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In vitro Binding Capacity of Cholesterol and Bile Salts by Partially Depolymerized Chitosans

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Abstract: Orthogonal design was used to optimize the binding capacity of Sodium Cholate (SC) and Sodium Deoxycholate (SD) by nine partially depolymerized chitosans obtained from a native chitosan using sodium nitrite ($NaNO_2$). The effects of sodium treatment on depolymerization of chitosan were investigated by measuring the molecular weight, viscosity and reducing sugar of chitosan. Depolymerization for 3 h reduced the molecular weight of the chitosan by 91% (26 kDa) compared to the native chitosan (458 kDa) with a decrease in viscosity of the chitosan solution and increased reducing sugar. The binding capacity of total cholesterol, SC and SD by chitosan samples were measured *in vitro* by enzymatic and HPLC methods, respectively. The optimized conditions were sodium nitrite (0.4%, w/v), acetic acid (1 and 2%, v/v), chitosan (3%, w/v) and reaction time (1 h) at room temperature, sodium chelate 69 μmol g^{-1} chitosan and deoxycholate125 μmol g^{-1} chitosan. The strongest binding capacity of sodium deoxycholate and total cholesterol was observed with partially depolymerized chitosan (average molecular weight 52 kDa) compared to the native chitosan.

Key words: Chitosan, partially depolymerization, cholesterol, bile salts, binding capacity

INTRODUCTION

Chitosan is derived by alkaline deacetylation from chitin, an abundant polymeric product of natural biosynthesis, found especially in crustaceans (Ylitalo *et al*., 2002). It can be defined as a dietary fiber since it is a polysaccharide, which cannot be digested by digestive enzymes of humans (Razdan and Pettersson, 1996; Gallaher *et al*., 2000). It is claimed to control obesity and to lower serum cholesterol levels (Jing *et al*., 1997; Wuolijoki *et al*., 1999; Gallaher *et al*., 2002). Many of the functions of chitosan are attributable to its cationic structure, the most important being its hypocholesterolemic action. Chitosan interferes with normal emulsification of neutral lipids, i.e., cholesterol and other sterols, by binding them with hydrophobic bonds. This electrostatic and hydrophobic bonding causes the formation of large polymer compounds, which are weakly broken down by the digestive processes in man (Ylitalo *et al*., 2002). Chitosan is also known to inhibit the absorption and enterohepatic circulation of bile acids, leading to the decrease of plasma cholesterol levels accompanied by an increase in compensatory oxidative synthesis of the bile acids from hepatic cholesterol (Thongngam and McClements, 2005).

It was reported that the hypocholesterolemic activity of chitosan was better when DD was high (90% deacetylated), which could have been due to the electrostatic force between chitosan and anionic as fatty acids and bile acids (Vahouny *et al*., 1983; Deuchi *et al*., 1995). Molecular weight is also

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related to the hypocholesterolemic effect of chitosan and the mechanism might be similar to that of certain dietary fibers, such as guar gum and pectin (Vahouny *et al*., 1983).

Although the influences of chitosans with different molecular weigh or viscosity on hypocholesterolemic action have been studied, results are inconclusive. LeHoux and Grondin (1993) reported that high molecular weight chitosan were found to be less effective as hypocholesterolemic than low molecular weight chitosan, but Sugano *et al*. (1988) and Chiang *et al*. (2000) demonstrated different results . A major reason for this discrepancy seems to be the difference not only in the molecular weight, but also in the deacetylation degree.

Unfortunately, the high viscous nature of chitosan restricts its use as a constituent of physiologically functional foods. This obstacle can be overcome by using chitosan derivatives, obtained by partial depolymerization, which retain positive properties of the original preparation (Thanou *et al*., 2001). Currently, low-molecular-weight chitosan is produced by chemical or enzymatic hydrolysis. In chemical depolymerization, various substances such as hydrochloric acid, nitrous acid, phosphoric acid and hydrogen fluoride have been used to obtain low-molecular-weight chitosan. Sodium nitrite is a common depolymerizing agent which easily depolymerizes chitosan under mild conditions. However, care must be taken during handling of liquid chitosan due to the susceptibility to further degradation. The objective of this study was to investigate the effects of sodium nitrite treatment on chitosan. Furthermore, the *in vitro* binding capacity of total cholesterol and bile salts (chelate and deoxycholate) of native and partially depolymerized chitosans were also assessed.

MATERIALS AND METHODS

Chitosan, derived from fresh shrimp shell, molecular weight 457.98 kDa; deacetylation degree 77.92%, used in this study was prepared by the method of degradation with acetic acid in the laboratory as earlier described by Zhou *et al*. (2006). Sodium chelate, sodium deoxycholate, sodium taurocholate and cholesterol were purchased from Sigma Company (St. Louis, MO, USA). Total cholesterol assay kit was purchased from Zhejiang Dongou Bioengineering Co. Ltd., China. All the other reagents that were used for the experiments were analytical grade.

Determination of Deacetylation Degree, Intrinsic Viscosities and Viscosity Average Molecular Weight

The degree of deacetylation (DD) was determined, according to a colloid titration method (Cho *et al*., 1998). Briefly, dried chitosan was dissolved in 0.2M CH_3COOH/0.1M CH_3COONa solution to a final concentration of 0.02% (w/v) and 0.0025 M polyvinyl sulfate potassium salt (PVSK) and 0.1% (w/v) Toluidine Blue (TB) were added, as titrant and indicator, respectively. The DD was calculated as follows:

$$NH_2\% = \frac{N \times (V_1 - V_2) \times M}{5 \times C} \times 100 \quad (1)$$

where, N is the concentration of PVSK (M), M is equivalent to 161.15 which is the molecular weight of a glucosamine residue, C is the concentration of chitosan (g mL^{-1}), V_1 is the volume of PVSK (mL) when titrating the chitosan and V_2 is the volume of PVSK when titrating the deionized water, which is a control (mL). Then,

$$DD\% = \frac{NH_2\%}{9.94\%} \times 100 \quad (2)$$

where, 9.94% is the theoretical NH_2 percentage of chitosan.

Table 1: The variables investigated and their levels

	Levels of each variable		
Variables	1	2	3
Concentration of chitosan (w/v %)	1.5	2.25	3.0
Concentration of acetic acid (v/v %)	1.0	1.50	2.0
Concentration of sodium nitrite (w/v %)	0.2	0.40	0.6
Depolymerization time (h)	1.0	2.00	3.0

The intrinsic viscosity ([η]) of each chitosan sample is determined using a capillary viscometer (Ubbelohde type, 30°C) at various polymer concentrations. Prior to measurement of flow times, chitosan solutions were dissolved in 0.1 mol L^{-1} CH_3COONa-0.2 mol L^{-1} CH_3COOH solution. The intrinsic viscosities [η] were calculated according to the following equation (Wang *et al*., 2005):

$$[\eta] = \frac{\eta_{sp} + 3\ln \eta_r}{4c} \quad (3)$$

where, η_r, η_{sp} refer to the relative viscosity and the incremental viscosity, respectively and c is the concentration of chitosan (g mL^{-1}).

The viscosity-average molecular weight was calculated using Mark-Houwink equation relating to intrinsic viscosity:

$$[\eta] = K_m M_v^a \quad (4)$$

where, $K_m = 1.81\times10^{-3}$ and a = 0.93 are the empirical viscometric constants that are specific for a given polymer, solvent and temperature (Khan *et al*., 2000).

Preparation of Partial Depolymerized Chitosans

Chitosan (1.5-3.0% w/v) dissolved in CH_3COOH (100 mL, 1-2% v/v) solution were partially depolymerized by chemical reaction for 1-3 h with 10 mL of $NaNO_2$ (0.1 to 0.6% in water) as shown in Table 1.

The aqueous solution of $NaNO_2$ was slowly dropped into the dissolved chitosan solution with stirring for 30 min, followed by holding for 0.5-2.5 h under magnetic stirring at room temperature. After reaction time, the partially depolymerized chitosans were precipitated by raising the pH to 9 with 4 M NaOH, then were washed thoroughly with 70% methanol, dialyzed for 24 h under stirring with three changes of water and freeze-dried (FD-5 freeze dryer. Shanghai, China).

Determination of Reducing Sugar Content

The reducing sugar content was determined using the 3, 5-dinitrosalicylic (DNS) reagent method (Miller, 1959). The intensity of developed color was measured at 510 nm using a UV-2100 spectrophotometer. D-glucosamine was used as a standard and the reducing sugar content expressed as mg mL^{-1} chitosan.

The percentage decrease in viscosity was calculated as follows:

$$\text{Viscosity decrease (\%)} = \frac{\eta_0 - \eta_t}{\eta_t}\times 100 \quad (5)$$

where, η_0 refers to the intrinsic viscosity of initial chitosan, η_t refers to the intrinsic viscosities of chitosan degraded solution at different times.

In vitro Cholesterol-Binding Capacity

The binding capacity of cholesterol with chitosan *in vitro* was measured by the method of Nagaoka *et al*. (2001) with some modifications. Cholesterol micellar solutions (1 mL) containing sodium taurocholate (10 mM), cholesterol (0.4 mM), oleic acid (1 mM), NaCl (132 mM), sodium phosphate (15 mM, pH 7.4) was prepared by sonication. Fifty-six milligrams of chitosan samples was added to 5 mL of micellar solution. Cholestyramine was used as a positive control and the micellar solution without chitosan was used as a substrate blank to calculate the recovery. Each sample was prepared in triplicate. The mixtures were then incubated for 2 h at 37°C in shaker water bath and then centrifuged at 16000 rpm for 20 min at 37°C. The supernatant was collected for the enzymatic determination of cholesterol. The binding capacity of chitosan was expressed as a milligram of bound cholesterol per gram of chitosan.

In vitro Bile Salts Binding Capacities

The *in vitro* binding procedure of native and depolymerized chitosans to bile salts was measured by the method of Hu *et al*. (2008). Each bile salt (as substrate) was dissolved in physiological saline (pH 6.5) to make a 2 μmol mL^{-1} solution. Fifty milligrams of the chitosan sample were added to each 5 mL bile salt solution and the individual substrate solution without samples was used as blank. Then tubes were incubated for 1 h at 37°C in shaking water bath. Mixtures were centrifuged at 60,000 g for 20 min at 10°C in an ultracentrifuge (Model J-26XPI, Beckman, USA). The supernatant was removed into a second set of tubes and frozen at -20°C for bile salts analysis. Bile salts were analyzed using HPLC (Model 1525, Waters, USA) on a Sun fire C_{18} column (4.6×150 mm i.d., 5 μm particle size, Waters, USA), maintained at 35°C. The injected sample volume was 10 μL for each bile salt. The bile salts were eluted with methanol: 0.04 g dL^{-1} formate acid (88:12) at a flow rate of 0.8 mL min^{-1} for 10 min. The absorbance of the eluate was monitored continuously at 220 nm (Model 2996 PDA detector, Waters, USA).

Statistical Analysis

Mean separation and significance for correlation were analyzed using the SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) software package.

RESULTS AND DISCUSSION

Effect of Sodium Nitrite on Molecular Weight, Viscosity and Reducing Sugar of Chitosan

Molecular weight is one of the key factors governing the functional properties of chitosan. In general, the intrinsic viscosity of linear macromolecular substances is related to the molecular weight or degree of polymerization (Chen and Tsaih, 1998). Average molecular weights of sodium nitrite-treated native chitosan were calculated from measured intrinsic viscosities using Eq. 4. Figure 1 shows the molecular weight changes of chitosan without and with sodium nitrite. The molecular weight of chitosan without sodium nitrite slightly decreased with depolymerization time (Fig. 1). The molecular weight of sodium nitrite-treated chitosan shows that the depolymerization occurred mainly in the first hour and then slowed down significantly (Fig. 1). This behavior was expected since the concentration of sodium nitrite decreased with the reaction time. The molecular weight of chitosan was significantly reduced by 81% (58 kDa) in the first hour of sodium nitrite treatment and further depolymerization for 3h reduced the molecular weight of chitosan up to 91% (26 kDa) compared to the native chitosan. On the other hand, depolymerization without sodium nitrite did not significantly affect the molecular weight of chitosan. Decrease in average molecular weight of native chitosan with sodium nitrite treatment also was reported by Mao *et al*. (2004). It is known that reduction of molecular mass of polymers is closely related to the reduction of polymer solution viscosity.

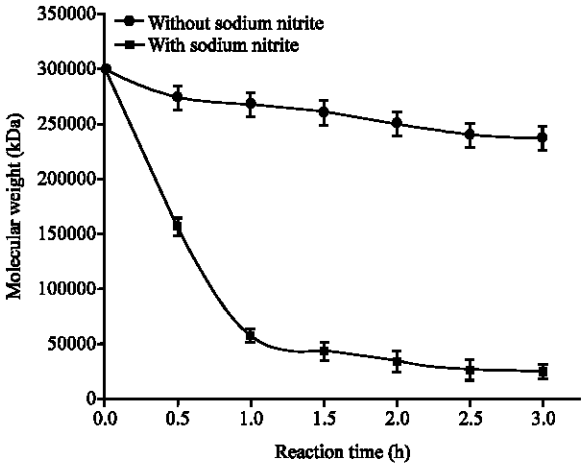

Fig. 1: Effect of reaction time on the average molecular weight of native chitosan. The measurements were carried out in triplicate and the results are expressed as Mean±SD. Chitosan (3%, w/v) was depolymerized in acetic acid (2%, v/v) with $NaNO_2$ (10 mL, 0.4%)

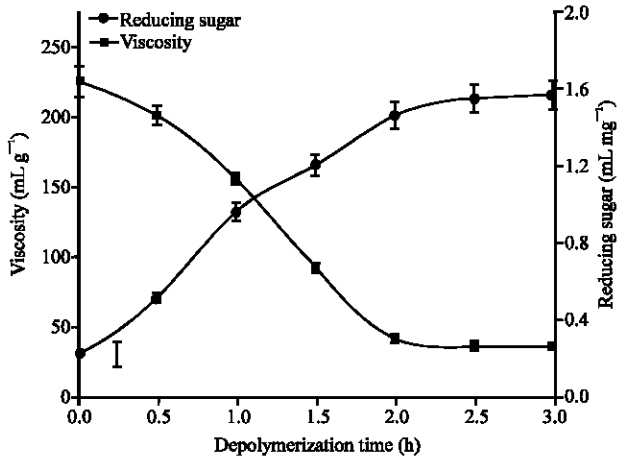

Fig. 2: Effect of reaction time on the intrinsic viscosity and released reducing sugar. The measurements were carried out in triplicate and the results are expressed as Mean±SD. Chitosan (3%, w/v) was depolymerized in acetic acid (2%, v/v) with $NaNO_2$ (10 mL, 0.4%)

Figure 2 shows the reduction in intrinsic viscosity and release reducing sugar content as a function of reaction time in the presence of sodium nitrite. Reduction of viscosity is already apparent within 0.5 h and it was followed by subsequent increase in reducing sugar content. Sodium nitrite treatment for 3 h was reduced the intrinsic viscosity of chitosan by 85% from 224 to 35 mL g^{-1}. The increase in reducing sugar content, although showing steady increased, was not dramatic as drop in viscosity. The rate of decrease in viscosity value and increase in reducing sugar observed in this study was comparable to those of chitosan samples reported by Wang *et al*. (2005) and Lee *et al*. (2005).

Optimum Conditions of *In vitro* Bile Salts Binding Capacities

In order to search for the optimum combination, the following four factors, concentration of chitosan (% w/v), concentration of acetic acid (% v/v), concentration of sodium nitrite (% w/v) and

Table 2: Experimental arrangement and test results[a]

Experiment No.	A	B	C	D	[b]Sodium chelate (μmol g^{-1} chitosan)	[b]Sodium deoxycholate (μmol g^{-1} chitosan)
1	1	1	3	2	56.6	49.6
2	2	1	1	1	61.6	70.8
3	3	1	2	3	68.8	108.4
4	1	2	2	1	59.2	108.6
5	2	2	3	3	41.2	51.0
6	3	2	1	2	58.2	94.0
7	1	3	1	3	47.0	105.2
8	2	3	2	2	59.8	124.8
9	3	3	3	1	57.0	110.0
[a]K_{1SC}	54.27	62.33	55.60	59.27		
[a]K_{2SC}	54.20	52.87	62.60	58.20		
[a]K_{3SC}	61.33	54.60	51.60	52.33		
*R_{SC}	7.13	9.46	11.00	6.94		
[a]K_{1SD}	87.80	76.27	90.00	96.47		
[a]K_{2SD}	82.20	84.53	113.93	89.47		
[a]K_{3SD}	104.13	113.33	70.20	88.20		
*R_{SD}	21.93	37.06	43.73	8.27		

[a]K_1, K_2, K_3 was the average summation of bile salts (chelate and deoxycholate) of each factor-level. *R was level difference ($R= K_{max}-K_{min}$). [b]Bile salts (chelate and deoxycholate) binding capacities were measured by HPLC and the values are the average of duplicate determinations (Hu *et al*., 2008)

Table 3: Average molecular weight and degree of deacetylation of native and partially depolymerized chitosans*

Experiment No.	M_v (kDa)	DD (%)
1	21.06±1.05	74.66±0.04
2	123.83±6.15	75.56±0.11
3	83.70±4.19	78.17±1.77
4	39.37±1.87	77.21±0.21
5	8.60±0.42	74.42±0.45
6	34.57±1.68	76.42±0.15
7	17.68±0.88	75.98±0.01
8	52.08±2.60	72.24±0.29
9	58.99±2.93	75.02±7.95
10	458.00±4.96	77.92±0.04

*Mean±SD of triplicate determinations. DD: Degree of deacetylation; M_v: Viscosity average molecular weight. Mv from 8.6-124 kDa represent partially depolymerized chitosan samples obtained by orthogonal test design in this study, whereas 458±4.96 kDa represents native chitosan sample

depolymerization time (h), were selected and their levels were modified through orthogonal experiment design L_9 (3^4) (Table 1). The final experiment results were shown in Table 2. The *in vitro* binding capacity of bile salts (chelate and deoxycholate) was a criterion of each test. According to the orthogonal method Wang *et al*. (2008), the analysis of variance for the experimental designs was calculated by summation of bile salts bound of each factor-level and level difference (R). Based on the magnitude order of R (Table 2), the R-values of sodium nitrite for both bile salts (11 and 43.73) were higher than that of acetic acid (9.46-37.06), chitosan (7.13-21.93) and time (6.96-8.27), hence sodium nitrite indicated significant influence. Compared to sodium nitrite and acetic acid, depolymerization time had a little influence. The order of influence of each variables on the binding capacities of both chelate and deoxycholate by partially depolymerized chitosans was quite similar C>B>A>D. In the third and eighth experiment (Table 2), the binding capacity of bile salts (chelate and deoxycholate) by partially depolymerized chitosans were better than other conditions. The results showed that sodium nitrite concentration of (0.4%, w/v), acetic acid concentration (1 or 2% v/v), chitosan concentration (3%, w/v) and treatment time 1 h at room temperature were the optimum conditions for binding capacity of sodium chelate and sodium deoxycholate, respectively.

In this study, molecular weight and degree of deacetylation of partially depolymerized chitosans were shown in Table 3. The M_v (kDa) and DD (%) of partially depolymerized chitosans obtained varied in the range of 8.6-124 Da and 69.52-77.70%, respectively. Figure 3 shows that the *in vitro*

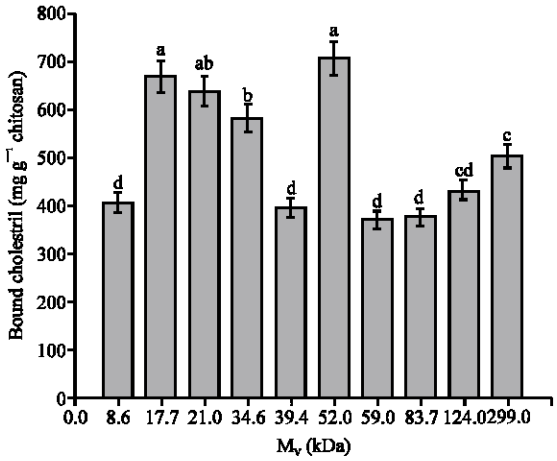

Fig. 3: Amount of *in vitro* cholesterol (mg) bound per g of chitosan as a function of average molecular weight. All chitosan samples were ground to small particle size (<150 μm) prior to analysis. All tests were conducted in triplicate. Values marked by the same latter(s) are not significantly different ($p<0.05$)

cholesterol-binding capacity of chitosans was in the range of 370-705 mg g^{-1} chitosan. Cholesterol-binding capacity of partially depolymerized chitosan 21 kDa (637 mg g^{-1} chitosan) was comparable to the binding capacities of both 17.7 kDa (667 mg g^{-1} chitosan) and 52 kDa (705 mg g^{-1} chitosan). As shown in Fig. 3, the average molecular weight of partially depolymerized chitosan samples increased the binding capacity was decreased from 17.7 to 39 kDa (395 mg g^{-1} chitosan) and increased from 59 kDa (374 mg g^{-1} chitosan) to 124 kDa (430 mg g^{-1} chitosan). The decreasing trend is a clear consequence of depolymerization of native chitosan by sodium nitrite, resulting in a reduction of chitosan NH_2 groups. There was no correlation between the *in vitro* cholesterol binding capacity and any of the physicochemical parameters for the chitosan samples. This implies that none of the physico-chemical characteristics indicated can be used to predict cholesterol binding capacity of partially depolymerized chitosan. The present results support those of previous workers (Zhou *et al*., 2006; Liu *et al*., 2008).

***In vitro* Binding Capacity of Sodium Chelate and Deoxycholate**

Table 2 shows the result of *in vitro* binding capacity of both sodium chelate and sodium deoxycholate by partially depolymerized chitosans. The values for sodium chelate and sodium deoxycholate binding were ranged between 41.2-69 and 49.6-124.8 μmol g^{-1} chitosan, respectively. Partially depolymerized chitosan sample 3 with 83.70 kDa was found to bind the biggest amount of sodium chelate in comparison with native chitosan (60 μmol g^{-1}). In addition, the binding capacity of deoxycholate (108.4 μmol g^{-1}) by sample 3 was significantly increased. On the other hand, the highest binding capacity against sodium deoxycholate was observed with depolymerized chitosan in sample 8, while partially depolymerized chitosan sample 5 exhibited significantly weaker binding capacity against both chelate and deoxycholate. It is observed that the sodium chelate and sodium deoxycholate of native chitosan (60-98 μmol g^{-1}) did not show any higher binding capacity and the high binding capacity of chelate was observed by high Mv partially depolymerized chitosan.

In agreement to the observation by Kim and Chun (1999), an individual chitosan sample had greater or similar absorbing capacity against deoxycholate than that against chelate under the experimental conditions. It is confirmed by this investigation as in Fig. 4a-d, in which chelate was the

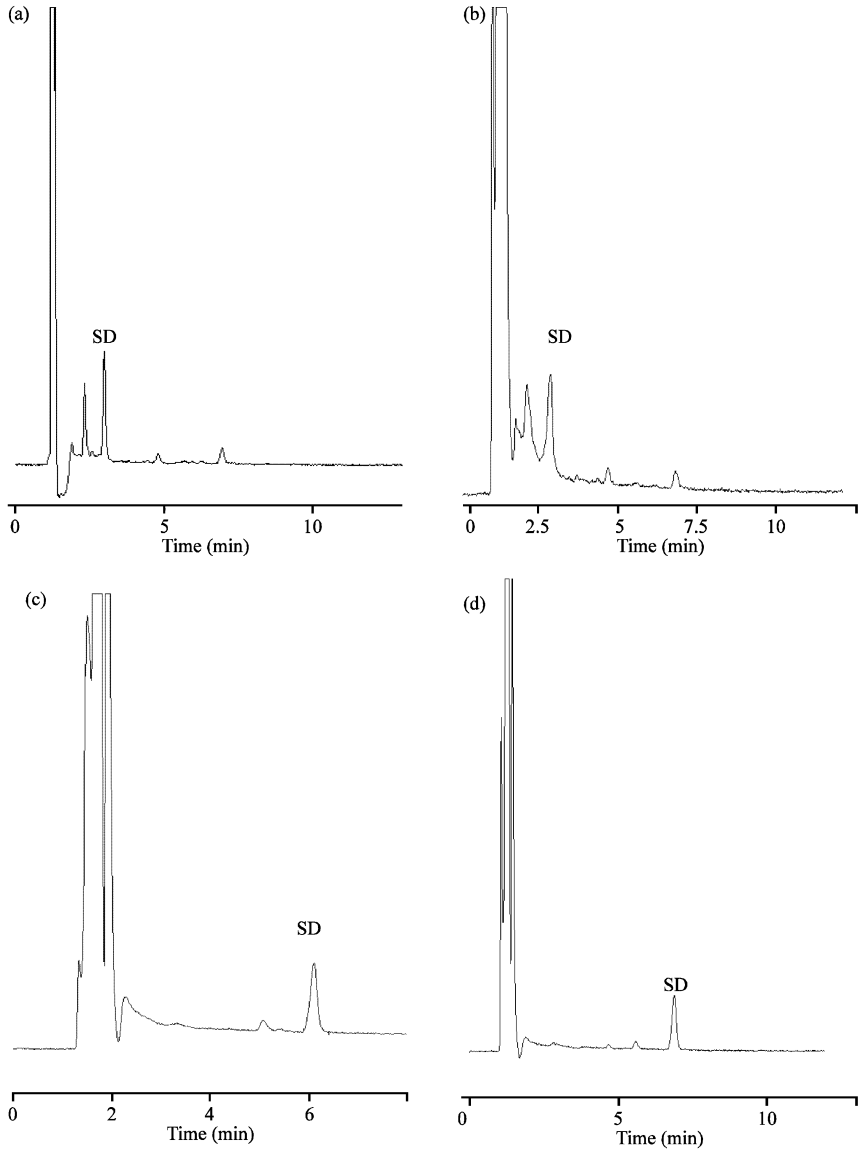

Fig. 4: Typical chromatograms for (a) Chelate Standard (SC), (b) sodium chelate plus PDC3, (c) sodium deoxycholate standard (SD) and (d) sodium deoxycholate plus PDC8

less bound, however deoxycholate was the best bound. This kind of adsorption behavior that the deoxycholate with 2 hydroxyl groups was adsorbed more than the chelate with 3 hydroxyl groups is similar to those of commercial bile salt adsorbents (Hagerman *et al*., 1973; Kim and Chun, 1999). Even though, the primary mode of interaction of bile-salt anions to chitosan is most probably electrostatic in nature, the secondary binding forces may play another role in binding bile-salt anions. These forces, being non-electrostatic in nature, would involve interactions between the hydrophobic regions of the adsorbate and adsorbent molecules. There was no linear correlation between individual binding of chelate and deoxycholate by chitosan under the experimental conditions. This observation was

supported by a earlier study of the bile salt-binding capacity of corn bran dietary fiber against chelate, chenodeoxycholate, deoxycholate and taurocholate (Hu *et al*., 2008). The binding mechanisms of bile salts by partially depolymerized chitosan might be different and the binding of any two bile salts might be not competitive.

CONCLUSION

Sodium nitrite treatment of chitosan resulted in a substantial decreased in molecular weight of chitosan solution, in viscosity and increased in reducing sugar content. R-value of both chelate and deoxycholate showed that binding of bile salts by partially depolymerization chitosans was influenced by the concentration of sodium nitrite and depended on type of bile salts. The binding capacity of deoxycholate was higher than that of chelate. There was not linear correlation between the bindings of any two bile salts by native and depolymerized chitosans, which indicates that the binding mechanisms of different bile salts by chitosan studied here might be different.

Due to the reduction of molecular weight and viscosity of chitosan by sodium nitrite, this information is important for further improving the total cholesterol binding capacity, bile salt-binding capacity and physico-chemical properties of partially depolymerized chitosans to promote their potential utilization in functional food and supplemental products.

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