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Full Length Research Paper

Depolymerization of chitosan by enzymes from the digestive tract of sea cucumber *Stichopus japonicus*

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A complex of enzymes was isolated in a preparation derived from the digestive tract of sea cucumber, *Stichopus japonicus*. Hydrolysis of chitosan using this enzyme preparation decreased its molecular weight (Mw), increased its water solubility and produced water-soluble chitosan (WSC). The conditions for hydrolysis were optimized to pH 6.0, temperature 45°C, 16 mg enzyme preparation (22.08 U of chitosanase activity) in a reaction solution (500 ml) containing 5 g chitosan and total reaction time of 3 h. The Mw of hydrolyzed chitosan was 1260 Da, and the WSC content in the resulting product and the yield were 96.7 and 95.4% (w/w), respectively. The structure of WSC was characterized using Fourier transform infrared (FTIR) spectroscopy.

Key words: Water-soluble chitosan, complex enzyme preparation, sea cucumber *Stichopus japonicus*, hydrolysis.

INTRODUCTION

Water-soluble chitosan (WSC) has many advantages when compared with ordinary chitosan; these include antifungal, antibacterial and antitumor activities (Kang et al., 2007). WSC can be synthesized by either chemical or enzymatic hydrolysis. Enzymatic hydrolysis methods are more advantageous than chemical approaches, because of mild reaction conditions, high specificity, no modification of sugar rings and mass production of WSC (Akiyama et al., 1995). Many enzymes with different original specificities, such as chitinase (Sutrisno et al., 2004), chitosanase (Ming et al., 2006), cellulase (Liu et al., 2005), pectinase (Kittur et al., 2003), pepsin (Roncal et al., 2007), papain (Lin et al., 2002), neutral protease (Li et al., 2005), lipase (Lee et al., 2008), α-amylase (Wu, 2011) and glucoamylase (Pan and Wu, 2011), have been evaluated for their ability to hydrolyze chitosan.

Sea cucumber has been used traditionally as a tonic food source in China as well as other Asian countries for thousands of years (Fu et al., 2005). The major edible part of the sea cucumber is its body wall that is mainly constituted by collagen and mucopolysaccharides, both of which have active functions in nutrition (Cui et al., 2007).

Furthermore, a number of enzymes, such as protease, lipase, esterase, glycosidase (amylase, cellulose, disaccharidases, invertase and chitinase) and phosphatase (Wang et al., 2008), have been isolated from the digestive tract of sea cucumber, and some of these enzymes have been reported to hydrolyze chitosan. Thus, in this study, we aimed to prepare WSC by hydrolyzing chitosan using a complex enzyme preparation isolated from the digestive tract of sea cucumber *Stichopus japonicus.* In addition, the hydrolytic conditions were optimized for the production process using the complex enzyme preparation, the product composition was examined and the product was characterized by Fourier transform infrared (FTIR) spectroscopy.

MATERIALS AND METHODS

Live sea cucumbers were purchased from a local market (Lianyungang, Jiangsu, China). Chitosan, with a degree of deacetylation of 91.7% and Mw 4.1 × 10^5 Da, was obtained from Nantong Biochemical Co. (Jiangsu, China). All other chemicals were of reagent quality.

Preparation of complex enzyme

Each experiment involved 50 sea cucumbers; these were sacrificed

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and dissected immediately thereafter. After removing the tissue contents, the digestive tracts were separated and rinsed with cold distilled water. These digestive tracts were minced and homogenized in 100 mmol/L cold Tris-HCl buffer (pH 6.0) and the homogenate was then centrifuged at 10,000 × *g* for 20 min. The complex enzyme preparation was obtained from the supernatant by freeze drying, and then stored at -80°C.

Determination of chitosanase activity in the complex enzyme preparation

The chitosanase activity of the complex enzyme preparation was determined according to the methods described previously by Pan and Wu (2011), although with slight modifications. Chitosan was dissolved in 1% (v/v) acetic acid (HAc) to make up a concentration of 1% (w/v) in solution and the pH was adjusted to 6.0 using 1 mol/L NaOH. To this solution, 0.5% (w/v) of the crude enzyme was added and the mixture was incubated at 45°C for 30 min; then, the amount of reducing sugar released during the reaction period was measured using the 3,5-dinitrosalicylic (DNS) reagent method (Miller, 1959). One unit of enzyme was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute. D-glucosamine was used as the standard reference.

Hydrolysis of chitosan by the complex enzyme preparation

Chitosan was dissolved in 1% (v/v) HAc solution to obtain a concentration of 1% (w/v), and the pH was adjusted to 6.0 using 1 mol/L NaOH. Then, 10 mg of the complex enzyme preparation was added into a reactor containing 500 ml of chitosan solution and the mixture was placed in a thermostatic water bath at 45°C for 3 h. Aliquots of the reaction mixture were periodically sampled, filtered, heated to 95°C for 15 min to terminate the reaction, and then analyzed.

Precipitation with ethanol

The reaction mixture was filtrated, concentrated to approximately 15% (w/v) in a vacuum rotary evaporator (Jiangyin Dry Equipment Manufacture Co., Ltd, Jiangyin, Jiangsu, China) at 65°C, and then precipitated with four volumes of ethanol. The precipitates obtained were dried at 60°C for 2 h.

Analytic methods

The pH of the solution was recorded using a digital pH meter (Model: PHS-3C, CD Instruments, China). Ash, moisture, total sugar and protein content of the samples were determined according to standard methods as described previously (Hou, 2004). The reducing sugars were estimated by the method of Somogyi and expressed as the dextrose equivalent (DE) value (Nelson, 1944). DE values were used as an index of the enzymatic activities in the reaction mixture. The molecular weight of hydrolyzed chitosan was determined by the method described by Wu et al. (2009), but with slight modifications and using a high-performance gel-filtration chromatograph (LC-10A, Shimadzu, Japan) on an Ultrahydrogel size exclusion column (LKB-Prodokter, AB, Bromma, Switzerland) that was capable of detecting substances with molecular weights from 10^3 to 10^6 Da. In the size-exclusion chromatographic studies, a 0.1 mol/L HAc/0.1 mol/L NaAc eluent was used at a flow rate of 0.9 ml/min, and detection accomplished with a highly sensitive refractive index detector (Model ERC-7515 A, ERC Inc., Japan). Detector calibration was done with known concentrations of a commercially available dextran (Sigma Aldrich Fluka, USA). For the analysis, triplicate aliquots of 20 µl volume were injected into the ambient temperature column after filtration through a 0.45 µm millipore filter. The multi-channel chromatography data station (Version 144A, 1993 to 1997 Ampersand, Ltd.) was used for data collection. FTIR spectra of representative WSC samples were collected in KBr pellets on a Nicolet Nexus FTIR 470 spectrophotometer across a wavelength range of 400 to 4000 cm^{-1}.

RESULTS AND DISCUSSION

Chitosanase activity in the complex enzyme preparation

The chitosanase activity of the complex enzyme preparation was 1380 U/g. This activity was determined for chitosan; therefore, the complex enzyme preparation has been used to depolymerize β-glycosidic bonds in chitosan.

Effect of time on chitosan hydrolysis

Time-course analysis of chitosan hydrolysis by the complex enzyme preparation was carried out for a period of 3.5 h. As shown in Figure 1, DE shows a sharp increase with time for 2.5 h, a slower increase over 2.5 to 3 h, and no further increase is observed after 3 h. The optimum reaction time was thus judged to be 3 h.

Effect of pH, temperature and amount of the complex enzyme preparation on chitosan hydrolysis

The pH and temperature of the reaction mixture can play pivotal roles in the activity of the complex enzyme preparation and subsequently affect hydrolysis of chitosan. The amount of the complex enzyme preparation may also be important for efficient hydrolysis, and therefore, effect of pH, temperature and amount of the complex enzyme preparation on chitosan hydrolysis was investigated. The optimal hydrolytic conditions were determined as pH 6.0 (Figure 2) and temperature of 45°C (Figure 3) with 16 mg (22.08 U of chitosanase activity), the complex enzyme preparation in the reaction mixture (Figure 4). However, optimal conditions were reported for hydrolysis of chitosan with enzyme having chitosanase activity at pH 4.0 (Lin et al., 2002), 4.5 (Pan and Wu, 2011; Roncal et al., 2007), 5.0 (Wu, 2011), 5.3 (Liu et al., 2005), 5.4 (Li et al., 2005) and 5.6 (Lin and Ma, 2003), and temperatures of 40°C (Roncal et al., 2007), 45°C (Lin et al., 2002), 50°C (Lin and Ma, 2003; Li et al., 2005; Wu, 2011), 55°C (Lee et al., 2008; Pan and Wu, 2011) and 58°C (Liu et al., 2005) previously. These differences in reported optimal pH values and temperatures may be attributed to the differences in substrates, enzyme sources and reaction time. Although,

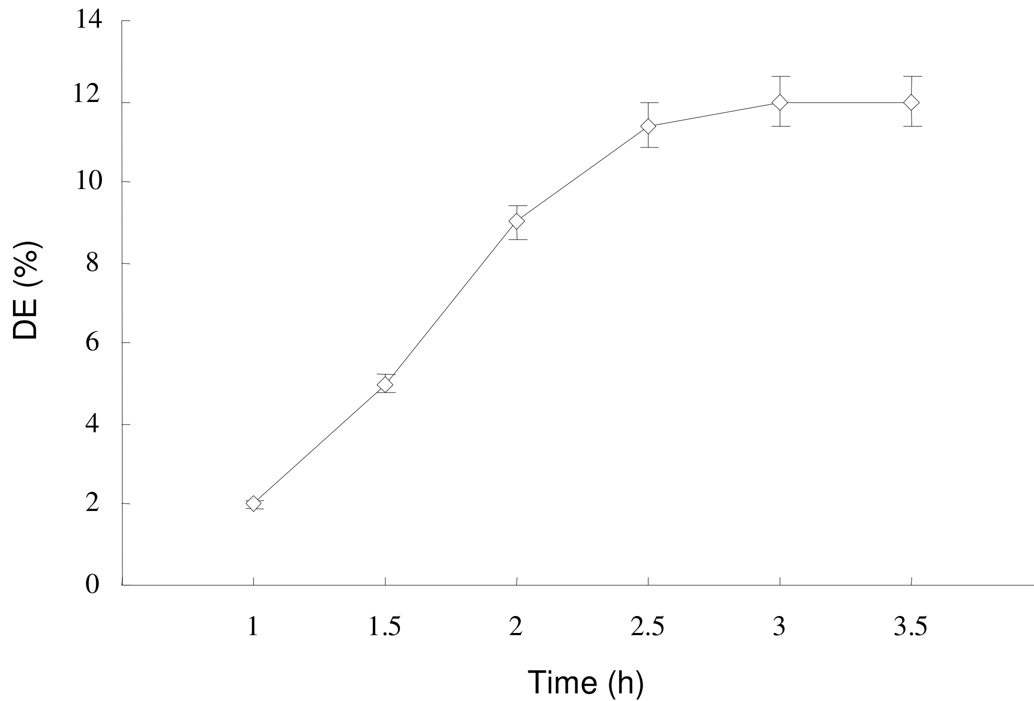

Figure 1. Effect of time on chitosan hydrolysis by the complex enzyme preparation. Data are shown as mean ± SD (n = 3).

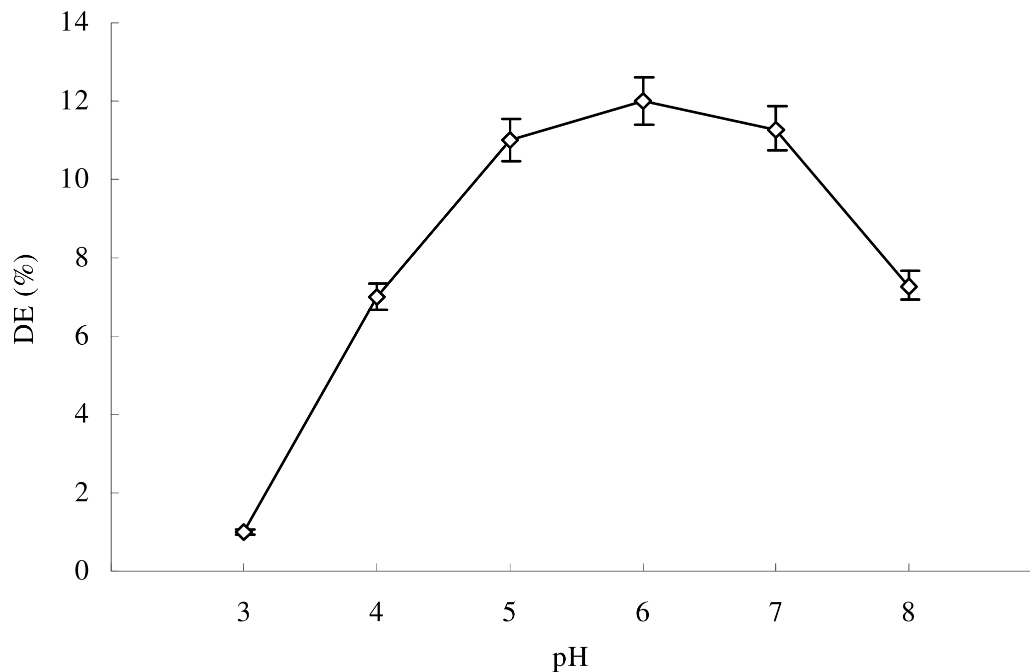

Figure 2. Effect of pH on chitosan hydrolysis by the complex enzyme preparation. Data are shown as mean ± SD (n = 3).

the maximum DE of the hydrolysate was achieved at a pH of 6.0, it was interesting to note that considerable DE values were obtained across a relatively wide pH range of 5.0 to 7.0, probably due to the nature of different

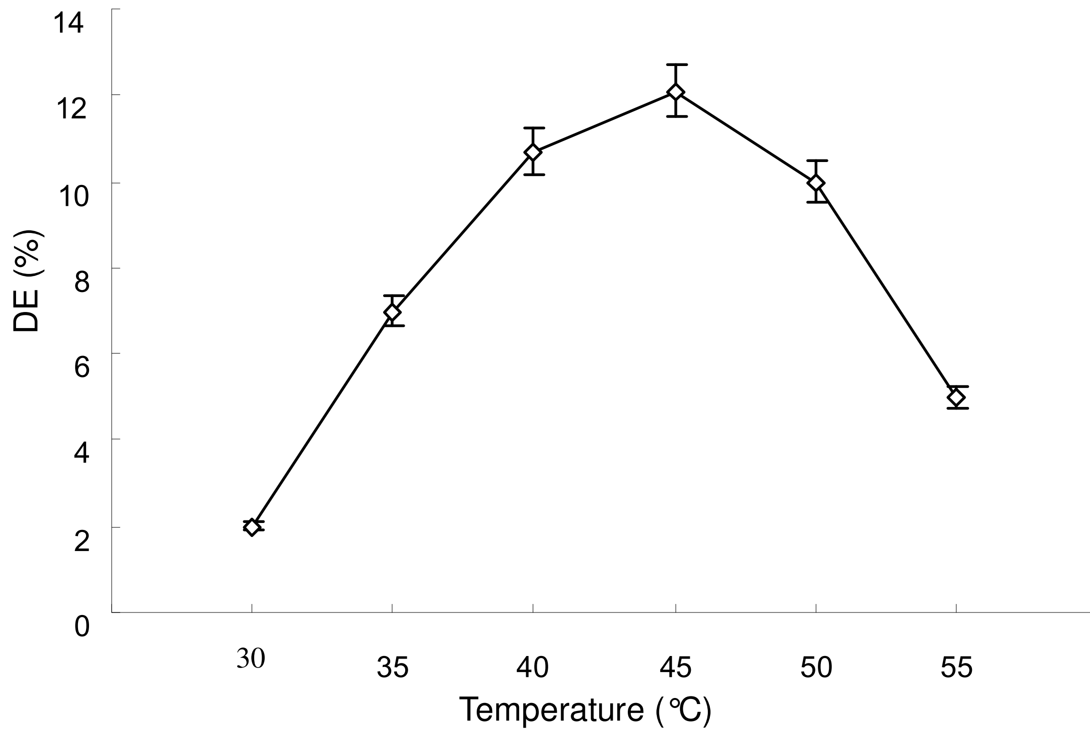

Figure 3. Effect of temperature on chitosan hydrolysis by the complex enzyme preparation. Data are shown as mean ± SD (n = 3).

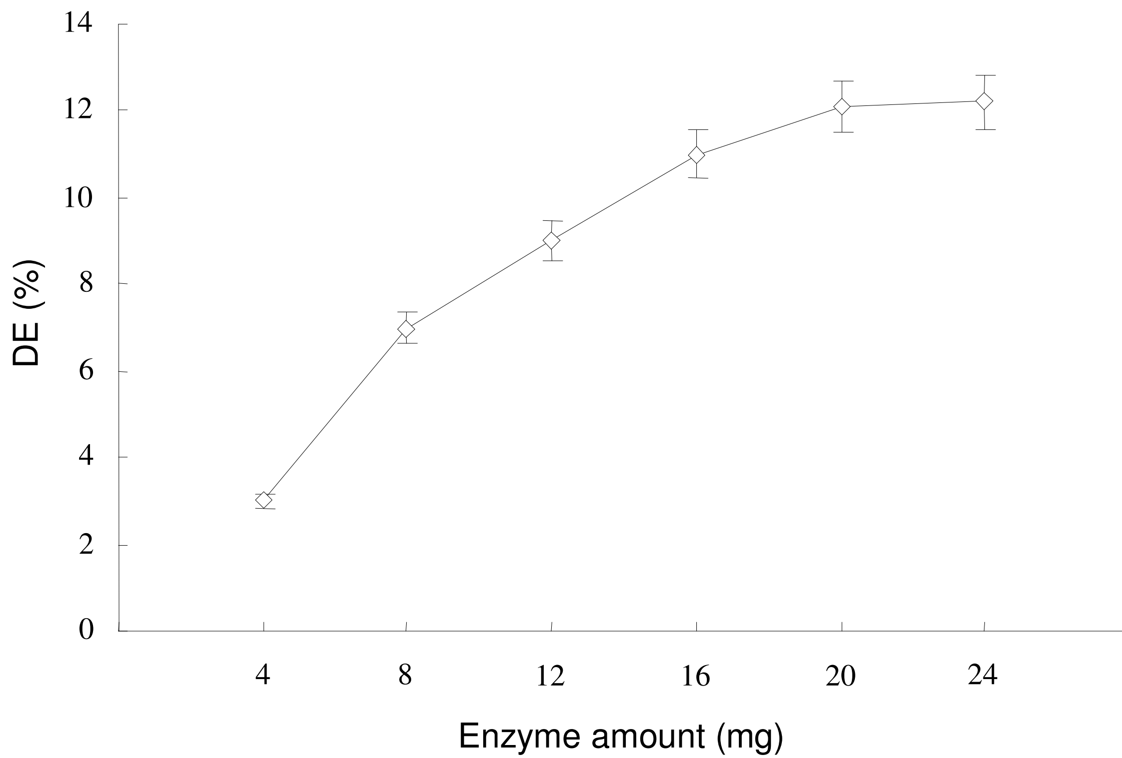

Figure 4. Effect of enzyme amount on chitosan hydrolysis by the complex enzyme preparation. Data are shown as mean ± SD (n = 3).

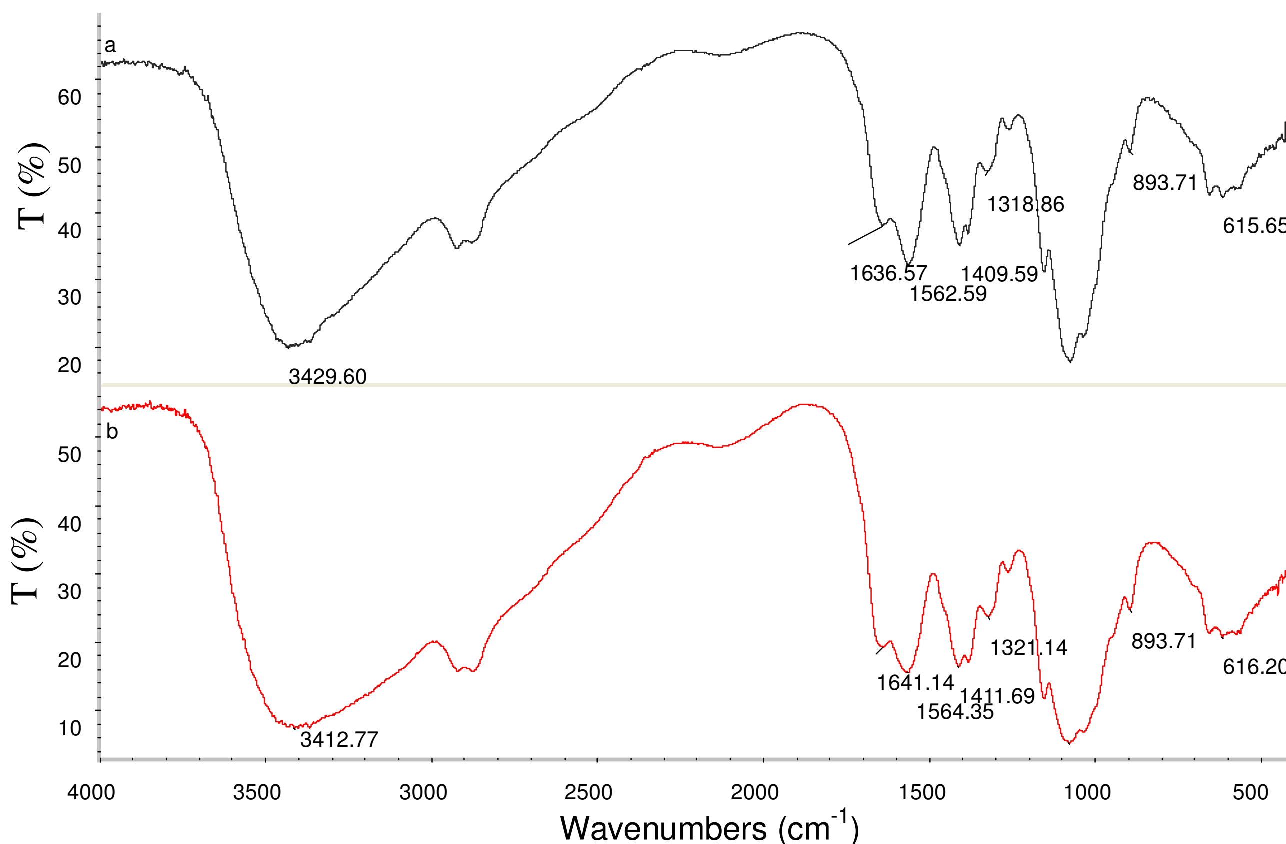

Figure 5. FT-IR spectrum of original chitosan (a) and WSC (b).

enzymes having variable chitosanase activity.

Characterization of the product

The FTIR spectrum of the WSC product obtained during this experiment and the original chitosan are almost identical, indicating that the major composition of this product is chitosan (Figure 5). The molecular weight of chitosan decreased to 1260 Da under optimal hydrolytic conditions. The composition of ash, moisture and total sugar were 0.2, 3.2 and 96.7% (w/w), respectively. Therefore, the purity of the WSC product was very high, and the yield of WSC was 95.4% (w/w). All product samples were white powders and soluble in alkaline and acidic water solutions as well as in pure water.

Conclusions

WSC can be prepared by hydrolyzing chitosan using a complex enzyme preparation isolated from the digestive tract of the sea cucumber, *S. japonicas*, under optimal reaction conditions of pH 6.0, temperature 45℃, with 16 mg complex enzyme preparation in 500 ml of reaction mixture (22.08 U of chitosanase activity), and reaction time of 3 h, to obtain maximum DE. The products of this reaction comprised of chito-oligosaccharides. The WSC content in the product and the WSC yield were 96.7 and 95.4% (w/w), respectively. All product samples were white powders and soluble in alkaline and acidic water solutions as well as in pure water.

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