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Partial Depolymerization of Chitosan with the Aid of Bromelain

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Abstract: Commercial bromelain was applied in the study to partially depolymerise chitosan to produce oligochitosan. The enzymatic reactions were determined by the liberation of reducing sugar and the reduction in viscosity. The suitable reaction conditions for reaction was found at 2 h, pH 5, 1.0% w/v substrate, 7% w/w enzyme and 50°C. At this optimum condition, bromelain was able to reduce the viscosity of chitosan to 84%. The freeze-dried oligochitosan obtained was off-white in colour. The hydrolysate is easily solubilised in water (at room temperature), up to 1% w/v, gaving a clear solution. At higher concentration, suspension started to form and marked precipitation begin to appear at a concentration of 1.5%.

Key words: Chitosan, partial depolymerization, protease, oligochgitosan, viscosity, solubility

INTRODUCTION

Chitosan [β(1,4)-2-amino-2-deoxy-D-glucan] is a unique polysaccharide obtained from deacetylation of chitin [β(1,4)-2-acetamido-2-deoxy-D-glucan]. Its functions and physicochemical properties depend on the degree of deacetylation and polymerization[1]. Chitosan dissolves in aqueous solution of organic acid and forms a high-viscous solution. But, it becomes insoluble when the pH increases to about 6.5. For example, the viscosity of 0.2% chitosan solution prepared by dissolving chitosan in a 0.8% sodium acetate and 1.2% acetic acid was found as high as 9-16 (μsp/c). Therefore, it is difficult to prepare a high-viscous chitosan solution[2].

Chitosan is an important natural biopolymer. Its applications are versatile in food processing, agriculture, biomedication, biochemistry, sewage effluent treatment and can be used as membrane and microcapsule, because of its biological activity, biocompatible and biodegradable with low toxic[3]. However, its utilities in those fields are limited by its insolubility in water because of its high molecular weight[4]. Chemical or enzymatic degradation of chitosan polymer is able to produce low molecular weight and water-soluble oligochitosan[5]. Oligochitosan is very useful because of its specific biological activity, such as antifungal, antibacterial[6], antitumour, immunity effect to cancer[7] and protection from infection of some pathogen in rat[8].

Oligochitosan can be prepared by partially hydrolysing the chitosan with concentrated hydrochloride acid[9]. However, acidic hydrolysis was found to produce only small amount of oligochitosan, but high in D-glucosamine monomer. Besides, acidic hydrolysis of chitosan can induce ring opening and this can lead to the production of toxic components because of the chemical changes during the reaction[10]. In the enzymatic hydrolysis, Uchida *et al.*[6] reported that chitosanase from *Bacillus* spp. was capable of producing high amount of oligosaccharides (DP 2-6) and small quantity of D-glucosamine. Enzymatic process performed better than the chemical reaction because of its specificity and ease in the fractionation of product compared to the acidic hydrolysis[11].

Ilyina *et al.*[4] reported that the most effective enzyme to hydrolyse chitosan was chitinase and chitosanase, which can be found in fungus, bacteria and plant. However, the commercial application of chitinase and chitosanase were limited because these enzymes are scarce and thus, very expensive. However, Yalpani and Pantaleone[11] has reported that several enzymes, such as glucosanase, lipase, tannase and protease, have chitosanolitic activities and showed similar and sometime

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batter, degradation activities than chitinase and chitosanase enzymes.

The objective of this study is to evaluate the degradation of chitosan using commercially available bromelain. As an acidic protease, the pH range for reaction of bromelain is in the range of 5.0-8.0[12].

MATERIALS AND METHODS

Materials: Chitosan powder (batch No. 00/200/27 and PM 100) was purchased from Chito-chem (M) Sdn. Bhd. Bromelain (EC 3.4.22.32) B4882 in powder form were supplied by Sigma, USA. All chemicals used are of reagent grade.

Determination of reducing end: The degree of enzymatic activity for chitosan were determined by the formation of coloured compound after reaction of the liberated reducing group and Dinitosalicylate (DNS), monitored at 530 nm[13]. The content of reducing sugar in chitosan hydrolysate was calculated by referring to the standard curve of standard sugar (glucose) and the final result is expressed as mg reducing sugar/g chitosan.

Determination of viscosity of chitosan: Viscosity of chitosan before and after enzymatic hydrolysis was measured using Brookfield Digital Viscometer (Model DV II) with spindle No. 3 at 50 rpm. Percentage of reduction in viscosity of chitosan was also calculated.

Enzymatic hydrolysis: Initial assay was performed generally in accordance to parameter suggested in the technical information of bromelain, as supplied by SIGMA. Chitosan solutions, 1% w/v in 200 mL of acetate buffer were hydrolysed with 5% w/w bromelain (enzyme/chitosan) at pH 4.6. The reactions were run at 25°C for 1 h. Each reaction was compared with control, where the enzyme was not added. Based on this basic combination, assays were performed to determine the optimum condition for depolymerization of chitosan at different enzyme concentrations (1, 3, 5, 10 and 15%), substrate concentrations (0.5, 1, 1.5 and 2%), pH (3, 4, 5 and 6), temperatures (30, 40, 50, 60 and 70°C) and reaction times (10, 20, 40, 60, 90, 120, 180 and 240 min).

Freeze drying of chitosan hydrolysate: Chitosan hydrolysate was freeze-dried using laboratory freeze dryer (Labconco LYPH-LOCK 6) at -50°C under vacuum condition of 100 mbar.

Determination of solubility of chitosan hydrolysate: Solubility of chitosan hydrolysate in water were determined by carefully weighing the dried hydrolysate in batches of 50 mg. The hydrolysate was then dissolved in 1 mL distilled water (with gentle stirring) until a point is reached where the powder failed to dissolve completely (forming suspension) after 15 sec of thorough stirring in a vortex.

Statistical analysis: Data obtained were analysed using variance analysis method (ANOVA). Duncan's Multiple Range Test was used to determine the significant differences at the confidence level of 95% ($p<0.05$). All analysis was done with the aid of a computer software program of Statistical Analysis System (SAS).

RESULTS AND DISCUSSION

The effect of enzyme concentration: The recommended assay condition of the bromelain was at pH 4.6, 25°C and using specialised substrate (N-α-CBZ-L-lisin p-nitroenol ester). Due to the change of substrate and the general changes of reaction conditions, the optimum reaction conditions need to be investigated.

Increasing in enzyme concentration from 1 to 15% w/w enhanced the reducing sugar liberation and the viscosity reduction of chitosan solution. The most significant drop in viscosity was observed 7% enzyme concentration and continued until 10% (Fig. 1). Beyond this concentration, no significant drop of viscosity was observed. The trend of viscosity drop was followed by an

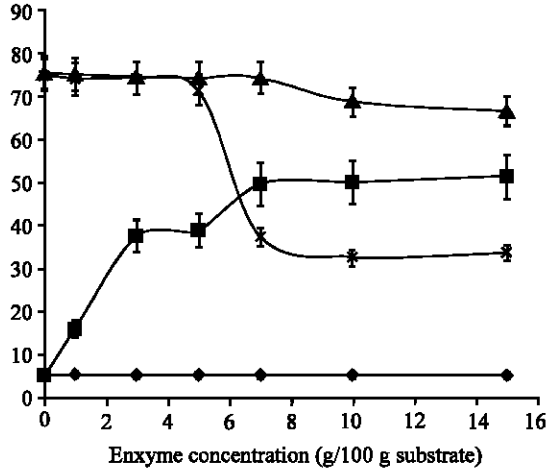

Fig. 1: The effects of enzyme concentration on the release of reducing ends and change in viscosity of the chitosan after reaction with bromelain. Each point is the average±SD at $p<0.05$ (n=3) Viscosity of control (▲), viscosity of reaction product (×), reducing ends of control (◆), reducing ends of reaction products (■)

increase in reducing ends values (DNS values). The highest DNS value (49.97 mg reducing group/g chitosan) was observed at 7% enzyme concentration.

Effect of concentration of substrate: Chitosan is soluble in dilute organic acid solution at pH range of 3 to 6.5. Thus, substrate was dissolved in acetate buffer pH 4.6 to capitalise on the solubility characteristics of chitosan and also to follow the recommended pH for bromelain reaction. Figure 2 showed that the most efficient depolymerization using 7% enzyme concentration occurred at 1% substrate concentration, where the lowest viscosity (44.12±3.2 cps) was reached. This was also followed by an increase in DNS value. However the magnitude of increase in DNS value is not proportional to the decrease in viscosity. At the end of assay, there is a slight increase in viscosity and the final viscosity is 55.82±5.2 cps which is about 1/3rd of the initial viscosity. This data indicate that the reaction of bromelain on chitosan is also endopeptidase in nature, producing mainly oligochitosans. Repeated experiments showed similar trends and consistency. Reaction on 2% chitosan was very slow. This could be due the high viscosity (almost gel-like), which inhibit free movement (collision) of substrate and enzyme to affect reaction. It was reported that the activity of chitinase from *Streptomyces* spp. No. 6 was inhibited at more than 0.5 mg mL^{-1} chitosan[14]. Fenton and Eveleigh[15] found that chitinase from *Penicillium islandium* was inactivated by chitosan oligomer produced from the hydrolysis.

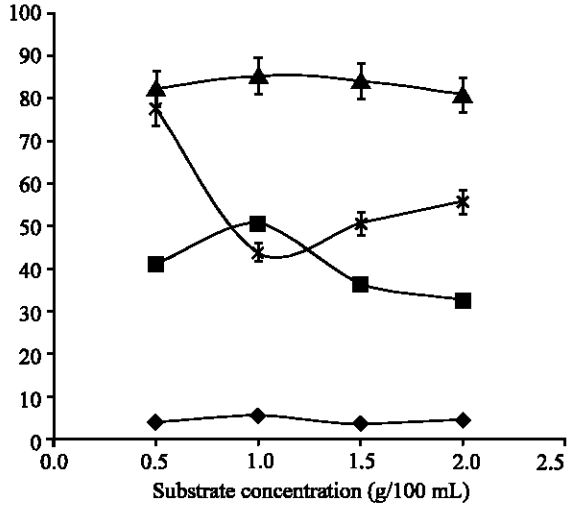

Fig. 2: The effects of substrate concentration on the release of reducing ends and change in viscosity of the chitosan after reaction with bromelain. Each point is the average±SD at $p<0.05$ (n=3)
Viscosity of control (▲), viscosity of reaction product (×), reducing ends of control (◆), reducing ends of reaction products (■)

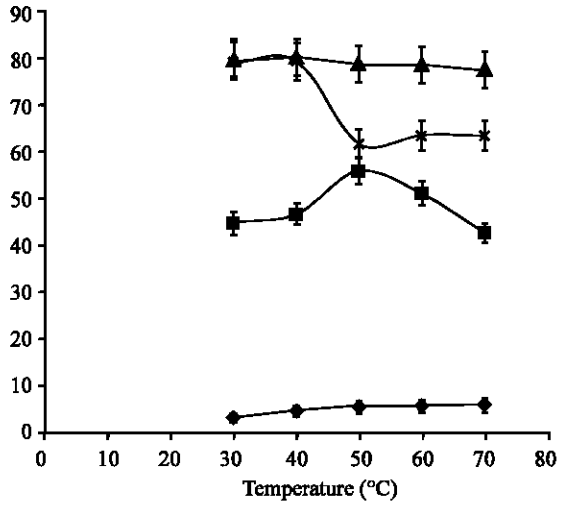

Fig. 3: The effects of reaction temperature on the release of reducing ends and change in viscosity of the chitosan after reaction with bromelain. Each point is the average±SD at $p<0.05$ (n=3)
Viscosity of control (▲), viscosity of reaction product (×), reducing ends of control (◆), reducing ends of reaction products (■)

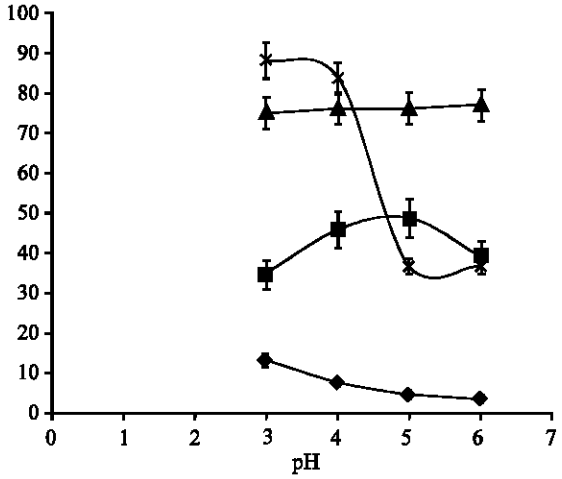

Fig. 4: The effects of pH on the release of reducing ends and change in viscosity of the chitosan after reaction with bromelain. Each point is the average±SD at $p<0.05$ (n=3)
Viscosity of control (▲), viscosity of reaction product (×), reducing ends of control (◆), reducing ends of reaction products (■)

The effect of temperature: Figure 3 shows that the optimum temperature for the reaction bromelain on chitosan was 50°C (using 1% substrate, 10% enzyme, 1 h reaction, pH 4.6). At this pH, the solution reached its lowest viscosity (61.5±1.8 cps) and highest DNS value

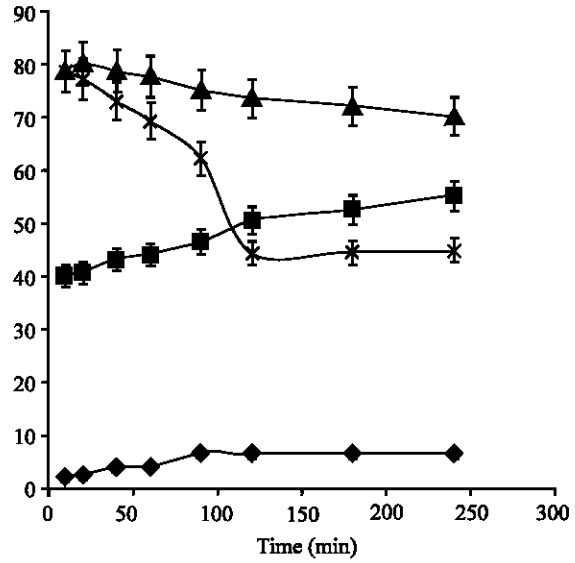

Fig. 5: Effect of reaction time on the release reducing ends and viscosity of chitosan after reaction with bromelain. Each point is the average±sd at $p<0.05$ (n= 3)
Viscosity of control (▲), viscosity of reaction product (×), reducing ends of control (◆), reducing ends of reaction products (■)

(56.2±3.2 mg reducing sugar/g chitosan). It is logical to think that higher temperature (higher energy) is required to depolymerase a complex substrate.

The effect of pH: Enzymatic hydrolysis had been studied at different pH of 3, 4, 5 and 6. The use of low pH is unavoidable to favour chitosan solubility. As shown in Fig. 1-4, all control reactions were not static. Some acidic degradation of chitosan appeared to have taken place even in the absence of enzyme. This is not surprising as a similar observation was made by Muzzarelli *et al.*[16], where acetate buffer solution was able to reduce the viscosity of chitosan to 40% at pH 3.98 and 12% at pH 5.5. Figure 4 showed that the optimum pH for bromelain reaction on chitosan was at pH 5, where the lowest viscosity value was reached (35.7±1.3 cps) and DNS value was the highest (48.7±2.6 mg reducing sugar/g chitosan).

The effect of reaction time: Figure 5 shows the reaction profile over time. Reduction of viscosity is already apparent even after 10 min of reaction and it was followed by subsequent increase in DNS value. Until the 90th min, the decrease in viscosity continued at a steady rate. On the 120th min, there was a sudden drop in viscosity. Beyond this point, the change in viscosity was not significant. The increase in DNS value, although showing steady increase, was not as dramatic as the drop in viscosity. This phenomenon again can be interpreted as an indication that the degradation was endo-type where the final product are mainly oligo-chitosan which can still contribute to the viscosity of the solution (44.89±2.6 cps) even after 240 min of reaction. The initial viscosity was 78.71±4.1 cps. An exo-type reaction will produce monomers, which cannot offer any meaningful contribution to the viscosity of the solution.

The solubility of chitosan hydrolysate: The native chitosan is not soluble in distilled water, but soluble in dilute organic acid solution, such as acetic acid (pH 3-5), to form clear solution. The dried hydrolysate obtained was off-white in colour, which quickly turns brownish upon prolonged exposure to air. The highest solubility of chitosan hydrolysate in this study was 1.0% w/v. At this concentration and below, the hydrolysate dissolved fairly easily, to give clear solution. Beyond this level and up to 1.5 g/100 mL, suspension started to form, even after vigorous shaking, but without heating.

The relatively cheap commercial bromelain was found to be capable of partially degrading the chitosan under mild and controllable environment. The enzymatic hydrolysis of chitosan using bromelain was optimally run for 2 h, at pH 5, 1% w/v substrate, 7% w/w enzyme and 50°C. At this optimum condition, the viscosity of chitosan was reduced to 84%. Repeated trials consistently showed similar profile, indicating the specificity of the reaction. The maximum solubility of chitosan hydrolysate in water at room temperature was 1.0% w/v.

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