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A comparative study on depolymerization of chitosan by proteolytic enzymes

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Abstract

Proteolytic enzymes such as pepsin, papain and pronase caused depolymerization of chitosan, a co-polysaccharide of glucosamine and *N*-acetyl glucosamine residues linked by β-1,4-glycosidic bonds. The pH optima of these enzymes towards chitosanolysis were different from that towards their own substrates, indicating the involvement of pH-sensitive conformational changes during specific and non-specific activities. The depolymerization reaction obeyed Michaelis-Menten kinetics and K_m and V_{max} values indicated higher affinity of pepsin towards chitosan. The chitosanolytic products were low molecular weight chitosans (LMWC, a major product), chitooligomers (COs) as well as monomers. Low molecular weight chitosans had molecular weight in the range, 4.1–10.0 kDa depending on the reaction time. FT-IR indicated a decrease in the degree of acetylation of LMWC. GPC and HPLC of COs showed a degree of polymerization of 2–8 with a preponderance of di- to hexamer including monomers.

Keywords: Chitosanolysis; Proteases; Non-specificity; Low molecular weight chitosans; Chitooligomers

1. Introduction

Chitosan is a derivative of chitin, a by-product found in the offals of seafood processing industry. It is a copolymer of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) linked by β-1,4-glycosidic bonds and has a mean molecular mass of over 1 mDa, which corresponds to a chain length of approximately 5000 monomeric units, but there exists considerable variation between different batches (Synowieck & Khateeb, 2003). Chain length and degree of acetylation (DA) of chitosan are the most important factors influencing its functional value and biological activities. In spite of several potent bio-functionalities of chitosan, its effects in vivo are still ambiguous because of the low absorption on organism owing to its high molecular weight, which imparts high viscosity, even at low concentrations (Shon, 2001). Hence, an efficient in vivo utilization of chitosan requires its depolymerization into various low molecular weight products. The latter overcome these drawbacks and in addition, especially the chitooligomers, are useful as antitumor and immuno-stimulating agents.

Low molecular weight chitosans (LMWC) with an average molecular weight in the order of 5–20 kDa seem to have enhanced functional-biochemical significance compared to chitosans of higher molecular weights. According to Kondo, Nakatani, and Hayashi et al. (2000), 20 kDa chitosans prevent progression of *diabetes mellitus* and exhibit higher affinity for lipopolysaccharides than 140 kDa chitosan. LMWC of 5–10 kDa showed stronger growth inhibitory effect on several pathogens including *Fusarium oxyporum*, *Phomopsis fukushi*, *Alternaria alternata* (Felt, Carel, Bachni, Buri, & Gumy, 2000). Ikeda et al. (1995) demonstrated that LMWC, with an average molecular weight more than 5 kDa prevented the rise of serum cholesterol of rats fed cholesterol-enriched diets for 14 days. LMWC also reduced the incidence of early pre-neoplastic markers of colon carcinogenesis (Torzsas, Kendall, Sugano, Iwamoto, & Rao, 1996). Suzuki et al. (1986) found that chito-hexamer suppressed Sarcoma-180 and Meth-A tumor growth in mice. Chitotriose exhibited maximum inhibitory

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effect towards angiotensin converting enzyme (ACE) (Shon, 2001), whereas *N*-acetylglucosamine (GlcNAc) showed beneficial effect during the treatment of osteoarthritis and gastritis (Richardson, 1999).

Chitosanolysis can be achieved by chemical means using HCl, H_2O_2 and HNO_2 that, however, do not lend themselves to easy control and often results in modification of the products. Enzymatic means based either on specific (chitosanase) or non-specific enzymes (Cheng & Li, 2000; Ogawa, Chrispinas, Yashida, Inoue, & Kariya, 2001; Santos, Guirardello, & Franco, 2001) are the preferred alternatives. However, the high cost and unavailability of chitosanase in bulk, inhibits its use in industrial scale. Recently, several commercial non-specific enzymes were found to catalyze the cleavage of glycosidic linkages in chitosan, although their purity was in doubt (Kittur, Vishu Kumar, & Tharanathan, 2003; Pantaleone, Yalpani,& Scollar, 1992; Yalpani & Pantaleone, 1994). Whatever may be the mechanism of this non-specific catalysis, the reaction is of interest as they are commercially feasible and the enzymes are inexpensive and easily available. Another advantage of non-specific chitosanolysis is the production of LMWC in higher yields due to their low specificity or non-specificity. Though reports are available on the unusual susceptibility of chitosan to various enzymes, information regarding the conditions for optimum depolymerization is lacking. The present study fills the gap in providing kinetic parameters of non-specific proteases in chitosan depolymerization and obtaining products in higher yields. The latter were subjected to structural characterization with regard to molecular weight (M_W) and degree of acetylation (DA), which together play an important role in their solubility and bio-functionalities.

2. Materials and methods

2.1. Materials

Enzymes (pepsin from porcine stomach mucosa-EC. 3.4.23; papain from papaya latex-EC. 3.4.22.2; pronase from *Streptomyces griseus*-EC. 3.4.24.4) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals used were of highest purity available.

2.2. Production and characterization of chitosan

Shrimp chitin was subjected to heterogeneous *N*-deacetylation to obtain chitosan (Kittur, Kumar, & Tharanathan, 1998), which was further purified by dissolving in 1% acetic acid followed by filtration to remove suspended particles and finally precipitated with 2% sodium carbonate. The precipitate (native chitosan) was washed, freeze-dried.

(a) Molecular weight (M_W) determination: M_W of chitosan was determined by (i) viscometry (Wang, Shuqin, Shuqing, & Wen, 1991) using Ostwald's viscometer and Mark-Houwink's equation, $(\eta) = KM_W^a$ where (η) = intrinsic viscosity, $K = 3.5 \times 10^4$, $a = 0.76$, and (ii) GPC on Sepharose CL-4B column (Sigma Chemical Co., St. Louis, MO, USA, bed volume-180 ml) pre-calibrated with dextran and chitosan standards using acetate buffer (0.5 M acetic acid + 0.2 M sodium acetate, pH 4.5) as the solvent/eluant (Sekiguchi et al., 1994).

(b) Fourier transform infrared spectroscopy: FT-IR spectral studies were performed on a Perkin Elmer spectrum 2000 spectrometer under dry air at room temperature using KBr pellets. Chitosan (4 mg) was mixed with 200 mg KBr and 40 mg of the mixture was pelletized. Reproducibility of the spectra was verified on two preparations and the degree of acetylation (DA) was determined using the formula, $(A_{1655\ cm^{-1}}/A_{3450\ cm^{-1}}) \times 100/1.33$, where A is the absorbance at these wavelengths, calculated from baseline drawing (Baxter, Michael, & Taylor, 1992).

2.3. Enzyme purity

Enzyme purity was determined by polyacrylamide gel electrophoresis (both native and SDS-PAGE $\%T = 10$ and 12.5, respectively, where T is the concentration of the monomers, and proteins were revealed by Coomassie Brilliant Blue) at pH 8.8 (Laemmli, 1970). Their M_W was calculated using protein markers.

2.4. Determination of the specific activity

Proteolytic activity was determined by treating the enzymes with 2.5% hemoglobin/casein at optimum conditions (Table 2) and estimating the TCA soluble peptides released (specific activity, Unit = absorbance at 280 nm/reaction time × mg protein in the reaction mixture). For chitosanolytic activity, enzymes were incubated with 1% chitosan in aqueous acetic acid (1%) in the ratio of 1:100 at optimum conditions. After arresting the reaction by heating the mixture and adding equal volume of 2 N NaOH followed by centrifugation, the reducing groups released in the supernatant was estimated by modified Schales method (Activity, Unit = μmoles of reducing equivalents released per minute per mg of protein at optimum conditions) (Imoto & Yagishita, 1971).

2.5. Kinetic study of chitosanolysis

The pH optimum was determined using chitosan solution (1%) at pH between 1.5 and 6.0 (pH was adjusted using 0.1 N HCl/0.1 N NaOH and above pH 6.5, chitosan was insoluble). The temperature optimum was determined by carrying out the reaction between 20 and 60 °C. The Michaelis constants, K_m and V_{max} were evaluated from

the double reciprocal plot of the initial velocity versus substrate concentrations.

2.6. Isolation and characterization of the products

Chitosan solution (1%, dissolved in 1% acetic acid and pH adjusted with 0.1 N HCl/NaOH) was treated with enzymes in the ratio, 100:1 (w/w), incubated for different periods at optimum conditions. The reaction was arrested by heat denaturing the enzyme and adjusting the pH of the reaction mixture to 7.0 using 2 N NaOH. The precipitate (LMWC) obtained after centrifugation was washed and dialyzed against water using a membrane having M_W cut-off 2 kDa (Sigma Chemicals Co, USA) and freeze-dried.

M_W of LMWC was determined by GPC on Biogel P30 column (Bed volume 100 ml) and HPLC on E-linear and E-1000 columns (3.9×300 mm, Waters Associates, Ireland) connected in series, using acetate buffer (0.5 M acetic acid + 0.2 M sodium acetate, pH 4.5) as the mobile phase and RI detector. Dextran standards (10–70 kDa) were used to calibrate the GPC and HPLC columns. LMWC were characterized for DA by IR spectroscopy.

The supernatant containing reducing groups (chitooligomeric + monomeric mixture) was concentrated by freeze-drying and subjected to HPLC on an aminopropyl column (3.9×300 mm, Waters Associates, Ireland) using acetonitrile: water (70:30) as the mobile phase at a flow rate of 1.0 ml min^{-1} and detected using a RI detector. Chitooligomers, GlcN and GlcNAc were used as standards (Sigma Chemical Co., USA).

3. Results and discussion

3.1. Characterization of chitosan

M_W of chitosan was found to be ~71±2 kDa by both viscometry and GPC and the DA was ~26% as determined by FTIR spectral data (Fig. 1). IR spectra also indicated chitosan to have a β-conformation, as evidenced by the appearance of a single broad peak at around 3371 cm^{-1}, which is characterized by the individual chains arranged in a parallel manner and resulting in weak hydrogen bonding network and hence an easy penetration by the enzymes.

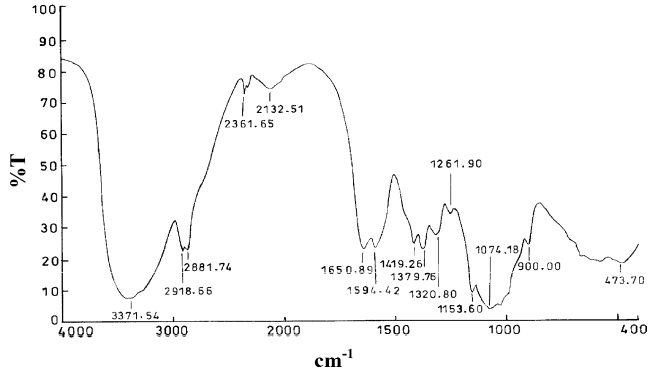

Fig. 1. Infrared spectrum of native chitosan.

Table 1
Activity of proteases towards chitosan as well as hemoglobin

Enzyme	Chitosanolysis (Units)[a]	Proteolysis (Units)[b]
Pepsin	4.98	13000
Papain	1.78	10.0
Pronase	1.16	4.14
Chitosanase (*Streptomyces griseus*)	50.0	–

[a] Unit = μmoles of reducing equivalents released/minute/mg of protein.
[b] Unit = absorbance at 280 nm/reaction time × mg protein in the reaction mixture.

3.2. Enzyme purity and M_W

Although many workers demonstrated chitosanolysis by several commercial non-specific enzymes, purity of the enzyme was in doubt (Pantaleone, Yalpani, & Scollar, 1992; Yalpani & Pantaleone, 1994). Earlier, we showed the association of chitosanolytic activity with an isozyme of pectinase from *Aspergillus niger* (Kittur, Vishu Kumar, Gowda, & Tharanathan, 2003). To rule out the above doubt, in the present study, the commercial proteolytic enzymes were subjected to native-PAGE, where pepsin and papain showed a major band along with minor ones and pronase showed a single band. Pepsin and papain were further subjected to GPC on Biogel P-30 (Bio Rad laboratories, CA, USA; column dimensions-90 cm length × 0.8 cm i.d., and elution performed with water) and the fraction corresponding to the major band was collected, concentrated and checked for its both proteolytic and chitosanolytic activities (Table 1). Purity was ascertained by SDS-PAGE (Fig. 2) and M_W calculated using protein markers were 34±1, 22±1 and 20±1 kDa for pepsin, papain and pronase, respectively.

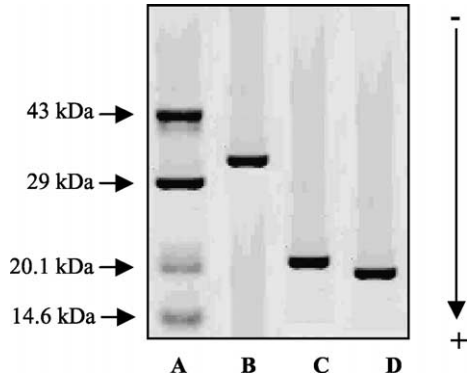

Fig. 2. SDS-polyacrylamide gel electrophoregram, (A) Protein markers, (B) Pepsin, (C) Papain and (D) Pronase.

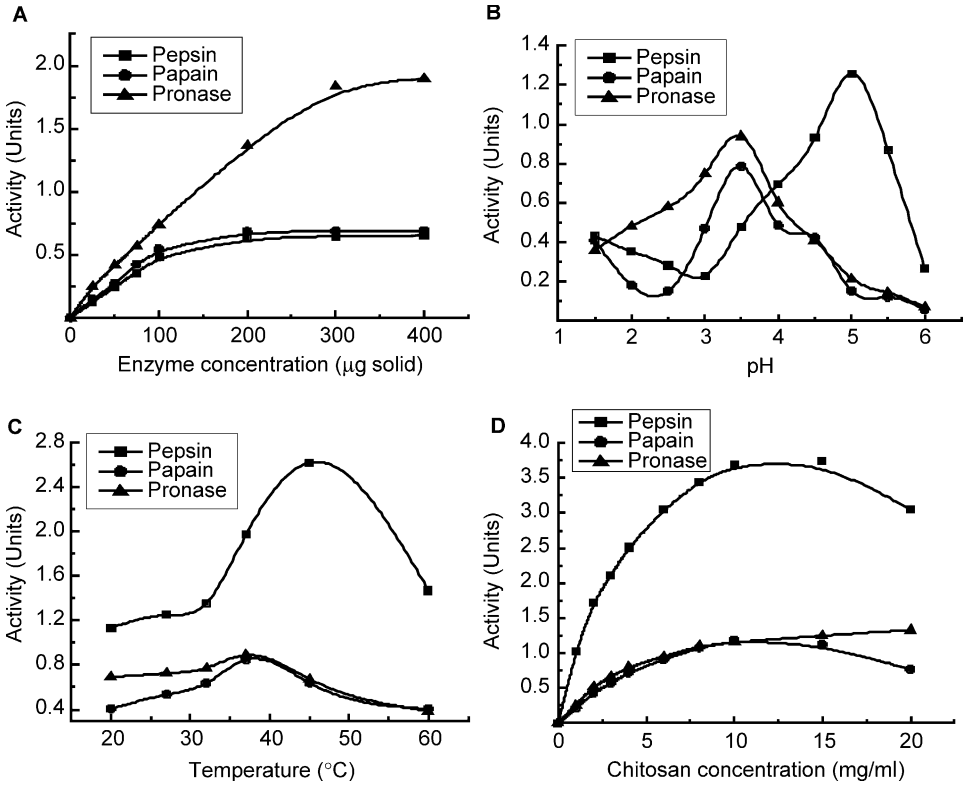

Fig. 3. Kinetic study of chitosanolysis by proteases, effect of enzyme concentration (A), pH (B), temperature (C) and substrate concentration (D).

3.3. Kinetic analysis of chitosanolysis by proteolytic enzymes

Fig. 3A depicts the effect of enzyme concentration on depolymerization of chitosan. Here, 1 ml chitosan solution (dissolved in 1% acetic acid, 10 mg, pH 3.4) was incubated with 25–400 μg of enzyme (GPC purified solid) at ambient temperature (27 ± 1 °C) for 1 h. The rate of catalysis increased in direct proportion to the concentration of the enzymes up to 100 μg enzyme, from which it could be concluded that chitosanolysis by these non-specific enzymes appears to follow Michaelis-Menten kinetics (Nordtveit, Varum, & Smidsrod, 1994). Pepsin and papain showed saturation at enzyme: chitosan ratio of 1:100 and it was 3:100 for pronase.

Table 1 represents the activity of the enzymes towards chitosanolysis as well as their own substrate (i.e. hemoglobin). Although proteolytic enzymes caused depolymerization of chitosan, chitosanase did not show any proteolytic activity towards hemoglobin. Compared to papain and pronase, pepsin showed better chitosanolytic activity. From the data, it was evident that there was no comparison between the specific and non-specific activities of the proteolytic enzymes, and even chitosanolytic activity of the latter were much lower than that of chitosanase. But the major advantage with non-specific enzymes was the production of LMWC in higher yields, which was lacking in chitosanase due to its specificity to produce oligomers of DP, 2-3, rather than LMWC. In addition, LMWC of desired M_W could be custom made by manipulating the reaction conditions.

Kinetic parameters of the non-specific chitosanolysis are given in the Table 2 and Fig. 3B and C represents the pH and temperature optima of the enzymes towards chitosanolysis. As chitosan is insoluble beyond pH 6.5, we could study

Table 2
Kinetic parameters of three proteases towards chitosanolysis

Enzyme	Optimum Chitosanolysis (non-specific activity)		Specific activity		Chitosanolysis	
	pH	Temp. (°C)	pH	Temp. (°C)	K_m [a]	V_{max} [b]
Pepsin	5.0	45	1.5–2.0	37	3.02	348.58
Papain	3.5	37	6.0–7.0	27	7.14	288.48
Pronase	3.5	37	7.5	37	5.21	138.55

[a] (mg ml^{-1}).
[b] nmoles min^{-1} mg^{-1}.

the effect of pH only in the range of 1.5–6.0. The extremities in the pH optima towards specific and non-specific activities could be due the different conformations. Close examination of the pH optimum curves of pepsin and papain showed, although not prominent, a slight increase in the activity in the pH range of 1.5–2.0 compared to pH 3.0 (Fig. 3B). This could be due to the presence of yet another conformation, active towards chitosanolysis. All the three proteases obeyed Michaelis-Menten kinetics (Fig. 3D) and their K_m and V_{max} values are given in the Table 2. K_m is the measure of the strength of ES complex; a higher K_m indicates weak binding, whereas the lower value, strong binding. Pepsin showed a lower K_m value indicating its higher affinity towards chitosan resulting in the maximum depolymerization as evidenced by its V_{max}.

Although papain continued its chitosanolytic activity up to 20 mg ml^{-1} of chitosan, concentrations beyond this was not suitable for study as the solution was highly viscous, thus affecting penetration by the enzyme. A lowering of chitosanolysis was observed in pepsin and pronase at chitosan concentration greater than 10–12 mg ml^{-1} suggesting the possibility of product or substrate inhibition (Fig. 3D). The possibility of product inhibition was ruled out by adding chitooligomeric mixture (0.1 mM), one of the products, to the reaction mixture, wherein there was no change in the activity, suggesting the susceptibility of the enzymes to substrate inhibition. Substrate inhibition has been described for a variety of enzymes and several mechanisms have been proposed to explain this effect (Clenland, 1979). The most convincing explanation could be that a second substrate molecule binds non-productively to the active site thereby inhibiting the productively bound one (Sanchez-Ferres, Franscisco & Francisco, 1993).

3.4. Characterization of the products—low molecular weight chitosan (LMWC)

The M_W of LMWC determined by GPC and HPLC were in accordance with each other (Table 3). Appearance of a single peak in the HPLC profiles was indicative of molecular homogeneity of the LMWC (Fig. 4). Unless stated otherwise, IR-spectra (Fig. 5) represent that of LMWC obtained after 5 h reaction time.

Pepsin showed chitosanolytic activity up to 5–6 h beyond which there was a decline. The M_W of LMWC obtained using pepsin was in the range of 4.6–10 kDa depending on the reaction time (1–5 h). Absence of a sharp and convoluted spectral band around 3600–3000 cm^{-1} in the IR-spectra of both native chitosan (Fig. 1) and LMWC (5 h samples, Fig. 5A) indicated the absence of free –OH groups and involvement of both -3-OH and –6-CH_2OH in intra- and intermolecular hydrogen bonding (Kittur, Vishu Kumar, & Tharanathan, 2003). The band above 3000 cm^{-1} was centered at 3371 cm^{-1} for native chitosan and 3380 cm^{-1} for LMWC. Such a shift to a higher frequency in the latter was indicative of an increase in the ordered structure (Focher, Naggi, Tori, Cosani, & Terbojevich, 1992). A peak near 1320 cm^{-1} corresponds to GlcNAc residues (Brugnerotto et al., 2001) and a decrease in its height in case of LMWC compared to native chitosan (Fig. 1 and Table 3) indicated decrease in the DA, which was further confirmed by progressive weakening of the band near 1655 cm^{-1} (amide I). HPLC characterization of the supernatant showed abundance of the monomers (both GlcN and GlcNAc) along with dimer to octamer (Fig. 6A), which was in good agreement with the GPC profile (chromatogram not shown).

Chitosan is characterized by four types of glycosidic linkages, viz., –GlcN–GlcN–, –GlcN–GlcNAc–, –GlcNAc–GlcN– and –GlcNAc–GlcNAc–. When treated with pepsin, there was a sharp decrease in the viscosity of chitosan solution indicating the endo- action of the enzyme, whereas the release of both GlcN and GlcNAc into the supernatant was indicative of its exo-action. When chitooligomeric mixture was treated with hexosaminidase, an enzyme specific for the release of GlcNAc from non-reducing end, there was an increase in the reducing equivalents, suggesting the action of pepsin on –GlcN–GlcNAc–/–GlcNAc–GlcNAc–, resulting in products with GlcNAc in the non-reducing end.

Papain showed chitosanolytic action even after 24 h of incubation, which is of interest during continuous production and isolation of depolymerization products. Immobilized papain preparations have successfully been used for chitosan depolymerization and after the reaction, retrieval of the immobilized enzyme for further use was possible, although the yield of LMWC and its M_W were low (Terbojevich, Cosani, & Muzzarelli, 1996). Surprisingly, although there was continuous activity, maximum depolymerization occurred only in the initial hour after which it

Table 3
Molecular parameters and percent yield of the products of non-specific chitosanolysis*

Enzyme	LMWC			Chitooligomers		Monomers (yield (%))
	M_w (kDa)*	Yield (%)	DA	DP	Yield (%)	
Pepsin	4.6–10.0	78–84	13–19	2–7	10–14	4–6
Papain	4.1–5.6	75–82	17–19	2–5	8–10	5–8
Pronase	8.5–9.5	74–80	14–19	2–6	12–15	2–4

*Range indicates different reaction time (1–5 h).

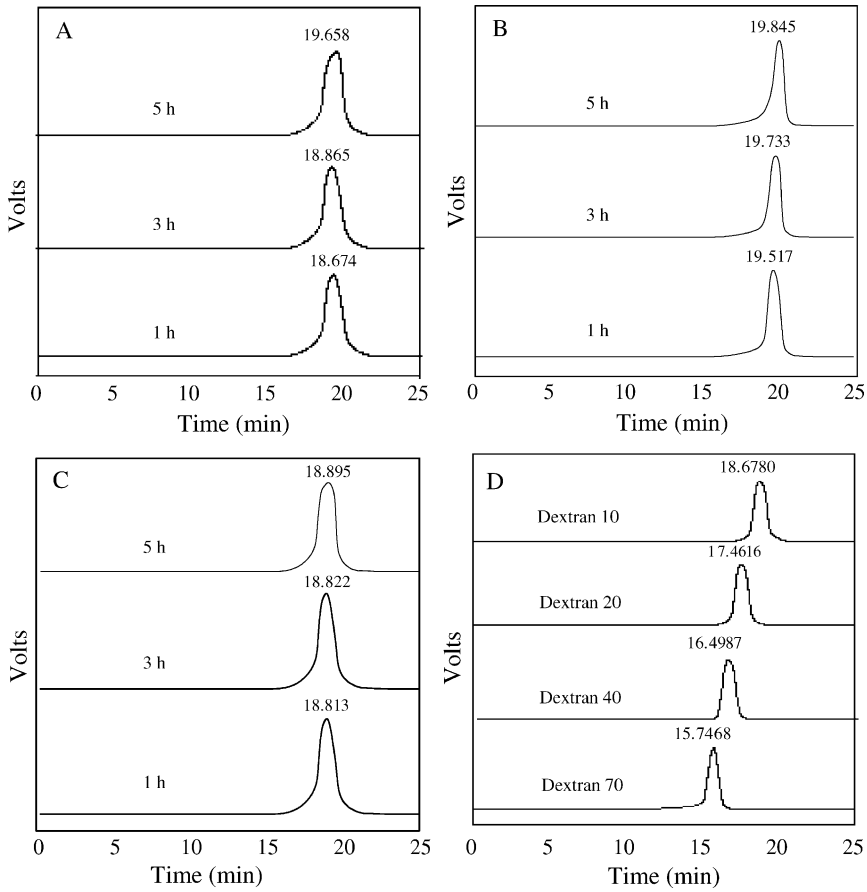

Fig. 4. HPLC of LMWC obtained using (A) Pepsin, (B) Papain, (C) Pronase and (D) Dextran standards (10–70 kDa).

was not much appreciable as evidenced by the M_W of the LMWC (Table 3) indicating the action of papain on chitosan of precise M_W. Of the three proteases used, papain showed maximum depolymerization of chitosan in the initial hour as evidenced by the M_W of LMWC. Like pepsin, papain also resulted in the decreased DA of LMWC (Table 3) as evidenced by the IR spectral data (Fig. 5B). Appearance of band at 3393 cm^{-1} in the latter was indicative of its orderliness and decrease in the 1320 cm^{-1} peak height further confirmed decrease in the DA (Fig. 5B). The supernatant showed both GlcN and GlcNAc along with dimer to hexamer (Fig. 6B), indicating the action pattern of papain to be similar to that of pepsin.

Pronase lost its stability at pH 3.5 (optimum pH for chitosanolysis) after 4 h of incubation and showed maximum depolymerization of chitosan in the initial hour (Table 3). IR spectra of LMWC (Fig. 5C) indicated an increase in the orderliness and a decrease in DA, in support of both deacetylation along with depolymerization. The supernatant oligomeric mixture contained mainly dimer to hexamer along with only GlcNAc (Fig. 6C). Addition of hexosaminidase to chitooligomeric mixture did not result in the release of more reducing groups, indicating pronase action on -GlcNAc–GlcN- type linkage and resulting in products having GlcNAc at the reducing end. This was further confirmed by using $(GlcNAc)_2$ and $(GlcNAc)_3$ as substrates, where the activities were found to be 0.09 and 0.18 Units, respectively. All these data supported the preference by pronase of GlcNAc at the non-reducing ends, i.e. -GlcNAc–GlcNAc type linkage, which results in the release of only GlcNAc and products with GlcNAc at the reducing ends. The results also document affinity of pronase towards chitosan with higher DA. Although the LMWC obtained showed a decrease in DA, it was essentially due to the release of mono- and oligomers into the supernatant indicating the action of proteolytic enzymes only on glycosidic bonds, leaving apart the *N*-acetyl groups intact.

3.5. Solubility of LMWC

Solubility plays an important role in the biological application of any compound and this study was performed for LMWC and chitosan according to the method described by Qin et al. (2003). Although the M_W of LMWC was <10 kDa, it was not readily soluble in aqueous medium (water-solubility of LMWC, M_W 9–10 kDa—72–75%, 4–5 kDa—58–63%), instead it required a very dilute acidic medium for complete solubilization (LMWC, 100% solubility in 0.01% acetic acid compared to 1% acetic acid for

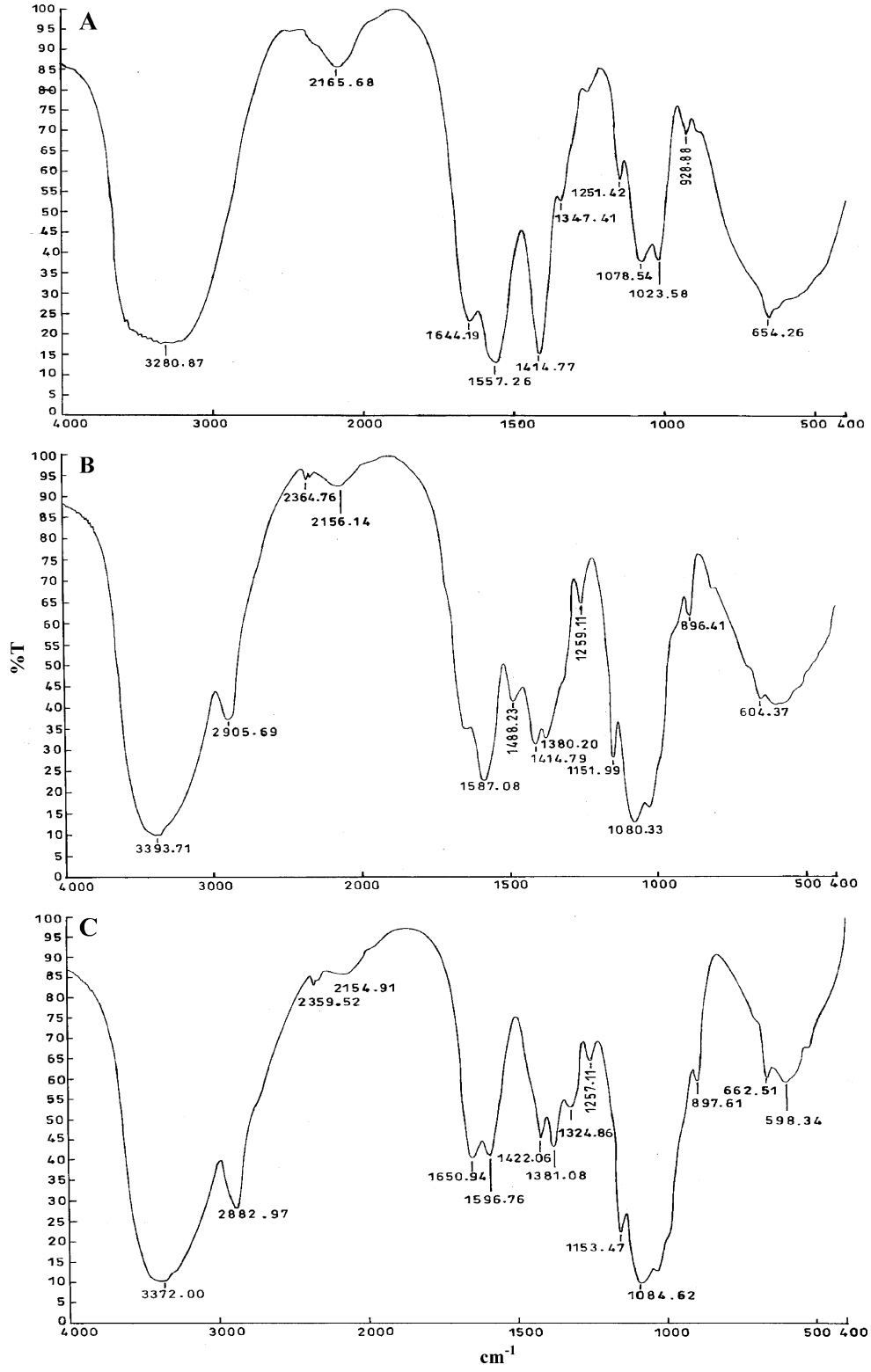

Fig. 5. IR-spectra of LMWC obtained using (A) Pepsin, (B) Papain and (C) Pronase after 5 h incubation.

chitosan and in 0.01% acetic acid, only 13% chitosan was soluble). The present observation was contrary to the earlier reports that decrease in the M_W of chitosan is associated with increased solubility, attributed to decreased intermolecular interactions, such as van der Walls forces and hydrogen bonds, provided that the DA did not change after degradation (Qin et al., 2003). But chitosanolysis by proteases resulted in the decreased DA as evidenced by

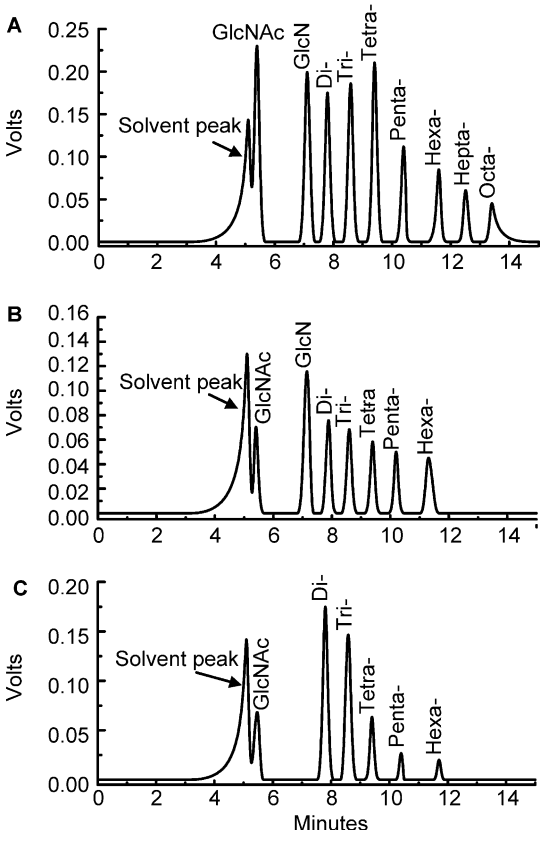

Fig. 6. HPLC of chitooligomeric mixture obtained using (A) Pepsin, (B) Papain and (C) Pronase.

the IR-spectra and hence the observed solubility could probably be due to the exposure and conversion of $-NH_2$ groups on LMWC to the sodiated form (Na^+, added to terminate the reaction), which could result in a different molecular conformation. Although, to arrest the chitosanolysis by proteases two volume of acetonitrile was used by Cheng and Li (2000) instead of equal volume of 2 N NaOH as in the present study, the drawbacks were the cost of solvent and flash treatment of the supernatant containing mono-oligomeric mixture, at elevated temperature, which results in Maillard products and hence difficulty in producing both LMWC and chitooligomers-monomers in one step. The use of acetonitrile in the present study resulted in the LMWC, which were readily soluble, in support of sodiated-form of LMWC. Although 0.01% acetic acid was necessary for LMWC solubilization, the pH of resulting solution was near neutrality ($\sim$6.8) and it did not hamper the biological activity of the LMWC (Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2003).

In conclusion, non-specific chitosanolysis by commercial proteases is of value for bulk production of low molecular weight degradation products, which find applications in various fields such as medicine, agriculture and biotechnology. It was possible to obtain the LMWC of desired M_W along with oligomers of higher DP by altering the reaction conditions. LMWC find applications mainly as antimicrobial and hypolipidemic-hypocholesterolemic agents whereas chitooligomers of DP$>$6 are useful as antitumor and antimicrobial agents.

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