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Some studies on characterization of three phase partitioned chitosan

Aparna Sharma, Kalyani Mondal, Munishwar Nath Gupta*

Department of Chemistry, Indian Institute of Technology, Hauz Khas, New Delhi 110016, India

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Abstract

Three phase partitioning (TPP) is generally carried out by adding ammonium sulfate and *t*-butanol to a solution of a macromolecule. Chitosan could be obtained as an interfacial precipitate with 88% yield by subjecting 0.2% (w/v) chitosan solution to TPP with 45% (w/v) ammonium sulfate, with an equal volume of *t*-butanol at 40 °C. TPP resulted in structural changes which could be seen in its UV spectra, FT-IR spectra and solubility characteristics. TPP-treated chitosan also showed decreased susceptibility towards hydrolysis by chitinase. Thus, TPP can be used as a useful way of altering the properties of chitosan.

Keywords: Biodegradability of chitosan; Three phase partitioning; UV and FTIR spectroscopy of chitosan

1. Introduction

Chitosan is obtained by partial deacetylation of chitin. The latter is a co-polymer of *N*-acetylglucosamine and glucosamine units and occurs naturally in all crustaceans. Chitosan is a nontoxic polymer and its derivatives find considerable applications in many areas (Muzzarelli, Mattioli-Belmonte, Pugnaloni, & Biagini, 1999; Roller & Covill, 2000; Wang, Ma, Wang, & He, 2001). Quite a few studies on its applications in bioseparation (Tyagi, Kumar, Sardar, Kumar, & Gupta, 1996; Roy & Gupta, 2001), biocatalysis (Roy, Sharma, & Gupta, 2002) and biomedical sciences (Muzzarelli & Biagini, 1993) have been reported. Separation (Keller, Friedmann, & Boxman, 2001), fractionation (Molyneux, 1983) and modification (Sashiwa, Makimura, Shigemasa, & Roy, 2000) of polymers are all useful objectives in polymer research. Three phase partitioning (TPP) promises to be a technique, which can be applied in all the three contexts. The focus in the present work is on characterization of chitosan after subjecting it to TPP.

TPP has been fairly successful in concentration and purification of proteins (Dennison & Lovrein, 1997; Sharma & Gupta, 2001a; Sharma & Gupta, 2001b; Singh et al., 2001). In this technique, a combination of ammonium sulfate and *t*-butanol is used to precipitate the macromolecule. The precipitate appears as an interface between lower aqueous and upper organic phases. Recently, we described that TPP can also be used for purification of commercial preparation of alginate (Sharma & Gupta, 2002). In this work, we extend the application of TPP to chitosan and investigate the changes in some of the properties of this carbohydrate polymer. This work, thus, focuses on TPP as a tool to alter the properties of carbohydrate polymers with chitosan taken as a model polymer.

2. Materials and methods

Chitosan (co-polymer of *N*-acetyl-glucosamine and glucosamine units) was purchased from Sigma (St Louis, MO, USA). All other chemicals used were of analytical grade.

2.1. Preparation of chitosan

Chitosan solution (50 ml, 1%, w/v) was prepared by dissolving 0.5 g of chitosan in 1% (v/v) acetic acid and then precipitating it by raising the pH to 8.0 by addition of 3 M NaOH. The precipitated chitosan was washed three times with 0.01 M Tris-HCl buffer, pH 8.0 and again solubilized

* Corresponding author. Tel.: +91-11-26591503; fax: +91-11-26581073.
E-mail address: mn_gupta@hotmail.com (M.N. Gupta).

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in 50 ml of 0.01 M acetate buffer, pH 5.0 to get the final concentration of 1% (w/v) (Senstad & Mattiasson, 1989).

2.2. Estimation of chitosan (phenol–sulfuric acid test)

Phenol solution (25 μl of 80%, v/v) and concentrated sulfuric acid (2.5 ml) were added to chitosan solution. The mixture was then kept at 25 °C for 10 min. Absorbance was read at 489 nm (Hirs, 1967).

2.3. Three phase partitioning of chitosan

Chitosan solutions (2 ml) (various concentrations, pH 5.0) were mixed with varying amounts of ammonium sulfate (w/v). Different volumes of *t*-butanol (v/v) were added to this solution, vortexed and incubated at a suitable temperature for 1 h. The upper layer of *t*-butanol was separated from lower aqueous layer by an interfacial precipitate of the polymer. The precipitated chitosan in the midlayer was suspended in 1 ml of 0.01 M acetate buffer, pH 5.0. The amount of chitosan precipitated was estimated by phenol–sulfuric acid test after dialyzing the precipitate in 0.01 M acetate buffer, pH 5.0. The amount of chitosan precipitated was calculated by taking the starting amount of chitosan as 100%.

2.4. Spectroscopy experiments

FT-IR was recorded on Nicolet-Protégé-460 spectrometer and UV spectra was recorded on Beckmann DU 640 spectrophotometer.

2.5. Purification of chitinase

Fresh cabbage leaves (250 g) were homogenized with 750 ml of 0.1 M acetate buffer, pH 5.2 in a mixer. After straining through four layers of cheese cloth, the turbid filtrate was centrifuged at 10,000*g* for 20 min to remove the insoluble substances The filtrate obtained was taken as crude chitinase extract (Chang, Lo, Wu, & Sung, 1992).

2.6. Determination of chitinase activity

Chitinase activity was determined as described by Chang et al. (1992). Chitosan and TPP-treated chitosan were used as substrates. One enzyme unit (U) is defined as the amount of enzyme which liberates 1 μmole of reducing sugar (calculated as *N*-acetyl glucosamine) per minute at 40 °C and at pH 5.2. The reducing end group produced was measured colorimetrically with dinitrosalicylic acid reagent.

3. Results and discussion

Fig. 1a shows the amount of chitosan precipitated with different starting concentrations of chitosan after TPP. The ratio of *t*-butanol to polymer solution was kept 1:1 (v/v) and temperature of 25 °C was used. The concentration of ammonium sulfate in these experiments was 30% (w/v). At 20% (w/v) ammonium sulfate concentration, while *t*-butanol and aqueous buffer formed two phases, substantial precipitation of chitosan was observed at the bottom of the vessel. No interfacial precipitation appeared in this case (data not shown). At 30% (w/v) concentration of ammonium sulfate, 50% chitosan appeared in the interfacial layer as white precipitate with 0.2% (w/v) chitosan solution (Fig. 1a). At 40, 45 and 50% (w/v) ammonium sulfate, chitosan again precipitated at the bottom of the aqueous phase and no interfacial precipitate was observed (data not shown). However, at 37 °C, chitosan formed three phases with wider range of ammonium sulfate concentration (Fig. 1b). The optimum concentration of ammonium sulfate was 45% (w/v). Thus, at 37 °C and with 1:1 (v/v) ratio of *t*-butanol to aqueous chitosan solution, 75% of the starting chitosan could be precipitated. Fig. 1c shows that variation of the ratio of *t*-butanol to the aqueous layer can also change the percentage of polymer precipitation. However, infact, 1:1 ratio turned out to be optimum. Keeping this ratio and other parameters constant, chitosan concentrations were varied (Fig. 1d). Again 0.2% chitosan concentration turned out to be the best choice. Changing the temperature to 40 °C improved the extent of precipitation to 88% (w/v) (Fig. 1e). Thus, by using 45%(w/v) ammonium sulfate, 1:1 ratio (v/v) of *t*-butanol and the aqueous phase, 0.2% (w/v) chitosan and temperature of 40 °C, 88% chitosan could be precipitated from its solution as an interfacial layer.

3.1. Changes in the properties of chitosan after TPP

Chitosan obtained after TPP (using conditions described for Fig. 1e) was investigated vis-à-vis some of its properties. One important property of chitosan is its property of pH dependent solubility. This reversibly soluble–insoluble nature has proved very useful in both bioseparation (Tyagi et al., 1996) and biocatalyst design (Roy, Sharma, & Gupta, 2002). Fig. 2 shows the pH versus the percent chitosan precipitated (w/v). It was found that for TPP-treated chitosan the curve shifted slightly towards more alkaline side. Thus at pH 6.0, chitosan shows 57% precipitation whereas TPP-treated chitosan is completely soluble. However, when both chitosan and TPP-treated chitosan are lyophilized, the behaviour of TPP-treated chitosan undergoes dramatic changes and it starts becoming insoluble even at pH 3.0 and is completely insoluble in the range of pH 6.0–7.0 (Fig. 2). The pH dependent solubility data, thus, can be summarized as follows: (i) For chitosan (untreated), lyophilization alone causes no change in the pH versus solubility curve. (ii) TPP alone does shift the curve towards alkaline side. (iii) For TPP-treated chitosan, lyophilization causes a shift in the curve towards lower pH side. This shift, in fact, is in the direction opposite to the shift caused by TPP treatment. (iv) Lyophilization

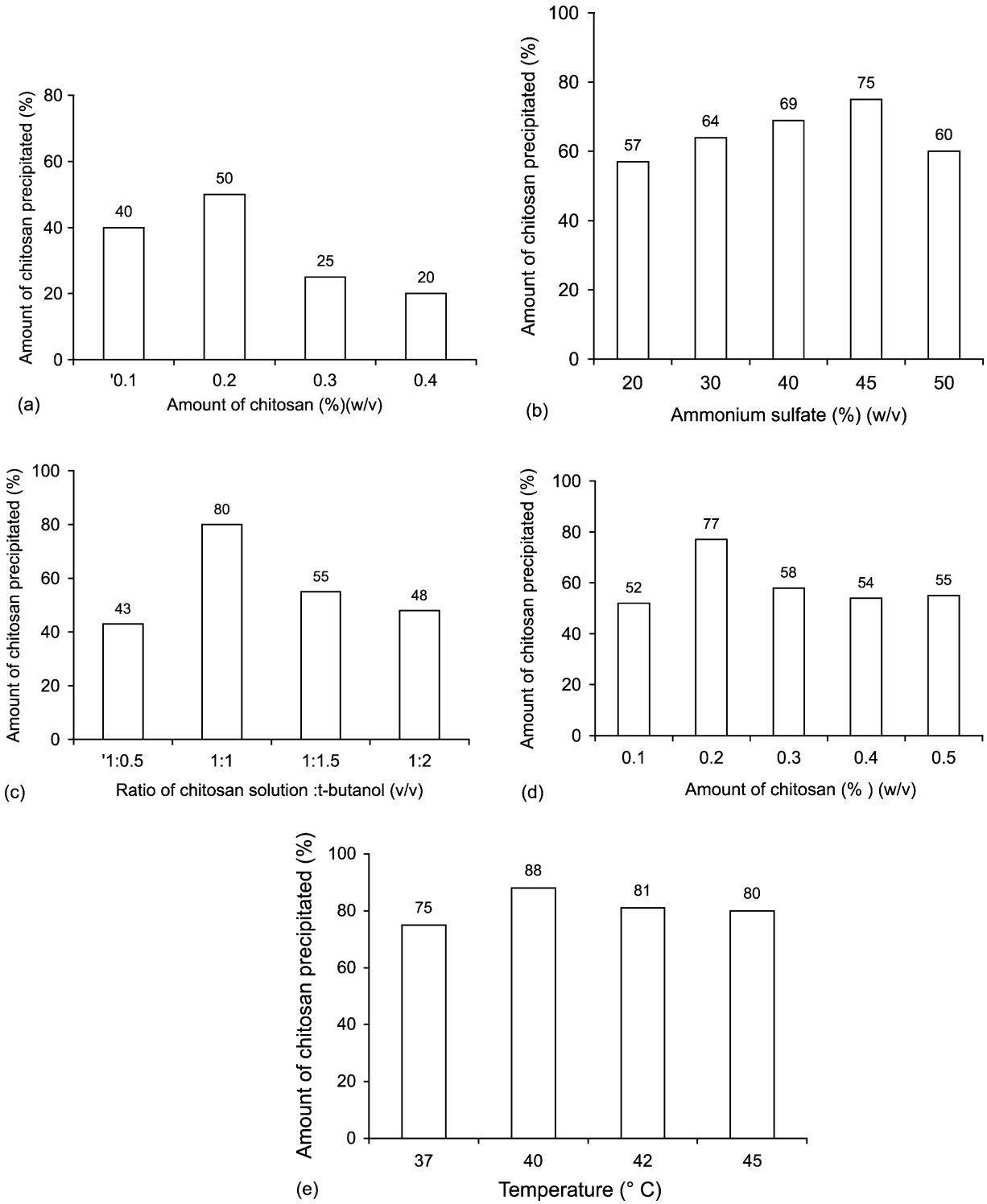

Fig. 1. Optimization of the conditions for precipitation of chitosan by TPP. (a) Ammonium sulfate (30%, w/v) was added to varying amounts of chitosan solutions (0.1, 0.2, 0.3 and 0.4%) (w/v). The ratio of chitosan solution to *t*-butanol was 1:1 in all the cases. The interfacial precipitate formed after keeping these systems at 25 °C for 1 h were collected. (b) Chitosan solutions (2 ml, 0.2%, w/v) were added to varying amounts of ammonium sulfate (w/v), mixed with 2 ml of *t*-butanol (v/v). The interfacial precipitate formed were collected after keeping these systems at 37 °C for 1 h. (c) Chitosan solutions (2 ml, 0.2%, w/v) were added to ammonium sulfate (45%, w/v), TPP was carried out at 37 °C by varying the ratio of polymer solution to *t*-butanol (v/v). (d) Ammonium sulfate (45%, w/v) was added to varying amount of chitosan solutions (0.1, 0.2, 0.3, 0.4 and 0.5%, w/v). The ratio of polymer solution to *t*-butanol was 1:1. The interfacial layer was collected after keeping these systems at 37 °C for 1 h. (e) Chitosan solution (2 ml, 0.2%, w/v) was added to ammonium sulfate (45%, w/v), mixed with 2 ml of *t*-butanol. TPP was carried out at different temperatures. In all cases, the precipitated solution was suspended in 0.01 M acetate buffer, pH 5.0. The amount of chitosan precipitated was estimated as described in 'materials and methods'. Each set of above experiments were carried out in duplicate which varied within ± 5%.

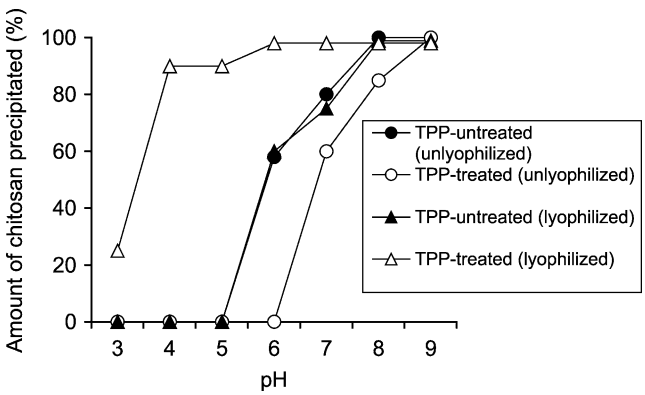

Fig. 2. pH induced chitosan precipitation. pH of 1 ml of (1%, w/v), chitosan and TPP-treated chitosan solutions were gradually changed by titration with 3 M acetic acid or 1N NaOH under continuous stirring. At different pH values samples were incubated for 30 min at 25 °C and centrifuged at 12,000*g* for 10 min. Unprecipitated chitosan was estimated in the supernatant using phenol–sulfuric acid assay.

followed by TPP is the only case in which drastic changes occur in the pH versus solubility curve. The pH dependent solubilization of chitosan is governed by protonation of free NH_2 groups in this partially deacylated polymer. Shifting of the curve towards low pH side implies that the pK_a of NH_3^+ group has shifted to lower pH. While abnormal ionizations are reported for some amino acid side chains in proteins (Means & Feeney, 1971), such dramatic change in NH_3^+ group has not been reported in polymers or even proteins. The reason for the unusually low pK_a needs to be investigated further.

The presence of magnesium ion concentration also influences the solubility of chitosan (Tyagi et al., 1996). Here also, chitosan and TPP-treated chitosan show different behaviour (Fig. 3). In this case also, solubility pattern changes when both samples are lyophilized (Fig. 3). The changes in magnesium ion concentration versus solubility curves are different upon lyophilization as well as upon TPP

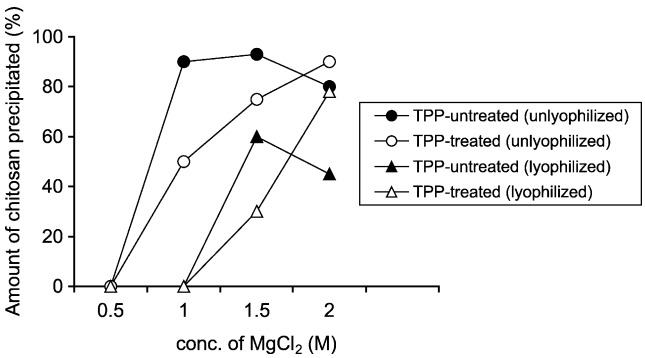

Fig. 3. Precipitation of chitosan with $MgCl_2$. Different concentrations of $MgCl_2$ (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M) were added to 4 ml of chitosan and TPP-treated chitosan solutions (to a final polymer concentration of 0.2%, w/v) in 0.01 M acetate buffer, pH 5.0. The mixtures were then incubated for 30 min at 25 °C for complete precipitation. After centrifugation at 12,000*g* for 10 min, unprecipitated chitosan was estimated in the supernatant using phenol–sulfuric acid assay.

as compared to the changes in pH versus solubility curve. This is not surprising. The precipitation by magnesium ions most likely follow a different mechanism from pH dependent precipitation. The interaction of metal ions with chitosan has been extensively studied although more work has been done with cations of transition elements (Muzzarelli, 1977; Rhazi et al., 2002). While chelation seems to be the main bonding with the latter, ion exchange and sorption also have been implicated in metal ion chitosan interactions (Muzzarelli, 1977). Rhazi et al. (2002), recently remarked 'There are no universally agreed mechanism for these processes'. To summarize, (A) In this case lyophilization alone shifts the curve of untreated as well as TPP-treated chitosan sample significantly towards higher concentration of magnesium ions. (B) TPP treatment for both unlyophilized as well as lyophilized samples also shifts the curve towards higher magnesium ion concentration. (C) Thus, in this case the result of lyophilization and TPP is cumulative. Thus, both lyophilization and TPP result in structural changes in chitosan which are not favorable for interaction with magnesium ions.

UV spectra of chitosan shows that it absorbs at 225 nm (Fig. 4) (Tyagi et al., 1996). The absorption around this region is due to the presence of amide linkages in chitosan. As chitosan is only partially deacylated chitin, it has enough of these linkages. The comparison of spectra-2 with spectra-1 and the comparison of spectra-4 with spectra-3 shows the effect of lyophilization on chitosan and TPP-treated chitosan. In both cases, lyophilization causes a shift in λ_{max} towards longer wavelength. The comparisons of spectra-3 with spectra-1 and spectra-4 with spectra-2 shows the effect of TPP-treatment on chitosan and

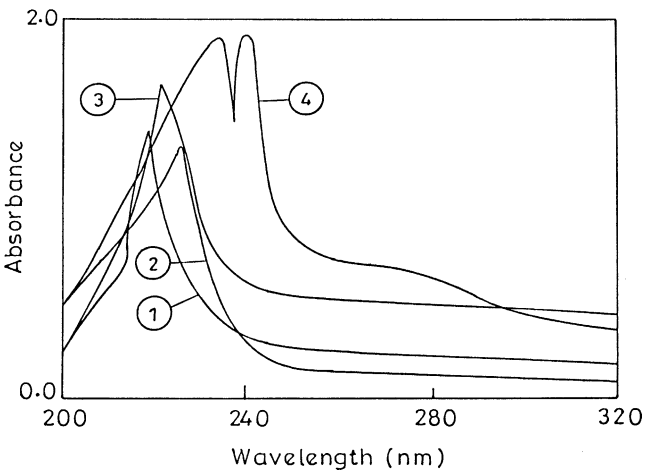

Fig. 4. UV spectra of chitosan solution. UV spectra of chitosan and TPP-treated chitosan solution was recorded. TPP of chitosan was carried out by adding 45% (w/v) ammonium sulfate to 2 ml of (0.2%, w/v), chitosan solution mixed with 2 ml of *t*-butanol. The interfacial precipitate obtained after keeping these solutions at 40 °C for 1 h was suspended in 0.01 M acetate buffer pH 5.0. Spectra 1, chitosan solution (unlyophilized sample); 2, chitosan solution (lyophilized sample); 3, TPP-treated chitosan (unlyophilized sample); 4, TPP-treated chitosan (lyophilized sample).

lyophilized chitosan. Again, in both cases, shifts of λ_{max} to longer wavelength can be seen. A remarkable effect is that lyophilization of TPP-treated chitosan causes splitting of the absorption maximum into two. This is unusual and needs to be investigated with other polysaccharides. Nevertheless, just like interaction with magnesium ions, the changes in UV spectra upon lyophilization and TPP are cumulative. The shifts to longer wavelength in UV spectra are known to be associated with increase in hydrogen bonding. Both inter- and intra-molecular hydrogen bonding are possible in these cases. The increased hydrogen bonding may also account for unfavorable interaction with magnesium ions.

Fig. 5a and b shows the FT-IR spectra of chitosan (Aiba, 1993). The IR bands of TPP-treated chitosan shifted to lower frequency. This again fits in well with increase in intra- or inter-molecular hydrogen bonding which decreased the frequency of amine and hydroxyl bonds.

The increase in intra or inter-molecular hydrogen bonding on exposure to organic solvent *t*-butanol is not surprising. With reduced water activity, hydrogen bonding with water is likely to be replaced with amino and hydroxyl groups of the molecule forming hydrogen bonding among themselves. It is worth noting that the data in the case of proteins indicate that exposure to organic solvents induces such intra-molecular hydrogen bonding (Gupta, 2000).

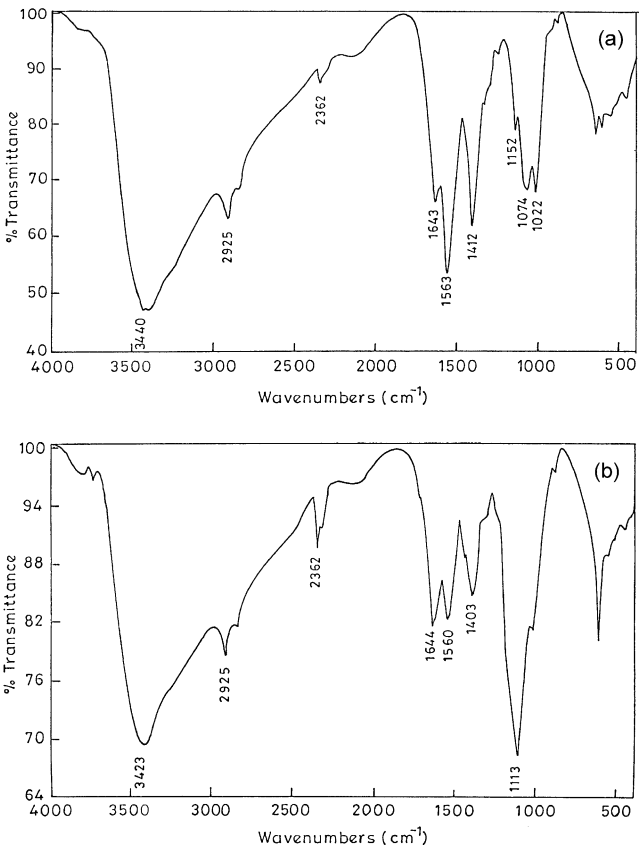

Fig. 5. Fourier-transform infrared (FTIR) spectra of chitosan; FT-IR spectra of chitosan (a) and TPP-treated chitosan solution (b). TPP of chitosan was carried out by adding 45% (w/v) ammonium sulfate to 2 ml of (0.2%, w/v), chitosan solution mixed with 2 ml of *t*-butanol. The interfacial precipitate obtained was suspended in 0.01 M acetate buffer collected after keeping these solutions at 40 °C for 1 h. Samples were lyophilized, mixed with KBr and homogenized in an agate mortar (1 mg of chitosan with 300 mg of KBr). The mixed powders were then pressed into pellets using a Perkin–Elmer kit and a 10-ton hydraulic pressure.

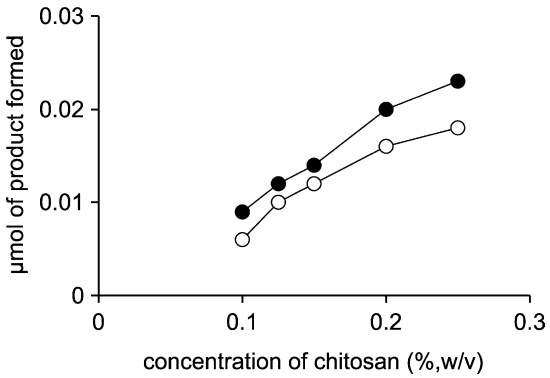

Fig. 6. Hydrolysis of chitosan by chitinase enzyme. Cabbage chitinase (10 U) was added to different concentrations of untreated chitosan (●-●) and TPP-treated chitosan solutions (○-○). The solutions were then incubated for 1 h at 40 °C. The amount of *N*-acetyl glucosamine formed was estimated colorimetrically by dinitrosalicyclic acid reagent.

3.2. Biodegradability of chitosan before and after TPP treatment

Fig. 6 shows the degradation of chitosan and TPP-treated chitosan by cabbage chitinase. The data indicates that after TPP treatment, chitosan is degraded less efficiently by chitinase. As indicated earlier, increased hydrogen bonding is likely to make the molecule somewhat more rigid and hence less susceptible to hydrolysis by chitinase. Again, such enhanced rigidity has been reported with proteins when exposed to the organic solvents (Gupta, 2000). Numerous industrial and biomedical applications of chitosan are reported (Muzzarelli et al., 1999). The decreased biodegradability of TPP-treated chitosan may have important applications in such areas. Thus, TPP treatment, per se appears to be a simple tool for altering biodegradability of the polymer.

4. Conclusion

To summarize, the present work shows that chitosan undergoes considerable changes in its solubility behaviour as a result of TPP. UV and IR spectroscopic data do indicate the structural changes in which this behavioral change originates. TPP treatment also results in decrease in the suspectiblity of chitosan to degradation by chitanase. Thus, TPP treatment may be a novel and simple tool to achieve desirable changes in properties of chitosan for some specific applications.

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