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Controlling chitosan molecular weight via bio-chitosanolysis

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ABSTRACT

An eco-friendly method was developed for bio-chitosanolysis in order to effect depolymerization and control the molecular weight (MW) of chitosan without changing other molecular structure. Hence biodegradation of chitosan was carried out under a variety of conditions using *Aspergillus niger* pectinase enzyme which is in common practice. The intrinsic viscosity and MW of chitosan were found to decrease by increasing the enzyme concentration, time of incubation and temperature up to a certain limit then leveled off except the temperature. Leveling off occurred at enzyme to chitosan ratio of 0.003 (w/w), pH 4.75 and temperature 55 °C for 30 min. Temperature higher than 55 °C deactivates the enzyme and results in marginal depolymerization of chitosan. IR spectra of chitosans having different molecular weights exhibit no obvious change in the degree of acetylation or other microstructural features. The need for chitosan with such tailored molecular weights will be emphasized in a forthcoming paper.

1. Introduction

Chitosan is the deacetyl derivative of chitin. The latter is the second most abundant polysaccharide next to cellulose. When chitin is deacetylated over about 60% it becomes soluble in dilute aqueous acids and is referred to as chitosan. Chitin is found in shells of crustaceans (e.g. shrimp, crab, and lobster) and in exoskeletons of mollusks and insects as well as in the cell walls of some fungi (Lim, 2002; Muzzarelli, 1977).

Chitin and chitosan are highly basic polysaccharides opposite to most of naturally occurring polysaccharides, e.g. cellulose, dextrin, pectin, alginic acid and agarose, which are neutral or acidic in nature. Their unique properties include polyoxysalt formation ability to form films, chelate metal ions and optical structural characteristics (Fouda, 2005).

Chitosan acquires a high antibacterial activity which advocates it for numerous applications (Kittur and Kumar et al., 2003; Lim & Hudson, 2003; Liu, Guan, Yang, Li, & Yao, 2001; Rinaudo, 2006). Molecular weight is very important parameters in many of such applications. For example the very high-molecular weight and, therefore, a very high viscosity of chitosan solution and the acidic media required to achieve solubilization in water (Roncal, Oviedo, de Armentia, Fernández, & Villarán, 2007), precluded its use in several biological applications.

Being a polymer, chitosan can be subjected to depolymerization (chitosanolysis) producing low-molecular weight chitosan (LMWC), oligosaccharides and monomers (Mourya & Inamdar, 2008). Chitosanolysis can be achieved by chemical means, e.g. HCl and H_2O_2 (Hebeish, Higazy, & El-Shafei, 2004; Vishu Kumar & Tharanathan, 2004), HNO_2 (Vishu Kumar & Tharanathan, 2004), and physical means such as microwave technology, electromagnetic radiation and sonication (Mourya & Inamdar, 2008).

As well as enzymatic means based either on specific chitosanase, or non-specific enzymes (Cabrera & Cutsem, 2005; Kittur, Kumar, Varadara, & Tharanathan, 2005; Kittur et al., 2003a; Roncal et al., 2007; Riccardo, Muzzarelli, Wenshui, Tomasetti, & Ilari, 1995; Vishu et al., 2004).

The high cost and unavailability of chitosanase in bulk inhibit its use in industrial scale (Vishu Kumar & Tharanathan, 2004). Alternatively non-specific activity of various enzymes on chitosan has been reported and includes hemicellulases (Cabrera & Cutsem, 2005), lipases (Riccardo et al., 1995), pectinases (Kittur et al., 2005; Kittur, Kumar, & Tharanathan, 2003a; Kittur, Kumar, Gowda, & Tharanathan, 2003b), cellulases (Roncal et al., 2007), proteases (Vishu Kumar & Tharanathan, 2004), lysozyme and β-(1 → 3) (1 → 4)-glucanases (Roncal et al., 2007). The advantages of non-specific chitosanolysis process are the production of LMWC in higher yields (Vishu Kumar & Tharanathan, 2004) in addition to the commercial feasible of the process. Furthermore, the enzymes are inexpensive and easily available.

Pectinases are a group of enzymes produced by a number of bacteria, yeast, fungi, protozoa, insects, nematodes and plants (Lozano, Manjon, Iborra, & Galvez, 1990; Whitaker, 1991). The best-

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known microbial producers of pectinase are different species of Aspergillus fungi, meanwhile some of the most popular (Debing, Peijun, Stagnitti, Xianzhe, & Li, 2006; Taragano, Sanchez, & Pilosof, 1997) are among the commercial pectinolytic enzymes. Preparations of the latter are obtained by the industrial cultivation of *Aspergillus niger*.

Current research focuses on establishment of eco-friendly conditions for preparation of chitosans with different MWs. Controlling the MW is affected through chitosanolysis using pectinase enzyme under the action of various parameters. Thus obtained products will be used as antibacterial for cotton textiles.

2. Experimental

2.1. Materials

High-molecular weight chitosan (HMWC) and Pectinase enzyme from *A. niger* (with activity 1.32 Units per mg protein) were purchased from Sigma-Chemical Company, St. Louis, MO, U.S.A. The other chemicals used in this study were of high analytical grade.

2.2. Biodegradation of chitosan

The enzyme was added to 250 ml of chitosan solution, [chitosan], 1% (in 0.1 M acetic acid/0.2 M sodium chloride) pH (3.0–9.5) in a conical flask with enzyme/chitosan ratio (0.0001–0.006/l g chitosan) (w/w). The reaction mixture was incubated at (30–70 °C) for (15–240 min) with constant stirring. After hydrolysis, the enzyme was inactivated at 100 °C for 10 min and the pH adjusted to 12 with NaOH to precipitate products with a high degree of polymerization (DP). The suspension was filtered and the insoluble residue was washed with double distilled water and dried to give low-molecular weights chitosan (Kittur et al., 2003a).

2.3. Determination of molecular weight

Chitosan samples of different molecular weights were dissolved in solution of (0.1 M acetic acid/0.2 M sodium chloride) (Kittur, 1992) at temperature not above 70 °C and left about 12 h with continuous stirring to obtain a 1% (w/v) solution. The chitosan solution was filtered to remove any undissolved matter. Molecular weight was determined by a viscometric method (Chen & Hwa, 1996; Zhang and Neau, 2001). Different concentrations of chitosan solution were prepared. The relative viscosity, η, of chitosan samples was measured using Ostwald viscometer at 30 ± 0.5 °C. Specific viscosity was determined using η sp = (η solution − η solvent)/η solvent. Intrinsic viscosity [η], is defined as reduced viscosity, η red, extrapolated to a chitosan concentration, C, of zero. [η] = (η sp/C)$C \rightarrow 0$ = (η red) $C \rightarrow 0$, where C, is in g/ml. Viscosity average molecular weight was calculated based on the Mark-Houwink equation $[\eta] = K\mathrm{MW}^{\alpha}$ (Ramachandran, Rajendrakumar, & Rajendran, 2004), where, K and α are constants that are independent of MW over a wide range of values. They are dependent on polymer, solvent, temperature and in case of polyelectrolytes the nature and concentration of the added low-molecular weight electrolyte, and K ($\mathrm{cm}^3\ \mathrm{g}^{-1}$) = 1.81×10^{-3}, $\alpha = 0.93$ for (0.1 M acetic acid/0.2 M sodium chloride) solvent (Kittur, 1992).

3. Results and discussion

3.1. Effect of different parameters on chitosanolysis by pectinase enzyme

3.1.1. Effect of enzyme concentration

Normally reactions are catalysed by enzyme concentrations which are much lower than substrate concentrations (Taylor, Green, & Stout, 1997). Chitosan solution of concentration 1% (in 0.1 M acetic acid, 0.2 M NaCl) was prepared and seven different enzyme:chitosan ratios ranging from 0.0001 to 0.006 (w/w) with activity (1.32 Units per mg protein) were used. Other reaction parameters such as pH, temperature and time were adjusted.

Table 1
Effect of pectinase enzyme concentration on chitosanolysis.

Enzyme:chitosan ratio (w/w)	Intrinsic viscosity η ($\mathrm{g\,dl^{-1}}$)	Molecular weight $\times 10^5$ (Da)
–	480	6
0.0001	290	4
0.0002	90	1.1
0.0004	74	0.9
0.0008	70	0.85
0.0016	64	0.77
0.003	60	0.72
0.006	60	0.72

Degradation conditions: [chitosan], 1% (in 0.1 M acetic acid, 0.2 M NaCl); temperature, 37 °C; time 4 h; pH, 4.7; (Da): Dalton; ($\mathrm{g\,dl^{-1}}$): gram/deciliter.

Table 1 shows the extent of chitosanolysis, expressed as intrinsic viscosity and molecular weight, as a function of pectinase enzyme treatment. Obviously the extent of chitosanolysis under the action of the pectinase enzyme decreases significantly by increasing the enzyme:chitosan ratio from 0.0001 to 0.006 (w/w) but with the certainty that the enzyme exhibits saturation at enzyme:chitosan ratio of 0.003 (w/w).

The decrement in chitosanolysis could be associated with better interactions between the pectinase enzyme and the chitosan at higher enzyme concentrations. That is, larger involvement of the chitosan molecules with the active sites on the pectinase enzyme as per key and lock mechanisms is highly favored and, results in enzyme–chitosan complex which breaks down to yield ultimately chitosan molecules with lower intrinsic viscosities and, therefore, lower molecular weights. This state of affairs seems to be valid until enzyme:chitosan ratio of 0.003 (w/w) is attained where saturation takes place as a consequence of perhaps full engagement of the chitosan at its own unaltered concentration with the active sites of pectinase whose concentration has become enough to accommodate the chitosan.

3.1.2. Effect of reaction time

The effect of time on chitosanolysis when chitosan was biodegraded by pectinase enzyme was investigated. Chitosanolysis was affected at 37 °C and pH 4.75 for a time ranging from 15 to 240 min. Table 2 shows the intrinsic viscosity and molecular weight of the biodegraded chitosan samples as a function of incubation time.

It is clear (Table 2) that the extent and rate of chitosanolysis, expressed as intrinsic viscosity and molecular weight, exhibit an initial fast rate followed by a slower rate during the first 30 min incubation. Incubation for 240 min marginally decreases the magnitude of intrinsic viscosity and molecular weight of the chitosans. Thus prolonging the incubation time more than 30 min is meaningless.

Table 2
Effect of time on chitosanolysis by pectinase enzyme.

Degradation time (min)	Intrinsic viscosity η ($\mathrm{g\,dl^{-1}}$)	Molecular weight $\times 10^5$ (Da)
15	100	1.2
30	74	0.9
45	70	0.85
60	65	0.79
240	60	0.72

Degradation conditions: [chitosan], 1% (in 0.1 M acetic acid, 0.2 M NaCl); enzyme:chitosan ratio 0.003 (w/w); temperature, 37 °C; pH, 4.75.

Table 3
Effect of pH on chitosanolysis by pectinase enzyme.

pH value	Intrinsic viscosity η (g dl^{-1})	Molecular weight × 10^5 (Da)
3	90	1.1
4.75	75	0.92
7	100	1.2
9.5	120	1.5

Degradation conditions: [chitosan], 1% (in 0.1 M acetic acid, 0.2 M NaCl); enzyme:chitosan ratio 0.003 (w/w); time 30 min; temperature, 37 °C.

The favorable effect of the incubation time could be associated with longer contact between the chitosan and the enzyme for better interactions. Time also helps formation of the chitosan–enzyme complex and dissociation of the latter to bring into focus the biodegraded chitosan products.

3.1.3. Effect of pH

Chitosan is not soluble in aqueous solution beyond pH 6.5 (Vishu Kumar & Tharanathan, 2004). This evoked the interest to study the chitosanolysis by pectinase enzyme at different PHs. Thus chitosan was subjected to the action of pectinase enzyme at pHs (3, 4.75, 7 and 9.5. The effect of the enzymatic treatment on the intrinsic viscosity and molecular weight of chitosan was investigated. The results obtained are set out in Table 3.

It is seen (Table 3) that chitosanolysis extent, expressed as intrinsic viscosity and molecular weight, relies on the pH of the treatment. The extent of chitosanolysis follows the order: pH 4.75 > pH 3 > pH 7 > pH 9.5. This order discloses that provides the most appropriate system for the interactions of the pectinase enzyme with chitosan. At this particular pH, chitosan seems to assume greater swellability, biocompatibility and high involvement and intimate association with pectinase enzyme whose biodegradability seems also to be in full swing at pH 4.75. That is, pH 4.75 offers the most favorable environment for both pectinase enzyme and chitosan for biodegradation to proceed easily. This seems not to be the case with neutral and alkaline pH's where lower swellability, and compatibility are encountered during the biodegradation process.

On the other hand, the moderate effect of chitosanolysis at pH 3 suggest that though this pH causes solubility of chitosan thereby rendering it more amenable for biodegradation, yet the pectinase enzyme at pH 3 assumes lower activity Also, molecular decreased from pH 3–4.75, then increased by increasing pH from 7 to 9.

3.1.4. Effect of temperature

In order to study the effect of temperature on chitosanolysis, the latter was performed at temperature ranging from 30 to 70 °C. Table 4 shows results of intrinsic viscosity and molecular weight for chitosan samples biodegraded by pectinase enzyme at the different temperatures. Evidently, biodegradation displays its maximal at 55 °C. Below 55 °C, that is, from 45 to 30 °C, the biodegraded chitosan samples acquire higher values. The same situation is encountered at 70 °C.

Table 4
Effect of temperature on chitosanolysis by pectinase enzyme.

Temperature (°C)	Intrinsic viscosity η (g dl^{-1})	Molecular weight × 10^5 (Da)
30	120	1.5
37	74	0.9
45	70	0.85
55	18	0.19
70	90	1.1

Degradation conditions: [chitosan], 1% (in 0.1 M acetic acid, 0.2 M NaCl); enzyme:chitosan ratio 0.003 (w/w); time 30 min; pH, 4.75.

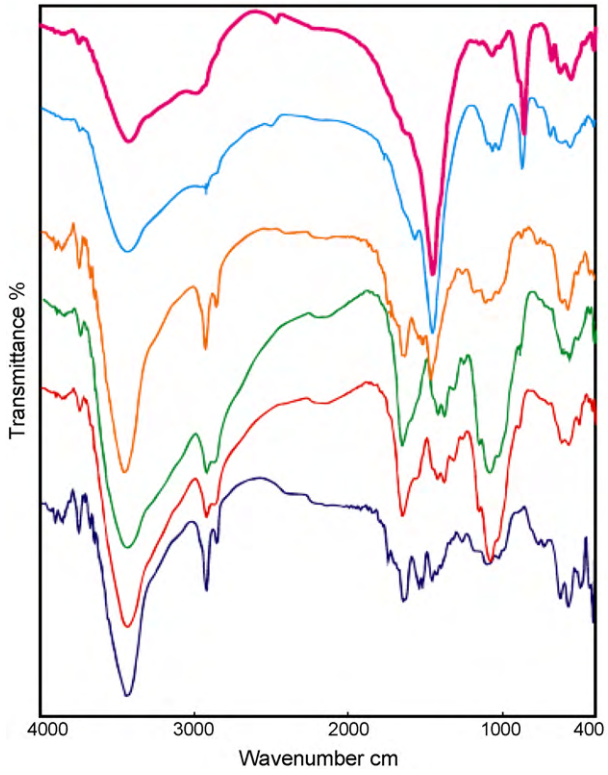

Fig. 1. IR spectra of the original chitosan and the degraded chitosan: (a) original chitosan 6 × 10^5 Da; (b) 4 × 10^5 Da; (c) 1.5 × 10^5 Da; (d) 1.2 × 10^5 Da; (e) 0.9 × 10^5 Da; (f) 0.19 × 10^5 Da. (a–f): From bottom to top.

The above findings signify that the activity of pectinase enzyme is optimal and so does the chitosan swellability and compatibility when chitosanolysis is conducted at 55 °C. Higher temperature (i.e. 70 °C) decreases remarkably the activity of the enzyme and detracts from its interaction with chitosan. The decreased pectinase activity is perhaps a manifestation of denaturation of the enzyme protein.

3.2. IR spectra of original chitosan and degraded chitosan

Fig. 1 shows the Infrared (IR) spectrums of the original chitosan (6_a) and the different MW chitosan samples 6_{a-f}. There were strong amino characteristic peaks of chitosan at around 3420, 1655, and 1325 cm^{-1}, and the peaks assigned to the saccharide structure were at 1152 cm^{-1} (C–H stretch), 1154 cm^{-1} (bridge-o-stretch), and 1094 cm^{-1} (C–O stretch). The spectrums of the chitosan samples (6_b) to (6_f) had no obvious difference with the original chitosan, and no difference between each of the biotreated samples. The results showed that chitosan samples prepared as per the method of pectinase hydrolysis had no obvious change in the degree of deacetylation (DD) and molecular structure, in accordance with previous reports (Liu et al., 2006).

4. Conclusion

Different parameters affecting pectinase-induced chitosanolysis were studied with a view to synthesize chitosan having different molecular weights. Results conclude that involvement of pectiase enzyme with chitosan through interactions and complexation and further dissociation of such complexes to yield biodegraded chitosan are a manifestation of the enzyme:chitosan ratio as well as pH, temperature and time of incubation. Varying any one of these parameters while keeping the others constant results in chitosan

with different molecular weights without obvious change in the degree of deacetylation and molecular structure of chitosan. Within the range studied chitosan having the lowest molecular weight could be prepared using enzyme:chitosan ratio of 0.003 (w/w), at pH 4.75 and temperature 55 °C for 30 min.

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