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Preparation of D-glucosamine by hydrolysis of chitosan with chitosanase and β-D-glucosaminidase

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ABSTRACT

Crude enzymes including chitosanase and β-D-glucosaminidase were obtained by centrifugal separation from the fermentation broth of *Microbacterium* sp. OU01. Then the crude enzymes are used to hydrolyze chitosan for producing D-glucosamine (GlcN). The effects of temperature, pH, substrate concentration, the ratio of enzyme to chitosan, and hydrolysis time on the productivity of GlcN were discussed. The experimental result showed that the optimal conditions were temperature 50 °C, pH 5.8, substrate concentration 20 mg/mL, the optimum ratio of enzyme to chitosan 1.5 U/60 mg. Under above conditions, chitosan was completely hydrolyzed in 5 h. These results provide a scientific material for the optimization process of enzymatic production of GlcN. What is more, thin layer chromatography and high performance liquid chromatography were used to analyze hydrolytic product, which was proved to be GlcN.

1. Introduction

D-Glucosamine (GlcN) is a monomer of chitosan with 100% deacetylation degree (DDA). Many biological, pharmaceutical and nutrimental effects of GlcN have been widely reported, such as liver protective effect and membrane stabilizing activity [1], application in osteoporosis treatment [2] and wound healing promoting by enhancing hyaluronic acid synthesis [3], etc. In brief, GlcN is receiving much more attention recently because of its easy absorption and excellent properties. At present, GlcN is mostly produced by chemical hydrolysis of chitosan, and it has some disadvantages, such as low yield, acidic waste by the usage of concentrated hydrochloric acid, chemical modifications of glucose ring and so on. Although several studies have been reported on GlcN production using fungal and engineered *Escherichia coli* fermentations [2,4–7], there has been a lot of interest in enhancing enzymatic production of GlcN. Chitosanases are a group of complex hydrolytic enzymes that catalyze depolymerisation of chitosan. Chitosan (with 100% DDA) degradation can be firstly hydrolyzed by chitosanase (EC 3.2.1.99) to oligoglucosamine chains. The oligomers are subsequently degraded to GlcN monomers by β-D-glucosaminidase (EC 3.2.1.165). In this context, the crude enzymes including chitosanase and β-D-glucosaminidase can be good alternative, which could hydrolyze chitosan (with 100% DDA) into GlcN. Several reports reveal that many microorganisms are excellent producers of extracellular enzymes of chitosanase and β-D-glucosaminidase [8–12]. At present, one-step hydrolysis of chitosan (with 100% DDA) to GlcN by the crude enzymes including chitosanase and β-D-glucosaminidase has not been reported. Based on our previous research, we got the crude enzymes including chitosanase and β-D-glucosaminidase through centrifugal separation from the fermentation broth of *Microbacterium* sp. OU01 [11]. In this paper, our purpose was to find out an effective, low cost, and eco-friendly enzymatic method and optimize enzymatic hydrolysis process for the production of GlcN.

2. Materials and methods

2.1. Microorganism and cultivation conditions

In our previous research, we isolated a strain which was further identified as *Microbacterium* sp. by the morphological and biochemical properties along with 16S rRNA partial sequence analysis. The strain was deposited in our laboratory and named *Microbacterium* sp. OU01 [11].

2.2. Culture condition for crude enzymes production

For the production of crude enzymes, *Microbacterium* sp. OU01 was grown in a liquid medium (1% colloidal chitosan, 0.13% $MgSO_4 \cdot 7H_2O$, 0.14% K_2HPO_4, 0.03% KH_2PO_4, 0.5% NaCl, 0.3% yeast extract, 1.87% $(NH_4)_2SO_4$, and 0.1% glucose) in shaken flasks at 30 °C, pH 6.3, and 150 r/min for 96 h. The mixture was centrifuged at 6000 × *g* for 10 min and the supernatant (containing the crude

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enzymes) was collected and stored at −20 °C for further experiments. All the experiments were performed in three sets and the average values are being reported.

2.3. Crude enzyme activities

Chitosan with 95% degree of deacetylation was used as the substrate in the crude enzyme assay. Crude enzyme activity was determined by quantitative estimation of the reducing sugars produced from chitosan. The reaction mixture contained 0.9 mL of 1% soluble chitosan, 0.1 mL of diluted crude enzyme solution, and 1 mL of 0.2 mol/L sodium acetate buffer (pH 5.8). The reaction tubes were incubated at 50 °C for 15 min. The reaction was arrested by addition of 1.5 mL dinitrosalicylic acid reagent followed by heating for 5 min. The colored solution was then centrifuged at 10,000 × *g* for 5 min and absorption of the supernatant was measured at 590 nm along against the control (blank) with glucosamine–hydrochloride as the calibration standard. One unit of crude enzymes was defined as the amount of enzyme that could liberate 1 μmol of reducing sugar as GlcN per min under the conditions described above. Three replications were performed per analysis.

2.4. Determination of the reducing sugar content

The production of enzymatic hydrolysate was determined by using the modified dinitrosalicyclic acid (DNS) method with glucosamine–hydrochloride as the calibration standard [13]. Chitosan is hydrolyzed completely when the reducing sugar content is constant.

2.5. Analysis of hydrolytic product of chitosan

For the analysis of product released from chitosan, the reaction mixture containing 0.3 mL of chitosan and 1.4 mL of citrate phosphate buffer of pH 5.6 was incubated with 0.3 mL of crude enzyme (the content of enzyme is enough and the chitosan can hydrolyze completely) at 50 °C for 2 h and 3 h. After incubation for predetermined time, the reaction was stopped. The incubation mixture and standard GlcN were applied onto a thin layer chromatography (TLC) plate and developed by n-propanol:water:ammonia water (7:2:1, v/v/v), respectively [14]. The same reaction mixture and standard GlcN were applied on Chrompack reverse phase high performance liquid chromatography (HPLC) column. HPLC was adopted with C_{18} column. Acetonitrile–H_2O (5:95 at 0 min and pure acetonitrile at 30 min, linear gradient elution) was used as mobile phase and detective wavelength was set at the flow rate of 1.0 mL/min at 210 nm. It would be judged whether hydrolytic product of chitosan by the crude enzyme is GlcN after comparing the corresponding chromatography of TLC and HPLC.

2.6. Optimization conditions of preparation of GlcN by the crude enzyme

A typical enzymatic reaction for production of GlcN was carried out by incubating the crude enzyme and the chitosan substrate. The exact set of conditions for each experiment was specified in the footnote of each figure. At each time point, a portion of the reaction mixture was sampled and determined the reducing sugar content. The content of reducing sugar was proportional to that of hydrolytic product when chitosan was hydrolyzed completely, so we took the content of reducing sugar as the yield of hydrolytic product in optimizing the conditions of preparation of GlcN by the crude enzymes.

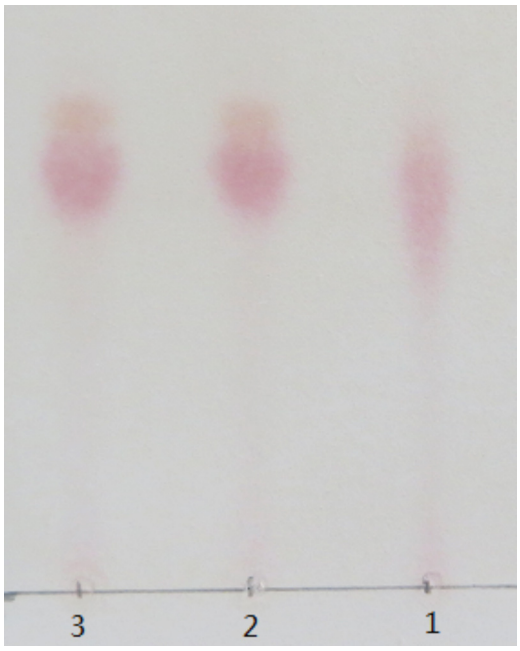

Fig. 1. TLC analysis of GlcN produced by hydrolysis chitosan by crude enzyme. Lane 1: GlcN standard; lane 2: chitosan incubated with enzyme for 2 h; lane 3: chitosan incubated with enzyme for 3 h.

3. Results and discussion

3.1. Analysis of hydrolytic product of chitosan

The hydrolytic product of chitosan was analyzed by silica TLC. After chitosan was hydrolyzed completely, hydrolytic product was detected, as indicated in TLC plate (Fig. 1). HPLC analysis also showed similar results (data not shown). At 2 h incubation, a peak corresponding to GlcN was observed in the chromatogram. With incubation time increasing (3 h), the production of GlcN also increased, as was evident by the increase in peak area of the chromatogram. This fact confirms that both chitosanase and β-N-glucosaminidase exist in the crude enzymes which hydrolyze chitosan (with 100% DDA) to GlcN by cooperation. Chitosanase is responsible for hydrolyzing polymeric chitosan chains into smaller chitooligosaccharides which are further hydrolyzed into GlcN by β-N-glucosaminidase. The similar result was reported in our previous research [11].

The production of GlcN by the crude enzymes including chitosanase and β-N-glucosaminidase was not reported at present. But the production of N-acetyl-D-glucosamine (GlcNAc) by enzymatic hydrolysis was reported. A result was reported by Sashiwa et al. [15] who found that the production of GlcNAc from chitin by using crude enzymes obtained from *Aeromonas hydrophila* H-2330. The similar result was also reported by Jamialahmadi et al. [16]. In addition, Binod et al. [17] used endochitinase and chitobiase from *Penicillium aculeatum* NRRL2129 and *Trichoderma harzianum* TUBF927, respectively, for production of GlcNAc. Their study showed that the enzyme preparation must contain both endochitinase (endochitosanase) and chitobiase (β-N-glucosaminidase) for one-step hydrolysis of chitin (chitosan) to GlcNAc (GlcN).

3.2. Effect of temperature on GlcN production

Fig. 2 shows the effect of substrate concentration on the content of reducing sugar. The optimal temperature is 50 °C. We have reported that the crude enzymes included two enzymes (chitosanase and β-D-glucosaminidase). And the optimal temperature