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Preparation of *N*-acetyl-D-Glucosamine Using Enzyme from *Aspergillus* sp.

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Abstract

N-acetyl-D-glucosamine (GlcNAc) is one of the basic constituents of mammal cartilage, which is known to help repair deteriorating cartilage and relieve pain and inflammation in osteoarthritis patients. The preparation of GlcNAc from squid pen β-chitin by using *Aspergillus sp*. chitinase in enzymatic hydrolysis is studied. The cultivation of the fungus in colloidal chitin minimum media at pH 3.5 and 40°C for 5 days gives the highest chitinolytic activity of 3.1 U/mL. The crude enzyme obtained from the the cultivation is used in the hydrolysis of milled squid pen β-chitin (50μm) at pH 4 (acetic acid 2M) and 45°C using 22 U of enzyme per gram of chitin. After 2 days of hydrolysis, GlcNAc is isolated in 65% yield by precipitation in ethanol from a charcoal decolorized aqueous solution.

Key words: chitosan, fungi, food supplement, glucosamine, monosaccharide, sugar

Introduction

Chitin, a linear polymer of β-1, 4-linked *N*-acetyl-glucosamine, is the second most abundant biopolymer next to cellulose. It is a major structural component of insect exoskeleton, shells of crustaceans and fungal cell walls. Chitin is broken down by endo– and exo–enzymes currently known as chitinases (EC3.2.1.14) and β-*N*-acetylhexosaminidase (EC3.2.1.52), respectively. In nature, these enzymes are produced by bacteria, fungi, and actinomycetes, among others. Chitinases are a group of complex hydrolytic enzymes that catalyze depolymerisation of chitin. Chitin degradation is generally started by random cleavage within the chain of chitin by chitinases to release oligomers of *N*-acetyl glucosamine chains (GlcNAc). The oligomers are subsequently degraded to monomeric GlcNAc by β-*N*-acetylhexosaminidase which progressively breaks down chitin and chitooligosaccharides from the non-reducing end of the molecule. The hydrolytic products of chitin have gained special interests in agriculture and food industry. Oligo-chitins have been proposed as anti-microbial agents, promoters of plant growth, elicitors of plant resistance, enhancers of the immune responses and agents against malignant growth.[1] Acid hydrolysis of chitin using hydrochloric acid is an industrial process for the production of glucosamine hydrochloride salt (GlcNHCl), one of the most popular food supplements and drugs prescribed for osteoarthritis patients.[2] To avoid there are probably several side effects of inorganic salt and its salty taste, a non-salt form of GlcNAc which has pure sweet taste has been proposed to be used as an alternative for the same applications.[3] A few reports on preparation of GlcNAc using a mild reaction condition of an enzymatic process have emerged in literature in recent year.[4,5,6,7] All those reports involve the use of bacterial enzymes. Although extracellular contents secreted from several fungi[5,6,11,14,15] including *Aspergillus* fungus[8,12] have been shown to possess high chitinolytic activity, their application for preparation of GlaNAc from chitin has not yet been reported. Here we would thus like to report an option of utilization of the crude enzyme from *Aspergillus* fungus for preparation of GlcNAc in multigram scale.

Materials and Experimental Procedures

Materials

Chitin from squid pen chitin (β-chitin) was purchased from Taming Enterprise. Co., Ltd. (Thailand). The chitin was ground by ultra-centrifugal mill (retsch, zm1000, Germany) at the

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Metallurgy and Materials Science Research Institute, Chulalongkorn University, to give puffy fibrous chitin with a diameter of about 50 μm and length of about 100 μm.

Microorganism and Cultivation

Aspergillus sp. was isolated by Assoc. Prof. Dr. Hunsa Punnapayak, Department of Botany, Faculty of Science, Chulalongkorn University. The fungus was stored on potato dextrose agar (PDA) slant at 4°C prior to use. The cultivation of the fungus was carried out on PDA at 40°C for 3 days. The PDA (diameter 0.5cm × 7 pieces/100mL) containing fungal filament was inoculated into the culture medium containing KH_2PO_4 (0.1% w/v), $MgSO_4.7H_2O$ (0.05% w/v), $FeSO_4.7H_2O$ (0.005% w/v), urea (0.3% w/v) and colloidal chitin (2% dry weight). The culture content was kept within the pH range of 3.5-5.0 and incubated in a shaking incubator at 40°C and 150 rpm. After 5 days, the culture content was centrifuged at 3500 rpm for 15 mins and filtered to supply the crude enzyme as a filtrate.

Chitinolytic Activity

The enzyme assay was determined according to Schale's method.[8,9] The crude enzyme solution (25 μL) was mixed with the substrate suspension containing 100 μL 0.5% colloidal chitin and 275 μL citric acid/disodium hydrogen phosphate buffer (0.5 M) with pH 4.0. Distilled water (1100 μL) was added to bring the total volume to 1.5 mL and the mixture was incubated at 45°C for 30 mins. After the incubation period, the K_3FeCN_6 solution (2mL of 0.1% w/v in 0.5 M Na_2CO_3) and the reaction mixture was heated in a boiling water bath for 5 mins. The absorbance (A) of the solution was measured by UV-Vis spectrometer (Beckman, DU 650, U.S.A) at 420 nm. The decoloration (ΔA) of K_3FeCN_6 is calculated from A_0-A where A_0 is the blank absorbance when the predenatured enzyme was used. Standard GlcNAc was used to calibrate the enzyme activity. One unit of chitinase activity is defined as the amount of sugar, liberated by enzymatic hydrolysis per minute, with reducing (decoloration) activity equivalent to 1 μmol of GlcNAc under the assay conditions.

The chitinolytic susceptibilities of different chitin substrates were determined by the enzyme assay method using 10 mg of each substrate (colloidal chitin, mill β-chitin, swollen β-chitin and α–chitin). The hydrolysate was centrifuged at 3000 rpm for 15 minutes and the supernatant was used for decoloration determination.

The optimum pH and temperature of the enzyme were determined by enzyme assay method using colloidal chitin (100 μL) as the substrate incubated in the citric acid/disodium hydrogen phosphate buffer (0.5 M) with pH range of 3 – 5.5, and at the temperature range of 35°C to 55°C.

Enzymatic Hydrolysis of Chitin

Squid pen chitin (5 g) was mixed with crude enzyme (110 U) and the total volume was adjusted to 200 mL by DI-water. The pH of the mixture was adjusted by 1 M acetic acid to pH 4. The mixture was incubated at 45°C for 2 days. After the incubation period, the reaction mixture was heated in the boiling water bath for 15 minutes to denature the enzyme. The mixture was filtered to remove solid residue and the filtrate was decolorized by activated charcoal. The clear filtrate obtained from filtration of the decolorized hydrolysate was concentrated to produce crude product. The light yellow crude was washed with absolute ethanol under 30 minute stirring at room temperature and filtered. White solids here obtained and dried under vacuum to produce the final product, GlcNAc.

Results and Discussion

Enzyme Preparation

Figure 1 shows chitinolytic activity secreted from the cultivation of *Aspergillus sp*. in colloidal chitin minimum media at pH 3.5 and 40°C. The highest chitinolytic activity was obtained on the 5th day of incubation. Figure 2 shows the chitinolytic activity of the crude enzyme produced from various cultivation pH. The pH in the range of 3.0-5.5 only slightly affected the chitinolytic activity with its maximum activity of 3.1 U/mL at pH 3.5.

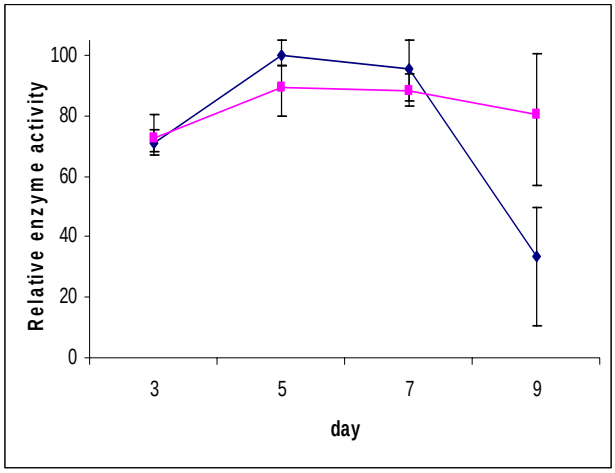

Figure 1. Chitinolytic activity of crude enzyme obtained from *Aspergillus sp*. at various incubation times. Incubation pH = 3.5 and temperature = 40°C.

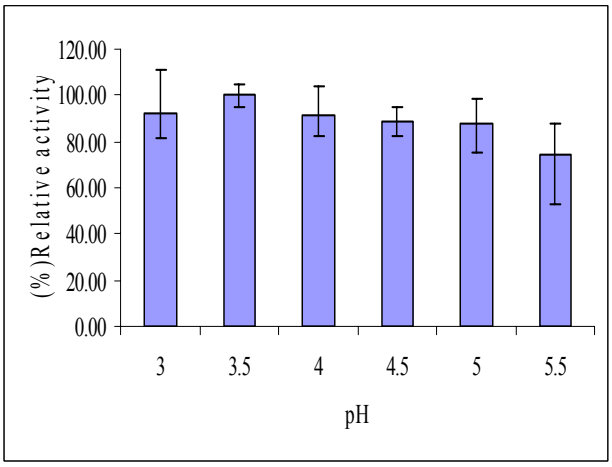

Figure 2. Chitinolytic activity of crude enzyme obtained from *Aspergillus sp*. Incubated in colloidal chitin minimum media at various pH. Incubation time = 5 days and temperature = 40°C.

Chitinolytic Susceptibility of Various Chitin Substrates

The substrate specificity of the enzyme was also tested on four types of substrates, i.e. milled β-chitin, milled α-chitin, swollen β-chitin and colloidal chitin. While milled β-chitin, swollen β-chitin and colloidal chitin exhibited relatively similar hydrolytic susceptibility toward this enzyme, milled α-chitin was a rather poor substrate for the enzyme (Figure 3).

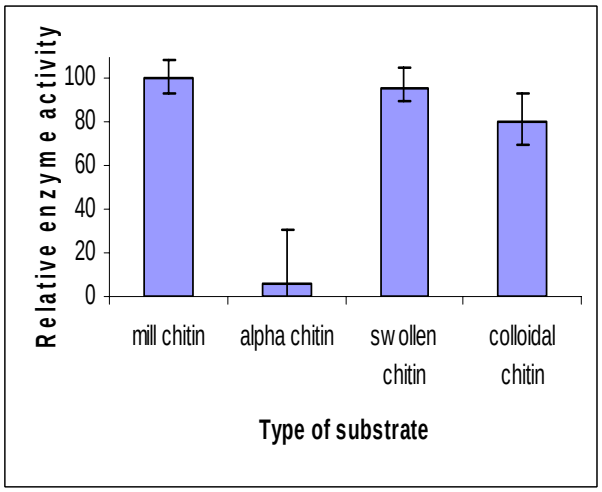

Figure 3. Chitinolytic susceptibility of various chitin Substrates toward the crude enzyme from *Aspergillus sp.;* pH = 4, temp. = 45°C, hydrolysis time = 30 min

The milled β-chitin used as a substrate for optimizing the hydrolysis condition was a fibrous material with approximately 50 μm in diameter and 100μm in length. With enzyme/chitin ratio of 22 U/g, the crude enzyme showed highest activity at pH 4 and at temperature of 50-55°C (Figure 4). Although the highest activity was observed at 50-55°C, it is important to test if the enzyme is stable at this high temperature range as it is known that most enzymes are less stable at higher temperature. The stability of the enzyme was studied by incubating the enzyme at pH 4.0 and various temperatures for 63 hrs. The chitinolytic activity of the enzyme was assayed at each time interval. The enzyme was relatively stable at 40 and 45°C retaining at least 90% of its activity up to 63 hrs (Figure 5). However the enzyme lost its activity quickly at 50°C retaining only 50% of its activity after 63 hrs. of incubation. Hence, the optimal temperature for both activity and stability should be 45°C. At the optimum pH and temperature, the hydrolysis was generally completed within 2 days as most of the chitin substrate disappeared.

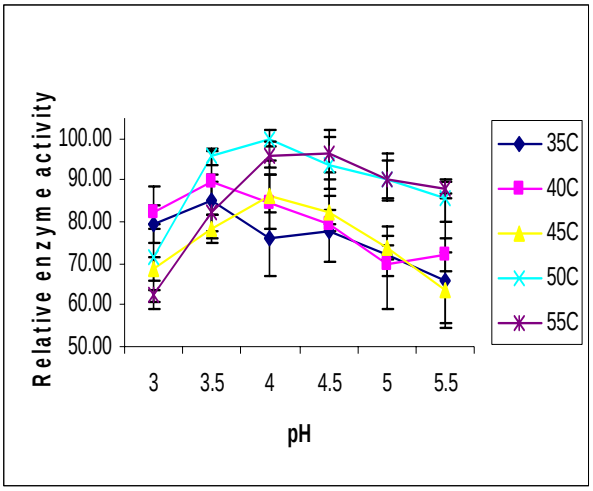

Figure 4. Relative chitinolytic activity of the crude enzyme on colloidal chitin tested at various pH and temperatures ; hydrolysis time = 30 mins.

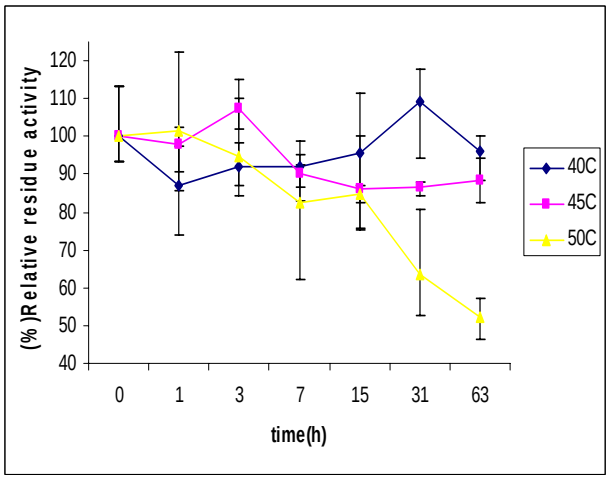

Figure 5. The time dependence of chitinolytic activity of the crude enzyme from *Aspergillus sp*. Incubated in pH 4 buffer at various temperatures showing the stability of the enzyme.

N-acetyl-glucosamine was isolated from the hydrolysate after 2 days of the hydrolysis according to the method described in the experimental section to afford GlcNAc in 65% isolated yield. The purity of GlcNAc was confirmed by ^{1}H-NMR (Figure 6) and HPLC against standard GlcNAc (Fluka). The results showed that GlcNAc isolated from the hydrolysate was over 70%.

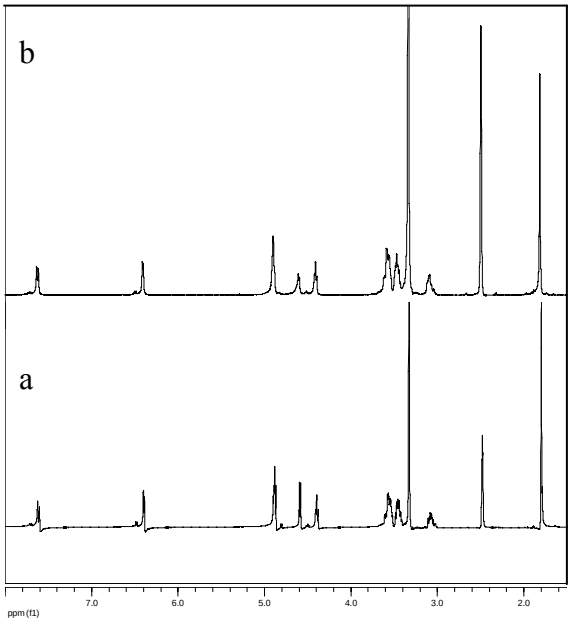

Figure 6. 1 H-NMR of GlcNAc a) standard and b) isolated GlcNAc prepared from the hydrolysis

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