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Immobilization of a protease on modified chitosan beads for the depolymerization of chitosan

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ABSTRACT

Neutral protease was immobilized on chitosan (CS), carboxymethyl chitosan (CMCS), and *N*-succinyl chitosan (NSCS) hydrogel beads. And the biocatalysts obtained were used to prepare low molecular weight chitosan (LMWC) and chitooligomers. Weight-average molecular weight of LMWC produced by neutral protease immobilized on CS, CMCS and NSCS hydrogel beads were 3.4 kDa, 3.2 kDa and 1.9 kDa, respectively. The effects of immobilization support and substrate on enzymatic reaction were analyzed by measuring classical Michaelis–Menten kinetic parameters. The FT-IR, XRD and potentiometric determination results indicated decrease of molecular weight led to transformation of crystal structure, but the degree of *N*-deacetylation and chemical structures of residues were not changed compared to initial chitosan. The degree of polymerization of chitooligomers was mainly from 2 to 7. We observed a strong dependence of the immobilized enzyme properties on the chemical nature of the supports, which leads to different microenvironment of neutral protease and changes the hydrolyzing process.

1. Introduction

Chitosan's biofunctionalities are highly related to its molecular weight and degree of *N*-deacetylation (DD). Fairly recently, evidence is beginning to accumulate that the chitosan with molecular weight ranging 5–30 kDa were shown to possess superior biological activities compared to chitosan. Low molecular weight chitosan (LMWC) of 5–10 kDa were shown to have potential as DNA delivery system (Richardson, Kolbe, & Duncan, 1999). LMWC of 20 kDa were shown to prevent progression of *Diabetes mellitus* and had high affinity for some bacterial lipopolysaccharides than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). Tsai, Wu, and Su (2000) proposed the practical use of LMWC in the milk preservation and oral hygiene. And Tomida et al. (2009) reported that LMWC (<30 kDa) have impressive antioxidant ability to scavenge hydroxyl radicals and to reduce cupric ions than high molecular weight chitosan. Several studies revealed the anti-tumor potential of chitosan was dependent on molecular weight (Harish Prashanth & Tharanathan, 2005).

Generally, low molecular weight chitosan can be obtained by chemical means using HCl, H_2O_2, and $NaNO_2$ that, however, do not lend themselves to easy reaction control and often results in modification of the products. Physical means using sonication and shearing that require special equipment. And by enzymatic means, based either on specific enzymes, such as chitosanases (Hsiao, Lin, Su, & Chiang, 2008) or on unspecific enzymes, including cellulase (Qin et al., 2004), lysozyme (Lin, Lin, & Chen, 2009), lipase (Muzzarelli, Xia, Tomasetti, & Ilari, 1995), amylase (Wu, 2011), papain (Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002; Terbojevich, Cosani, & Muzzarelli, 1996), pepsin (Roncal, Oviedo, Armentia, Fernández, & Villarán, 2007) and pectinase (Shin-ya, Lee, Hinode, & Kajiuchi, 2001).

Among these enzymes, neutral protease, a kind of nonspecific enzyme, had been found to be able to hydrolyze chitosan efficiently and obtain LMWC with different weight-average molecular weight easily by prolonging the duration (Li, Du, & Liang, 2007; Li et al., 2005). But the use of enzymatic means has been limited due to their unstable nature and the resulting requirement of stringent conditions, such as a particular pH and temperature. And in hydrolysis reactions, purified enzymes can be rather costly and to discard them after each use is not economical. The application of LMWC and COS, which obtained by such enzymatic hydrolysis for biochemical and food area, is limited as a result of an undesirable level of chitosan pyrogenicity caused by the presence of protein of enzyme admixtures (Ilyina, Tikhonov, Albulov, & Varlamov, 2000). The utilization

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of immobilized enzyme offers advantages over free enzyme for the preparation of LMWC and COS free of protein admixtures and is more suitable for biomedical and food applications.

Chitosan and its derivatives are known as ideal support materials for enzyme immobilization (Juang, Wu, & Tseng, 2002; Muzzarelli, 1980) because of their many characteristics like improved mechanical strength, resistance to chemical degradation, avoiding the disturbance of metal ions to enzyme, and antibacterial property and so forth on. And at the same time, using chitosan and its derivatives hydrogel beads as immobilized carrier decreased the contamination of impurity and simplified the purification process (Orrego, Salgado, Valencia, Giraldo, & Cardona, 2010).

It is widely known that immobilization can change, e.g. the accessibility of the enzyme to the substrate or even the affinity of the enzyme towards a specific substrate due to change in the local environmental or altering the mobility of the protein. And immobilization may affect enzyme activity in a way that depends on the enzyme, substrate, products of the reaction, immobilization method and support (Akkava, Sahin, Demirel, & Tümtürk, 2009; Pessela et al., 2007).

The purpose of this paper, therefore, is to study the effect of immobilized support and enzymatic reaction substrate on the catalytic properties and hydrolysis process of the neutral protease immobilized on chitosan (CS), carboxymethyl chitosan (CMCS) and *N*-succinyl chitosan (NSCS) hydrogel beads.

2. Experimental

2.1. Materials

Chitosan was obtained from Aoxing Ocean Biochemical Co. (Zhejiang, China). The chitosan with the DD 75.3% and weight-average molecular weight (M_w) 410 kDa, as initial material from crab shells, was used to prepare the CMCS and NSCS. The chitosan (CS1) with a DD of 91.7% and M_w of 286 kDa, as an initial material from shrimp shells, was used to prepare chitosan with different DD and as the substrate to prepare LMWC. Chitosan with different DD were prepared according to the reference (Chen & Hua, 1996) and CS2 (DD 80.8%) and CS3 (DD 63.2%) were obtained. D-Glucosamine HCl was purchased from Seikagaku Corp. (Japan). All other chemicals were of reagent grade.

The neutral protease, derived from *Bacillus subtilis* As 1.398, was a product of Ningxia XiaSheng Industry Co. (China). The enzyme purification was achieved according to the reference (Su, Wang, Yao, & Yu, 2006) and modified as following: the enzyme solution was supplemented with solid ammonium sulfate to achieve 80% saturation under constant stirring. The solid enzyme was collected by centrifugation and redissolved in water. The solution was then loaded onto the Sephadex G-25 column and deionized water was used as the eluent. The eluate was monitored for protein by measurement of the absorbance at 280 nm. The main active fraction was collected as the purified neutral protease solution, followed by a concentration treatment with PEG-20000. Then the concentrated enzyme solution was lyophilized to get neutral protease powder. Neutral protease purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight of enzyme was calculated using protein markers. The SDS-PAGE (Figure not shown) showed a single band and molecular weight calculated was 33.8 kDa.

2.2. Preparation of CMCS

CS (10 g) suspended in 50% (w/w) NaOH (12 mL) was kept at −20 °C overnight. The frozen alkali CS was unfrozen naturally and then transferred to 2-propanol (120 mL). The mixture was stirred at room temperature completely and $ClCH_2COOH$ (12 g) was added slowly. After the mixture was stirred at room temperature for 2 h, heat was applied to bring the reaction mixture to 60 °C for another 2 h. After the reaction, the mixture was filtered to remove the reaction solvent, and the precipitate was dissolved in deionized water. Next, acetic acid was added to the solution to adjust the pH to 7.0. After it was dialyzed against deionized water for 3 days, the CMCS salt was filtered, and the filtrates were concentrated to about one-twentieth with a rotary evaporator under reduced pressure. The synthesized CMCS was precipitated and washed with ethanol and then collected after it was dried over phosphorus pentoxide *in vacuo*.

2.3. Preparation of NSCS

CS (10 g) was treated with 200 mL of 5% (v/v) acetic acid and stirred at room temperature. To the viscous solution formed, 800 mL of methanol was added. After the solution was stirred for 1 h, succinic anhydride (30 g) was added to the CS solution. The reaction mixtures became a gel within 30 min. After the solution was stirred for 24 h, the reaction mixture (containing a gel) was filtered to remove methanol and was then dispersed in 400 mL of deionized water. To obtain a sodium salt of the product, and adequate amount of NaOH was added to the reaction mixture to give a clear solution of pH 8–10. The solution was dialyzed against deionized water for 3 days and then filtered. The filtrates were concentrated to about one-twentieth with a rotary evaporator under reduced pressure and precipitated by the addition of ethanol. The precipitates were collected after they were over phosphorus pentoxide *in vacuo* to get NSCS.

2.4. Preparation of hydrogel beads

An amount of CS (3 g) was completely dissolved in 100 mL of 1% (v/v) acetic acid. This solution was extruded through a syringe needle into 125 mL of distilled water containing 15 g of NaOH and 25 mL of ethanol under stirring to form spherical gels. The solution was allowed to stand for 3 h; the spherical gels that formed the wet CS beads were removed by filtration and rinsed with distilled water until neutrality was reached. The beads had an average diameter of 2.7 mm.

CMCS and NSCS powder (3 g) were completely dissolved in 100 mL of distilled water. The mixture was extruded drop by drop with a syringe needle into 150 mL of 1% (w/v) $CaCl_2$ solution containing 50 mL of ethanol under stirring to form beads. The beads were allowed to harden in the $CaCl_2$ solution for 3 h. CMCS and NSCS hydrogel beads had average diameters of 1.5 and 2.2 mm, respectively.

2.5. Immobilization of neutral protease

For the activation of the chitosan and its derivatives supports, wet chitosan and its derivatives hydrogel beads (2 g) were treated with 10 mL of GA solution in a shaker for 12 h at room temperature. After they were washed with distilled water until the GA in the washings was not determined at 245 nm, the crosslinked chitosan and its derivatives hydrogel beads were stored at 4 °C until use. The GA-activated chitosan and its derivatives hydrogel beads were then immersed in 10 mL of 0.1 M citrate–phosphate (C–P) buffer solution containing a given amount of neutral protease, and the mixture was gently shaken for 4 h at 25 °C. The supernatant was removed, and the resulting chitosan and its derivatives hydrogel beads were washed with 0.1 M C–P buffer until the protein in the washings was not detected at 280 nm. The immobilized neutral protease were recovered from the solution and then stored at 4 °C. The