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Development of Coencapsulating Technology for the Production of Chitosanoligosaccharides

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Abstract To easily separate chitosanoligosaccharides by size exclusion, an coencapsulating technology of substrate and enzyme was developed. The membrane was composed of alginate and a divalent cation such as calcium. Chitosan and chitosanase were enveloped in this membrane and the product released to medium by size exclusion. The capsule was stabilized in a 2% acetic acid solution (pH 5.0) containing 0.145 M $CaCO_3$. The leakage of substrate caused by the agitation speed was controlled by increasing alginate and $CaCO_3$ concentrations. The lower limit of the alginate concentration and the agitation speed were 0.5% and 40 rpm, respectively. Membrane thickness and capsule diameter were 10 μm and 2.5 mm, respectively. By TLC analysis, the composition of chitosanoligosaccharides were mainly 3-6 mers. The molecular weight distribution of the released oligosaccharides ranged from 262 to 3624 Da by GPC.

Keywords : chitosanoligosaccharide, coencapsulation, size exclusion

INTRODUCTION

Many biological polymers have been used for the immobilization and encapsulation of enzyme and mammalian cell, because of their advantages in biotechnology, biochemistry and medicine [1]. Encapsulation technology was mainly used for obtaining high cell density and separating substrate and enzyme. The encapsulation is based on the electrostatic interaction of a polymeric anion and a polymeric cation. Alginate, a natural polysaccharide extracted from brown algae, contains glucuronic and mannuronic acids. Alginate networks can be formed by divalent cations such as calcium and barium [2]. One of the important functions of the biopolymer membrane is its size exclusive separating ability. It was reported previously that the use of microencapsulated hybridoma cells in bioreactors allowed higher cells densities compared with suspension cell culture [3], and a higher purity of product [4], and facilitated the separation of cells from the medium. In addition, the product yield was increased [5] and easily separated. The above mentioned application requires microcapsules with a stable and reproducible size exclusion [6]. So, several attempts to modify the size exclusion ability of membranes have been performed [7]. However, this encapsulating technology was applied to isolated enzyme or cells in capsule. In the case of a biopolymer digesting system, the separation of the final product and substrate is very difficult. Chitosan is a deacetylated material of chitin obtained from natural resources. It has many biological activities such as, antimicrobial, anticancer, and immunostimulating, but it only dissolves in acidic condition. Its hydrolysates, chitosanoligosaccharides, have similar biological activity and are dissolved in neutral conditions. Therefore, the requirement for chitosanoligosaccharide as a food additive and healthy material is increased. Generally, chitosanoligosaccharides are produced by treatment of chitosan with acid. This method produces mainly chitobiose. Recently, chitosanase from microorganisms was used for the production of chitosan oligomer. In this case, the separating process of chitosan oligomer, chitosan, and enzyme, is very difficult and complex, and also, the enzyme is very expensive. So, we developed another encapsulating technique, coencapsulation, for the easy separation of substrate and enzyme from product. The objective of this study was the development of a coencapsulating method of substrate and enzyme in an alginate capsule, and allow release of product into the medium by its molecular weight. In this system, the substrate was chitosan and the enzyme chitosanase. The stability of the prepared capsules, influence of the agitating speed, and other conditions of the reaction system were also investigated.

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MATERIALS AND METHODS

Materials

Chitosan (M.W. = approximately 20,000, DAC = 81%,

cps = 2.9) was obtained from Sehwa Co. (Yosu, Korea). Sodium alginate (approx.= 3,500 cps) was purchased from Sigma. Chitosanase (from *Bacillus* sp. GM44, Endo-type) was obtained from Dr. Yong-chul Shin, Gyeongsang National University, Chinju, Korea. All other biochemical reagents were of analytical grade. The optimal pH and temperature of chitosanase were 5.0 and 40°C, respectively [8]. Chitobiose, chitotriose, and the other chitosanoligosaccharides were purchased from Seikagaku, Tokyo, Japan.

Substrate Leakage Estimation at Various Alginate Concentrations and Agitation Speeds

Ten milliliter of 4.0% (w/v) chitosan solution (pH 5.0) containing 0.05 M $CaCl_2$ was prepared, and then drawn into a 15 mL disposable syringe with a 24, 3/4 (0.48 mm) gauge needle. The solution was dropped into 100 mL of 0.2-0.8% (w/v) sodium alginate solution (pH 5.0) at 4°C with stirring. The capsules were allowed to harden for 10 min. They were then washed with distilled water, and transferred into a 2% acetic acid solution (pH 5.0) under 40°C. The leakage of substrate from the capsules according to alginate concentration and agitation speed (30, 40, 50, 60 rpm) was determined using the DNS assay method. Samples were taken up to 16 h at regular intervals and the amount of substrate released from the capsules determined. One mL of sample was mixed with 1 mL of enzyme solution (0.2 unit/mL). The mixture was incubated at 50°C for 10 min in shaking water bath. The reaction was stopped by the addition of 66 μL of 10 N NaOH. The reaction mixture was centrifuged at 3,000 g for 20 min. Reducing sugars in the supernatant were determined by the DNS method [9].

Stability of the Prepared Capsules According to pH Adjusting Salt Solutions

Stability of capsules containing 4% of chitosan solution was performed in 2% acetic acid solution at 40°C. The pH of this reaction reagent was adjusted at pH 5.0 by the addition of 10 N NaOH, 0.145 M $CaCO_3$, 0.01 M NH_4OH, and 14.8 M NH_4OH. The molecular weight of chitosan was about 20.0 kDa. The leakage of substrate was determined by the above DNS method, and also, enzyme itself was encapsulated using the above method. The leakage of enzyme was estimated by the use of substrate solution. One unit of the enzyme was defined as the amount of enzyme producing 1mole of reducing sugar per min; D-glucosamine was used as the standard.

Coencapsulating Technology

Ten milliliter of 2.0-4.0% (w/v) chitosan solution (pH 5.0) containing 0.05 M $CaCl_2$ was mixed with 20 unit of enzyme solution at 4°C, and with other enzyme concentrations. The mixture was dropped into 100 mL of 0.5-0.8% sodium alginate solution (pH 5.0) at 4°C with steady stirring. The dropping instrument was a 15 mL disposable syringe with a 24, 3/4 (0.48 mm) gauge needle. The capsules were allowed to harden for 10 min, washed with distilled water, and transferred into a 2% acetic acid solution (pH 5.0). Membrane thickness and capsule diameter was measured using an Olympus IX50 microscope, and was 10 μm and 2.5 mm, respectively. The enzyme reaction was operated at 40 rpm and 40°C. The amount of chitosanoligosaccharides was measured DNS and the composition of chitosanoligosaccharides was measured by TLC.

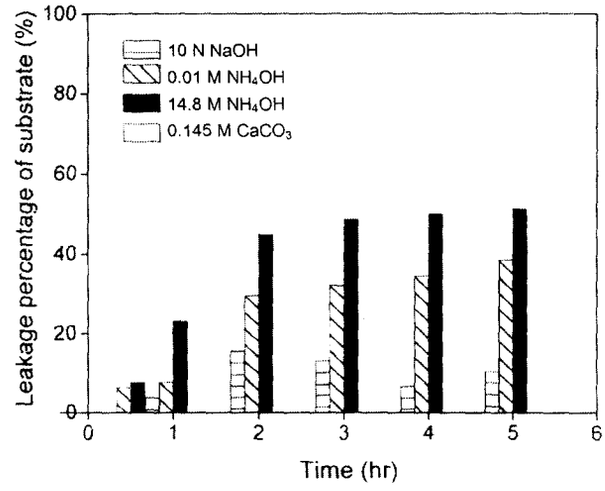

Fig. 1. Leakage percentage of substrate by pH in adjusting salt solutions.

Analysis of the Composition and Average Molecular Weight of Product

Thin layer chromatography (TLC) was carried out using a silica gel plate (silica 60 F254, Merck, USA) with *n*-propanol:ammonia water (32%) = (2:1, v/v). The spots were made visible by spraying with water-saturated *n*-butanol containing 0.1% ninhydrin, and by heating for 10 min at 160°C. The average molecular weight of oligosaccharide was determined by GPC system. The flow rate of the gel permeation chromatographic system (GPC, Shimadzu class-LC10A, Shimadzu Inc., Japan) was 0.6 mL/min and the delivery solvent was 5% acetic acid (pH 4.0). The column was a Ultrahydrogel 250 (Waters Inc., USA) and RI detector was used. The estimating standards were dextran series (M.W. 180, 1,500, 6,000, 11,300, 71,000, Fluka Inc., USA).

RESULTS AND DISCUSSION

Stability of Capsule according to pH Adjusting Salt Solutions

We developed the coencapsulating technique of chitosan and enzyme. By this technique, chitosanoligosac-

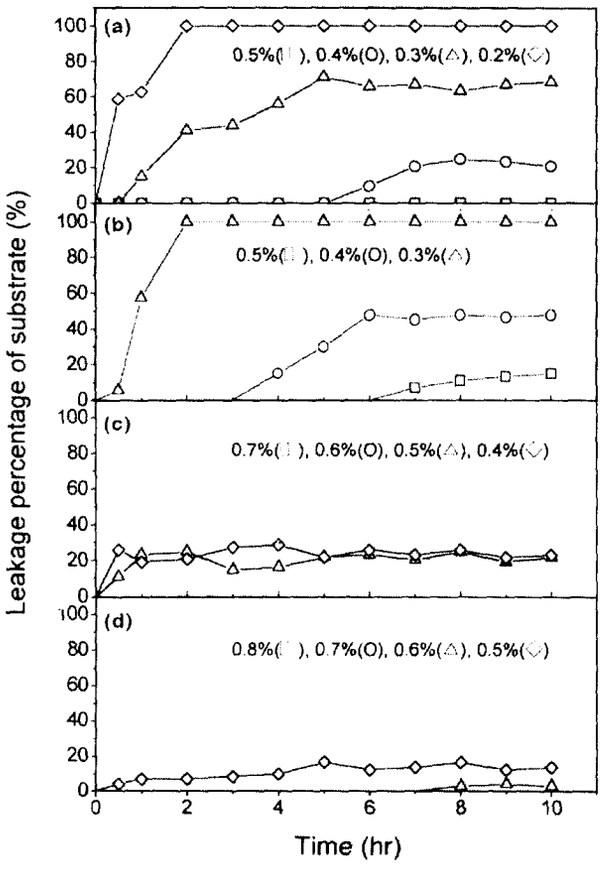

Fig. 2. Leakage percentage of substrate by alginate concentration and agitation speed. (a) 30 rpm, (b) 40 rpm, (c) 50 rpm, (d) 60 rpm.

charides were released into the capsule medium, and both enzyme and unreacted substrate remainded in the capsule. However, the holding ability of the capsule for substrate and enzyme is influenced by environmental conditions. So, we experimented with the leakage of substrate and enzyme according to the reaction solution properties. The optimum conditions of pH and temperature were pH 5.0 and 40°C, respectively. By Lee's experiment [10], the release rate of guaifenesin from a chitosan and alginate capsule was minimized at pH 4.8. Therefore, various pH adjusting salt solutions were tested for minimizing the leakage of substrate and enzyme from capsule made of alginate. Chitosan was encapsulated with alginate and this capsule was poured into 100 mL of a 2% acetic acid solution at 40°C. pH was adjusted to 5.0 by the addition of 10 N NaOH, 0.145 M $CaCO_3$, 0.01 M NH_4OH, and 14.8 M NH_4OH. Fig. 1 shows the leakage % of substrate according to the pH adjusted salt solutions. After 5 h, the leakage % was about 15% in 10 N NaOH, and about 50% in 14.8 M NH_4OH. However, the leakage of substrate was apparent after the addition of 0.145 M $CaCO_3$. The reason is capsule at above 0.2% of alginate.

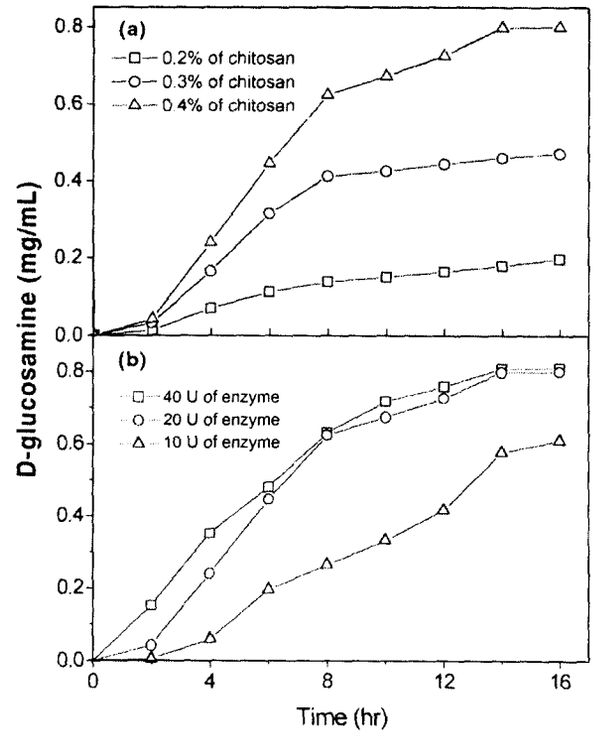

Fig. 3. The production of chitosanoligosaccharide by the coencapsulation system. (a) substrate concentration, (b) enzyme concentration.

Substrate Leakage at Various Alginate Concentrations and Agitation Speeds

To optimize the reaction conditions, substrate leakage from capsule at various alginate concentrations and agitation speeds was investigated. The capsules were prepared with 0.2-0.8% of alginate in 2% acetic acid solution (pH 5.0). The leakage % of substrate was determined by incubation at 40°C with agitation (30, 40, 50, 60 rpm). Fig. 2 shows the leakage % according to alginate concentrations and agitation speeds. The capsules made of 0.5% alginate did not show leakage of substrate over 10 h at 30 rpm and 6 h at 40 rpm. However, the leakage of substrate was significantly increased above 40 rpm. Capsules prepared with 0.6% of alginate also did not show the leakage of substrate at 10 h at 50 rpm and 8 h at 60 rpm. In the case of capsules prepared with 0.8% alginate, no leakage of substrate occurred after 10 h at 60 rpm. Above 60 rpm, capsules were significantly damaged by collision and agitation impact. So, we determined that the upper agitation limit was 60 rpm. And also, we determined the lower alginate concentration limit and agitation speed were 0.5% and 40 rpm. Because the leakage of substrate was only 15% after 10 h at 0.5% of alginate and 40 rpm. This leakage % was 18% at 20 h (data not shown).

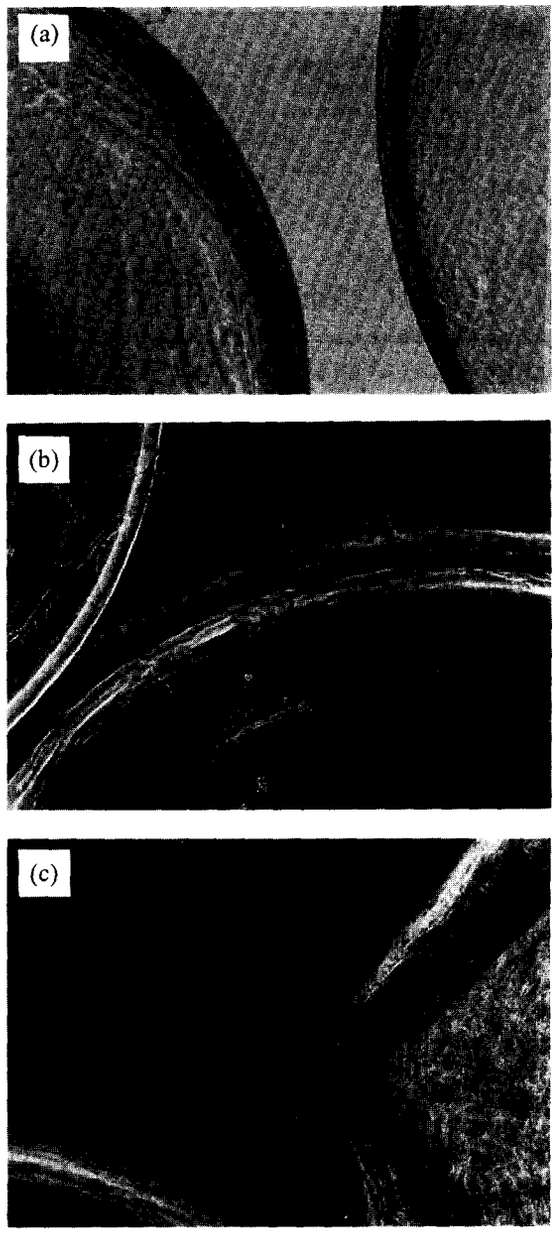

Fig. 4. Morphology of capsules at 0 h (a), 16 h (b, c).

Chitosanoligosaccharide Production by Coencapsulation System

Various concentrations of chitosan and chitosanase were coencapsulated with 0.5% of alginate. The agitation speed was 40 rpm. The production of chitosanoli that $CaCO_3$ adjusts pH and consolidates the alginate network. The enzyme is also encapsulated in the alginate. In this case, the enzyme was not released from the gosaccharide according to various concentrations of chitosan and chitosanase (20 unit) is shown in Fig. 3(a). It shows that the final concentration of chitosanoligosaccharide was 0.197 mg/mL at a chitosan solution final

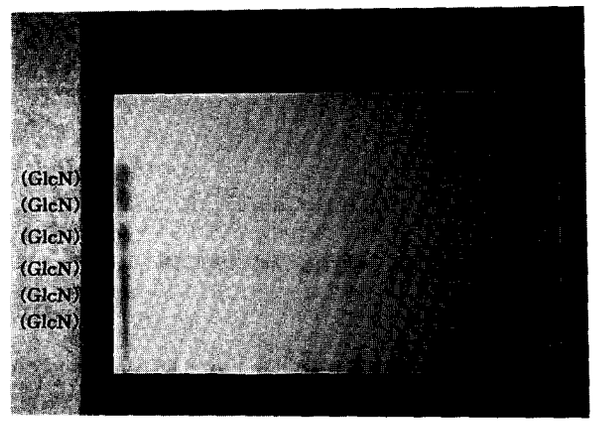

Fig. 5. Chitosanoligosaccharide compositions. 2%; 1(6 h), 2(8 h), 3(10 h), 3%; 4(6 h), 5(8 h), 6(10 h), 4%; 7(6 h), 8(8 h), 9(10 h). S; standard.

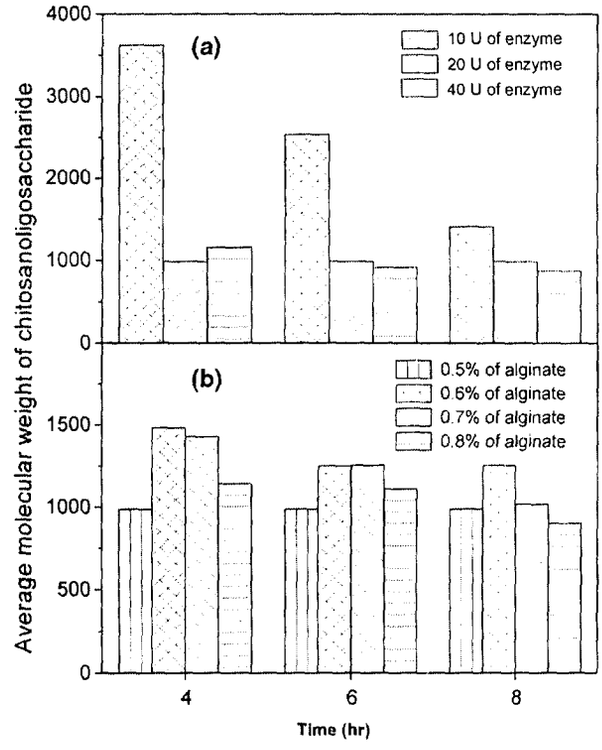

Fig. 6. Molecular weight distribution according to the enzyme (a) and alginate (b) concentration.

concentration of 2%, 0.471 mg/mL for 0.3% of chitosan solution, and 0.799 mg/mL for 0.4% chitosan solution. The production of chitosanoligosaccharide according to various concentration of enzyme is shown in Fig. 3(b). It shows that the final concentration of chitosanoligosaccharide appeared at 0.609 mg/mL at 10 U of enzyme solution, 0.799 mg/mL at 20 U of enzyme solution, and 0.808 mg/mL at 40 U of enzyme solution. To demonstrate the stability of capsules under the reaction condi-

tions, the morphology of capsules is shown (Fig. 4) at 0 h (a), 16 h (b), (c). In this case, the amount of capsule collapse increased at higher concentrations of chitosan. The reason may be that the hardness of the capsules is increased as chitosan concentration in increased. So, capsules became sensitive to collision impacts. By TLC analysis, the composition of chitosanoligosaccharides was mainly 3-6 mers (Fig. 5). GPC gave average molecular weight of oligosaccharides from 262 to 3,624 Da. Fig. 6(a) shows the molecular weight distribution according to enzyme concentration. In the case of 10 U of enzyme per reaction volume, the molecular weight of chitosanoligosaccharide was 3,624 at 4 h, 2,541 at 6 h, and 1,413 at 8 h. When 20 U and 40 U of enzyme were used per reaction volume, the molecular weight of oligosaccharide was about 1,000. As a result, low concentrations of enzymes produced high molecular weight oligosaccharides. However, in the case of Fig. 6(b), the molecular weight difference according to alginate concentration was of little significance.

In summary, coencapsulation of chitosan and enzyme offer the easy separation of product (oligosaccharide) and enzyme, and it is possible that encapsulated enzymes are reusable.

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