharides formed to N-acetylglucosamine. The results of chitinase purification are shown in Table I. The chitinase activity (fraction IV) was purified about 17 fold, but it was not homogeneous as judged with SDS-PAGE under nondenaturing conditions. The complete purification and characterization of chitinase will be published in elsewhere.

***Enzymatic degradation of N-acetylchitohexaose and colloidal chitin***

Chitinase from green onion cleaved chitohexaose

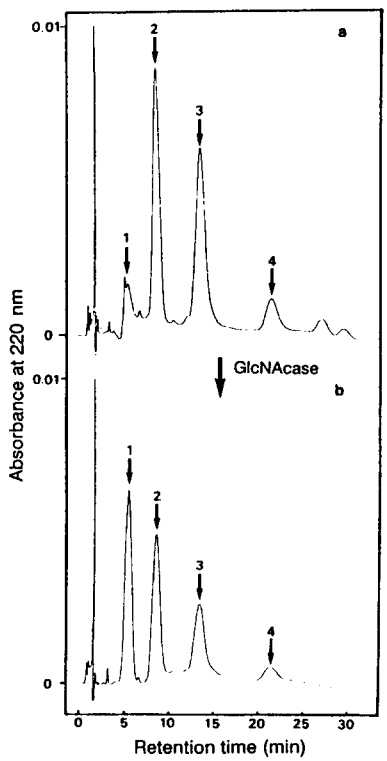


**Fig. 4. Demonstration of chitinolytic system in green onion using HPLC a) the reaction mixtures of chitinase and N-acetylchitohexaose b) after addition of N-acetylglucosaminidase to the reaction mixture (a).**
1) N-acetylglucosamine, 2) chitobiose, 3) chitotriose, 4) chitotetraose.

to chitotriose, chitobiose and chitotetraose with a ratio of 5 : 5 : 1 (a of Fig. 4). Regardless of duration of incubation, GlcNAc was only a minor reaction product. When this mixture was treated with GlcNAcase from green onion (fraction I of Fig. 3), it

was observed that N-acetylglucosamine was increased gradually (b of Fig. 4). Chitobiose, chitotriose and chitotetraose were decreased to next lower oligosaccharides. Chitinase also could hydrolyze colloidal chitin and yield mainly chitobiose and chitotriose on exhaustive depolymerization. The crude enzyme obtained from $(NH_4)_2SO_4$ precipitate depolymerized colloidal chitin to yield mainly N-acetylglucosamine (data not shown).

## DISCUSSION

The chitinase was partially purified from green onion. It did not show any GlcNAcase activity. The chitinolytic system from the mature tissues of green onion consists of two enzymes which succesively break down chitin to yield GlcNAc. The first enzyme acts on chitin, and the second further hydrolyze the products of the first. Chitinase from green onion is specific for chitin and glycol chitin. The results that chitinase and GlcNAcase could not bind to DEAE-Sephadex at pH 7.5 suggest that both enzymes are basic proteins. The chitinase activity could not be obtained accurately in the first three steps of purification in Table I, because GlcNAcase activity was also observed in each step except for gel-filtration stage. That might be reason why specific activity of endochitinase after purification was increased only 17-folds compared to supernatant. Although the supernatant was treated with 80% ammonium sulfate (saturated concentration), the amount of protein obtained was usually about 20-30%. It seemed that the rest of protein formed aggregation at that stage. It is not clear why specific activity was not increased compared to ammonium sulfate precipitation. It looks like enzyme was inactivated due to denaturatio ' of protein. This step shoud be improved in the uture for the large scale production.

The pattern of product' formation, as anlayzed by HPLC, indicates that enitinase act as an endochitinase. The GlcNAc detected might be formed by the endochitinase hydrolyzing chititriose to chitobiose and GlcNAc. It differs from the *Serratia* and *Streptomyces* chitinase[8,9,25] which produce N-acetylgucosamine and N-acetylchitobiose, as the main products, respectively.

To our knowledge, this is the first report in which the chitinolytic system has been examined in green onion. Green onion is available very easily around us. The very useful N-acetylchitooligosaccharides can be produced using chitinase from green onion.

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