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Preparation, water solubility and antioxidant activity of branched-chain chitosan derivatives

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ABSTRACT

Water-solubility at neutral or basic pH of chitosan was largely improved by specific attachment of carbohydrates to the 2-amino functions achieved by Maillard reaction or further reductive alkylation of Schiff bases. The characteristic physicochemical, rheological properties, and antioxidant activities of the derivatives were investigated. Experimental results indicated that the solubility of all the chitosan-saccharides before and after reducing had been greatly enhanced comparing to the native chitosan. The Schiff base typed chitosan–fructose derivative was highest at 13.2 g/L of all, and Schiff base typed chitosan derivatives existed better solubility, Ph stability and more effective scavenging activity against DPPH radical than N-alkylated chitosan derivatives. The degree of substitution (DS) of the chitosan derivatives increased with higher concentration of saccharide, increasing reaction time and temperature. The reduction of viscosity of chitosan derivatives decreased with increasing reaction time and temperature. The results suggest that the water-soluble chitosan derivatives produced through Maillard reaction may be promising commercial additive in cosmetics and food.

1. Introduction

Chitosan is a naturally occurring copolymer, one of the most abundant in the world, obtained from crustacean shells, insects, molluscan organs, and fungi (Jang, Kong, Jeong, Lee, & Nah, 2004). It consists unbranched chain of β-(1-4)-2-acetamido-2-acetamido-2-deoxy-β-D-glucose and β-(1-4)-2-amino-2-acetamido-2-deoxy-β-D-glucose as a repeating units. This polysaccharides has gained tremendous interest due to its excellent biological properties such as nontoxicity (Prabaharan, Borges, Godinho, & Mano, 2006), biodegradation (Sashiwa, Saimoto, Shigemasa, Ogawa, & Tokura, 1990; Shigemasa, Saito, Sashiwa, & Saimoto, 1994), biocompatible (Kurita, 1998), immunological (Song et al., 2009; Takashi et al., 1997), antibacterial (Tokura, Ueno, Miyazaki, & Nishi, 1997), and wound-healing activity (Kumar, Muzzarelli, Muzzareli, Sashiwa, & Domb, 2004). It has been widely applied in the fields of agriculture, environment, pharmaceuticals, medicines and industrial food processing (Chen et al., 2008; Ho, Mi, Sung, & Kuo, 2009; Liu, Nishi, Tokura, & Sakairi, 2001; Makoto et al., 2009; Zhang et al., 2009). However, chitosan-related applications are limited by its solubility only in diluted organic solutions such as formic, acetic, propionic, lactic, citric and succinic acid, as well as in a very few inorganic solvents, such as hydrochloric, phosphoric, and nitric acid at pH below 6.5 (Wang, Turhan, & Gunasekaran, 2004).

The intractability of chitosan lies largely in the rigid crystalline structure and the intermolecular hydrogen bonding caused by the acetamido or primary amino group residues (Nishimura, Kohgo, Kurita, & Kuzuhara, 1991). In an attempt to improve the water solubility of chitosan, many chemical modifications have been made to introduce hydrophilic groups by removing hydrogen atoms of the amino groups using acylation reaction (Sashiwa & Shigemasa, 1999), alkylation reaction (Chung, Tsai, & Li, 2006; Ma et al., 2008), quaternary reaction (Ignatova, Manolova, & Rasgkov, 2007; Verheul et al., 2008), carboxymethyl reaction (Sreedhar, Aparna, Sairam, & Hebalkar, 2007). Also there are other chemical and enzymatical modifications about chitosan (Sashiwa & Aiba, 2004).

The Maillard reaction is a well-known chemical reaction between an amino acid and a reducing sugar, usually requiring heat (Jokic, Wang, Liu, Frenkel, & Huang, 2004). Recently rheological characteristics and solubility of water-soluble chitosan derivatives derived from chitosan and saccharides have been demonstrated (Chung, Kuo, & Chen, 2005; Chung et al., 2006). The results indicate that the Maillard reaction is promising and facile for commercial manufacture of water-soluble chitosans. Thus, the development of a water-soluble chitosan is a prerequisite to successful industrial

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application. However, the fundamental information reported is not enough. Considering the further application of chitosan to food and additives for skin care in the future, intensive research is worthy.

In this paper Schiff-base type derivatives and N-alkylation of chitosan with different degrees of substitution (DS) of various saccharides (aldoses or ketoses) was prepared by Maillard reaction and further reductive alkylation as facile and versatile procedure. For the sake of choosing the saccharide which could most improve the solubility of chitosan. A serious of mono-, di-, saccharides including D-glucose, D-fructose, L-rhamnose, D(+)galactose, L-arabinose, D-mannose, maltose, and D-lactose were systematically screened by a water-solubility-based screening method established to estimate the solubility of the chitosan derivatives qualitatively for the very first time. And D-fructose turned out to be the best. Solubility, antioxidation activity, reduction of viscosity and structure of chosen derivatives were characterized in detail.

2. Experimental

2.1. Materials

Chitosan: 90% degree of deacetylation (DD) MW 105 000 (Zhejiang Aoxing Biotechnology Co. Ltd., Yuhuan, China). The reagents used were of analytical grade.

2.2. Preparation of water-soluble chitosan

90% DD chitosan was dissolved in 0.2 mol/L CH_3COOH solution (pH 3.6) to give a final chitosan concentration of 0.056 mol/L. Reducing saccharides (aldoses or ketoses) was added to the solution to give a final concentration of 0.056 mol/L to 0.56 mol/L. 15 samples were reacted at 70 °C for 1–7 days. Every other day, 3 samples withdrawn were centrifuged (8000 rpm, 15 min) and dialyzed against distilled water for 4–6 days (pH 6–7). After qualitative analysis of the solubility, each sample was reduced by 10.0 eq. ($-NH_2$) sodium borohydride at room temperature for 24 h. The Schiff base samples with highest solubility and every reducing sample were dialyzed (membrane tubing, molecular weight cutoff 12 000–14 000, Spectrum Laboratories, Savannah, GA, USA) against distilled water for 4–6 days and then freeze-dried and weighed.

2.3. Determination of yield, solubility and solution stability

The yield of water-soluble chitosan (chitosan-saccharide derivative) was expressed as the ratio of water-soluble chitosan to total added chitosan and saccharides.

For the first time a water-solubility-based screening method was established to estimate solubility qualitatively: the absorbance of half the concentration of the reaction solution at 600 nm was assumed as A_1, the absorbance of the reaction solution after adjusting to pH 7.0 at the same concentration was assumed as A_2. The relatively smaller of the value of ΔA ($=A_2 - A_1$) was, the higher solubility of the sample would be.

To estimate solubility quantificationally, 0.05 g of water-soluble chitosan was mixed with 5 mL distilled water, stirred for 5 h and then filtered through a 0.45-μm filter paper. Solubility was estimated from the change in filter-paper weight (Yalpani & Hall, 1984).

To estimate the solution stability of the water-soluble chitosan, 0.1 g was dissolved in 10 mL distilled water. The pH of the solution was monitored by adding 2 mol/L NaOH solution drop-wise until the change of the absorbance of the solution at 600 nm was higher than 0.1, which was deemed unstable (Yang, Chou, & Li, 2002).

2.4. Determination of degree of deacetylation (DD) and degree of substitution (DS)

To determine DD or DS of the water-soluble chitosan, 20 mg of the soluble variant was dissolved in 10 mL acetic acid (0.1 mol/L) and completely stirred for 1 h at room temperature. The mixture was diluted with 40 mL distilled water, then 5 mL of the diluted solution was withdrawn and one drop of 1% toluidine blue added as an indicator. Potassium polyvinyl sulfate solution (PVSK, N/400) was successively added until the titration end point was reached (Toei & Kohara, 1976).

Since the consumption of the N/400 PVSK (A mL, Eq. (1)) might correspond to that a of glucosamine unit in the water-soluble chitosan, the total weight of a glucosamine unit (X g, Eq. (1)) in the solution was obtained by (Eq. (1)):

$$X = \frac{1}{400} \times \frac{1}{1000} \times F \times 161 \times A \quad (1)$$

where F is the factor of N/400 PVSK, 161 is the molecular weight of the glucosamine, and 203 is the molecular weight of the N-acetyl-D-glucosamine. The water-soluble chitosan unit (Y g) is expressed as (Eq. (2)):

$$Y = 0.5 \times \frac{1}{100} - X \quad (2)$$

Thus, DD is calculated by (Eq. (3)), DS is calculated by (Eq. (4)), 90 is the DD(%) of chitosan:

$$\mathrm{DD}(\%) = \left[\frac{X/161}{X/161 + Y/203}\right] \times 100 \quad (3)$$

$$\mathrm{DS}(\%) = \frac{90 - \mathrm{DD}}{90} \times 100 \quad (4)$$

2.5. Determination of reactive extent of Maillard reaction

To assess the reactivity of the Maillard reaction, 3 mL solutions diluted 1 time from different chitosan-saccharide complexes were analyzed by measuring absorbance at 420 nm using a Beckman spectrophotometer (Liu, Chang, & Wu, 2003).

2.6. Evaluation of antioxidant activity

The DPPH (α,α-diphenyl-β-picryl-hydrazyl) scavenging activity of the samples was measured using the modified method of Yamaguchi et al. (Sun, Yao, Zhou, & Mao, 2008) 0.1 mL of ethanol solution of DPPH (0.1 mmol/L) was incubated with varying concentrations of test samples (0.1 mL). The reaction mixture was shaken well and incubated for 20 min at 30 °C and the absorbance of the resulting solution was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\text{Scavenging effect}\ (\%) = \frac{1 - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

2.7. Determination of reduction of viscosity

The reduced viscosity of the N-alkylated chitosan derivative in 0.2 mol/L CH_3COOH/0.1 mol/L CH_3COONa was determined using an Ubbelohde-type viscometer (Schott-Gerate, Mainz, Germany) with a capacity of 15–20 mL. The viscometer was suspended in a thermostatically controlled water bath (Model E200, Lauda Dr. R. Wobser GmbH & Co., KG, Germany) maintained at 30.0 ± 0.1 °C. Flow times were recorded electronically using photoreceptors mounted on the viscometer stand which could detect the passage of the solution meniscus, and the solvent flow time ratio of the kinematic relative viscosity was thus obtained. Because of the low concentrations

used (between 0.2 and 1.0 mg/mL), the density corrections for the different solutions.

2.8. Characterization of chitosan derivatives

IR spectra were recorded on FT-IR620 spectrometer (JASCO) in KBr discs by an average of 64 scans at a resolution of 4 cm^{-1}.

^1H NMR spectra were carried out on a Bruker AV600 MHz (Bruker, Rneinstetten, Germany). Chitosan derivatives were dissolved in D_2O.

X-ray diffraction spectrometry was obtained by using XD-3A powder diffraction meter with CuKα radiation in the range of 5–40° (2θ) at 40 kV and 30 mA.

3. Results and discussion

3.1. Screening of various Schiff base typed chitosan derivatives

To screen the most appropriate saccharide, 0.056 mol/L various reducing saccharides (D-glucose, D-fructose, L-rhamnose, D(+)galactose, L-arabinose, D-mannose, maltose, and D-lactose) were separately mixed with 0.056 mol/L chitosan (90% DD) solution and reacted at 70 °C for various periods. Surface functionalization of the lyophilized Schiff base type of chitosan derivatives with aldoses or ketoses via a heterogeneous system in water resulted in a soft and cotton-like chitosan containing mesopores. The longer reaction time caused the darker color of the derivatives formation.

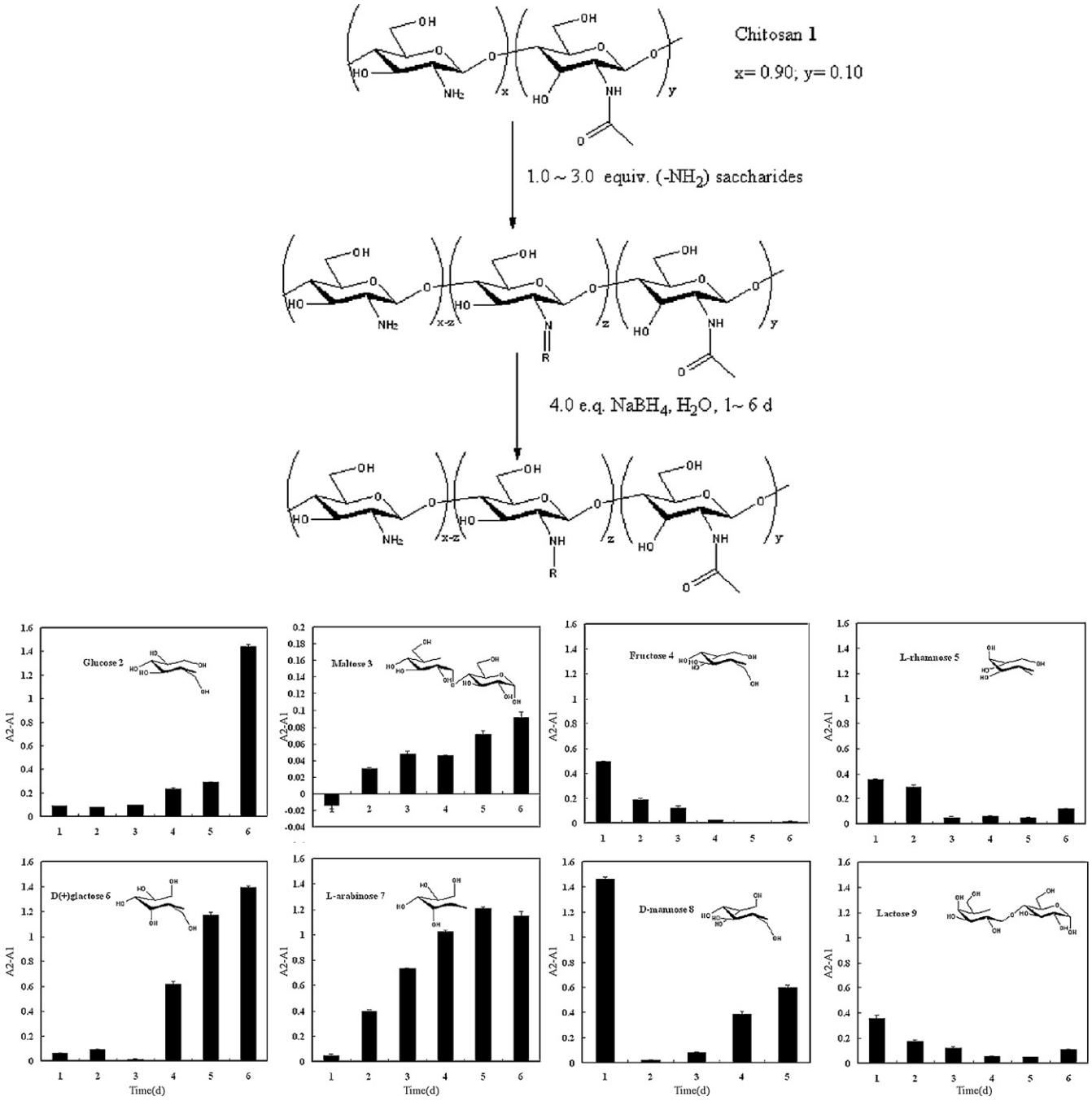

Fig. 1. The transitions and the value of ΔA of Schiff base derivatives modified by different saccharides. Reaction with 1.0 eq. (–NH_2) D-glucose (Glu70, **2**) or maltose (Mal70, **3**) or D-fructose (D-fru70, **4**) or L-rhamnose (L-rham70, **5**) or D(+)-galactose (D(+)-Gal70, **6**) or L-arabinose (L-ara70, **7**) or D-mannose (D-Man70, **8**) or D-lactose (D-lac70, **9**) for 6 days at 70 °C. The error bars indicate the standard derivation.

Table 1
Solubility, yield, degree of deacetylation (DD), and optimal reaction conditions for Maillard reaction.

Optimal reaction set			Property of chitosan derivative			
Chitosan	Saccharide	Operating condition	Yield (%)	Solubility (g/L)	DD (%)	pH stability*
DD 90%, 0.0056 mol	0.0056 mol, glucose	70 °C, 2d	40.7	5.9 ± 0.2	51.2	<7.5
DD 90%, 0.0056 mol	0.0056 mol, maltose	70 °C, 1d	29.6	7.3 ± 0.1	79.4	<7.5
DD 90%, 0.0056 mol	0.0056 mol, fructose	70 °C, 5d	58.6	11.46 ± 0.3	45.0	<13
DD 90%, 0.0056 mol	0.0056 mol, rhmnose	70 °C, 5d	20.0	5.0 ± 0.2	23.5	<10.0
DD 90%, 0.0056 mol	0.0056 mol, galactose	70 °C, 3d	52.0	5.0 ± 0.3	2.7	<7.0
DD 90%, 0.0056 mol	0.0056 mol, arabinose	70 °C, 3d	32.4	5.1 ± 0.1	0.0	<7.0
DD 90%, 0.0056 mol	0.0056 mol, mannose	70 °C, 2d	44.8	7.2 ± 0.2	29.3	<7.5
DD 90%, 0.0056 mol	0.0056 mol, lactose	70 °C, 4d	44.4	10.1 ± 0.2	19.7	<9.0

The values of yield and solubility with different superscripts within a column indicate significant differences ($P < 0.05$).
* pH stability represents the pH range for stable solubility of chitosan derivative.

In order to screen chitosan Schiff base derivatives modified by various saccharides in good water-solubility, a water-solubility-based screening method was established to estimate solubility qualitatively, the relatively smaller of the value of ΔA ($=A_2 - A_1$) was, the higher solubility of the sample would be. When chitosan reacted with different saccharides, the D-value of A_2 and A_1 indicated that the solubility of the chitosan derivatives increased with an increasing the reaction time, reaching a minimum on a particular day, and then gradually increased. The reaction conditions and the kinds of the reducing sugars decided the yield and the solubility of the chitosan derivatives as depicted in Fig. 1. These results were testified by determinating the solubilities quantificationally after lyophilization. Table 1 shows the basic properties of the chitosan derivatives at the optimized reaction conditions for the Maillard reaction. The solubility of Schiff base typed chitosan–fructose derivative was higher at 11.46 g/L in the fifth day depicted in Table 1 than other derivatives. It presumed that the relatively low rate of the chitosan–fructose and chitosan–lactose Maillard reaction were due to the Heyns rearrangement and isomerization, which is also the reason resisting formation of crystals and causing the high soluble derivatives (Wrodnigg & Stütz, 1999). In a word reaction time is the key for producing water-soluble chitosans.

3.2. Effect of reaction time, reaction temperature and concentration of saccharide on yield and solubility of Schiff base typed chitosan derivatives

The 0.056 mol/L chitosan (90% DD) was separately mixed with 1.0 eq. ($-NH_2$) or 3.0 eq. ($-NH_2$) of glucose or fructose and reacted at 40, 50, 60, or 70 °C for 1 day to 8 days. The yield and solubility of water-soluble Schiff base typed chitosan derivatives obtained from chitosan reacting with glucose (Fig. 2(A)), are shown at various temperature. Yield and solubility increased with relatively longer reaction time, reaching a maximum on the seventh day at 40 °C, or on the fifth day at 50 °C, or on the forth day at 60 °C, or on the second day at 70 °C. The maximal average yields solubility increased with higher temperature were 15.8%, 28.8%, 30.5%, and 40.7%, respectively. Also, the yield and solubility of water-soluble Schiff base typed chitosan derivatives obtained from chitosan reacting with D-fructose (Fig. 2(B)), are shown at various concentrations. Yield and solubility increased with relatively higher reaction concentration of D-fructose at 70 °C, reaching a maximum on the fifth day at the concentration of 0.0056 mol/L, or on the forth day at the concentration of 0.0168 mol/L. The maximal average yields solubility increased with higher concentration were 57.8%, and 94.3%, respectively. Though the increasing quantity did not enhanced the solubility of Schiff base-typed chitosan–fructose derivatives dramatically, the yield had been enhanced a lot. In addition, when chitosan reacted with glucose or D-fructose at 70 °C, the relatively longer time (>3 days with 1.0 eq. ($-NH_2$) glucose, >5 days with 3.0 eq. ($-NH_2$) D-fructose) resulted in the formation of more precipitates at pH 7.0, causing the increase of value ΔA ($=A_2 - A_1$), which indicated the lower solubility. The occurrence of the precipitates during the dialysis may probably due to the decrease in the ionic strength of dialysis solution or to the inappropriate hyper-attachment of saccharide to chitosan. Furthermore, the precipitates cannot dissolve in all pH of aqueous solution. In this study, vacuum drying reduced the solubility, while lyophilization most likely remained the solubility of the water-soluble chitosan. However a small part of low molecular derivatives (<8 kDa) lost from membrane tubing during the dialysis because of the bond cleavage, causing the decreasing of yield.

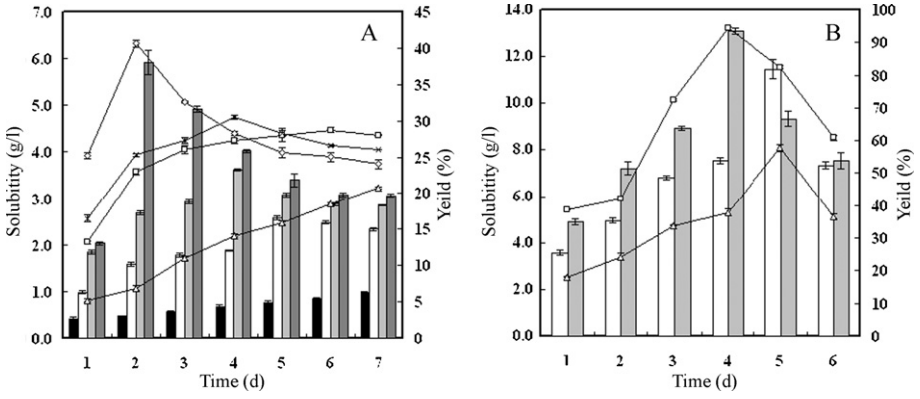

Fig. 2. Solubility (bars) and yield (circles) of chitosan derivatives. (A) Reaction with 1.0 eq. ($-NH_2$) glucose at 40 °C (Glc40, filled bars, (△) or at 50 °C (Glc50, open bars, (□) or at 60 °C (Glc60, filled with light grey, (×) or at 70 °C (Glc70, filled with dark grey, (◊). The error bars indicate the standard derivation. (B) Reaction with 1.0 eq. ($-NH_2$) D-fructose at 70 °C (D-fru70, open bars, (△) or with 3.0 eq. ($-NH_2$) D-fructose at 70 °C (D-fru70, filled bars, (□). The error bars indicate the standard derivation.

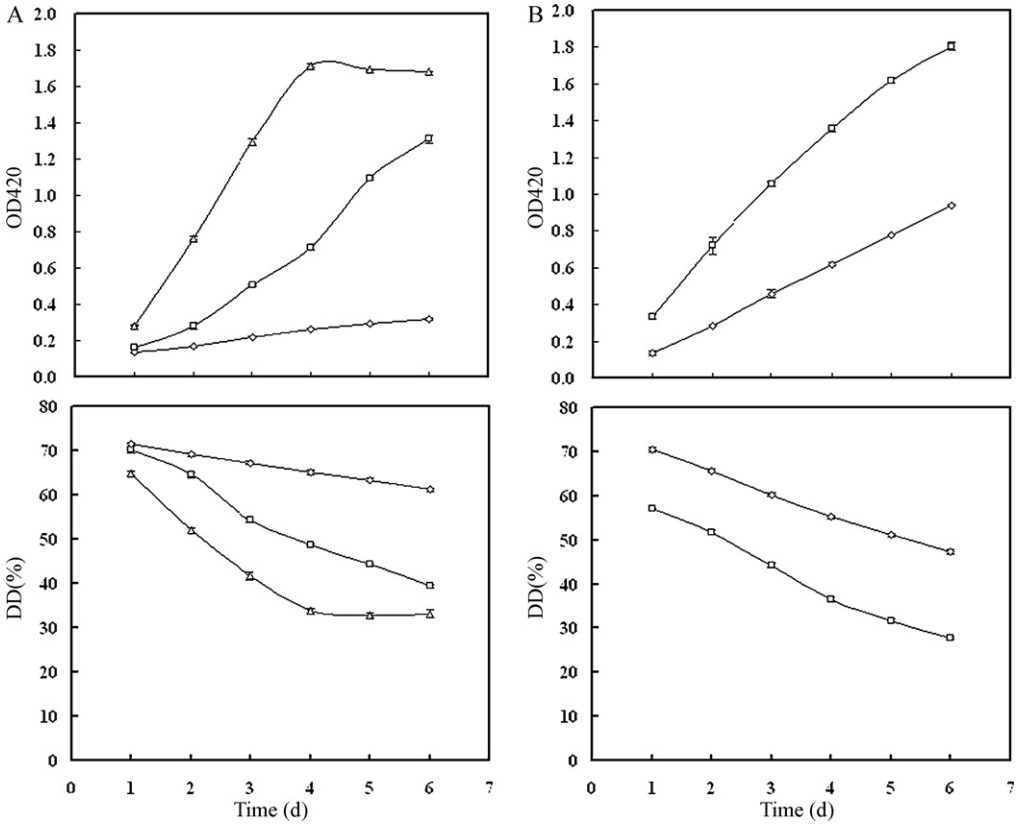

Fig. 3. Effect of reaction time and reaction temperature on absorbance and degree of deaceylation (DD) of water soluble Schiff base typed chitosan derivatives for 6 days. (A) Reaction with 1.0 eq. ($-NH_2$) glucose at 50 °C (Glc50, ◊) or at 60 °C (Glc60, □) or at 70 °C (Glc70, △). (B) Reaction with 1.0 eq. ($-NH_2$) D-fructose at 70 °C (D-fru70, open bars, ◊) or with 3.0 eq. ($-NH_2$) D-fructose at 70 °C (D-fru70, filled bars, □). The error bars indicate the standard derivation.

3.3. Effect of reaction time, reaction temperature, degree of deacetylation of chitosan, and concentration of saccharide on Maillard reaction

The extent of Maillard reaction between chitosan and saccharide can be determined from the absorption at 420 nm. Fig. 3 depicts that the absorbance increased with an increasing concentration of saccharide, reaction time, and reaction temperature. Furthermore, Fig. 3(A) shows that the absorbance of the derivatives produced from the chitosan and glucose at 70 °C levels off on the forth day and declines a little bit from then on, which dues to the precipitation dissolving out. The precipitation cannot even dissolve in acid aqueous solution, which suggests that the inappropriate hyper-attachment of saccharides to chitosan may probably make the solubility even worse. However, unlike the chitosan–glucose derivatives, the absorbance of the derivatives derived from chitosan and 1.0 eq. ($-NH_2$) D-fructose at 70 °C did not level off in six days (data not shown). Fig. 3(B) depicts that tripling the concentration of fructose resulted in an almost doubling of the effects on absorbance of chitosan derivatives or the rate of Maillard reaction. Respectively, the results of absorbance on the first day for the Schiff base typed chitosan–glucose, chitosan–rhamnose, chitosan–galactose, chitosan–arabinose, chitosan–mannose, chitosan–maltose, chitosan–fructose, and chitosan–lactose derivatives at 70 °C were 0.284, 0.387, 0.897, 2.614, 0.381, 0.100, and 0.136. Thus, it is suggested that 0.0056 mol/L aldose (D-glucose, L-rhamnose, D(+)galactose, L-arabinose, D-mannose, maltose) was sufficient to completely react with through Maillard reaction (data not shown). It presumed that the relatively low rate of the chitosan–fructose and chitosan–lactose Maillard reaction were due to the Heyns rearrangement and isomerization, which is also the reason resisting formation of crystals and causing the high soluble derivatives (Wrodnigg & Stütz, 1999). The results demonstrates that the maximum yield or solubility of Schiff base typed chitosan-saccharide derivatives is not proportional to the extent of Maillard reaction achievement, although high reaction temperature, long reaction time, and dense concentration favor its development (vanBoekel, 2006; Yasuko, Hiroko, & Tsukasa, 1999).

The extent of deacetylation of chitosan generally affecting the physical, chemical and biological properties of its derivatives (Huang, Fong, Khor, & Lim, 2005) is largely affected by reaction time and temperature. Hence, it is necessary to determine the degree of deacetylation of chitosan derivatives. In this study, the degree of deacetylation relates to the extent of Maillard reaction between chitosan and saccharide. The degree of deacetylation of the Schiff base typed chitosan derivatives is shown in Fig. 3. The results indicate that the degree of deacetylation is interralated to the reaction temperature and the concentration of saccharide. As depicted in Fig. 3(A), the degree of deacetylation of chitosan–glucose derivatives is relatively low at relatively high temperature, and first decreases at 70 °C with reaction time and then levels off on the forth day, which is marching the tendency of Maillard reaction. Fig. 3(B) shows that the degree of deacetylation of the chitosan–fructose derivatives decreased with increasing concentration of saccharide. Compare with chitosan–glucose derivatives, chitosan–fructose derivatives need higher temperature and concentration to succeed the same degree of deacetylation.

3.4. Effect of reaction time, reaction temperature, DS, and concentration of saccharide on reduction of viscosity

Fig. 4 displays the effect of temperature on the reductive viscosity of water-soluble chitosan, obtained from Schiff base typed chitosan–glucose/fructose derivatives in 0.2 mol/L

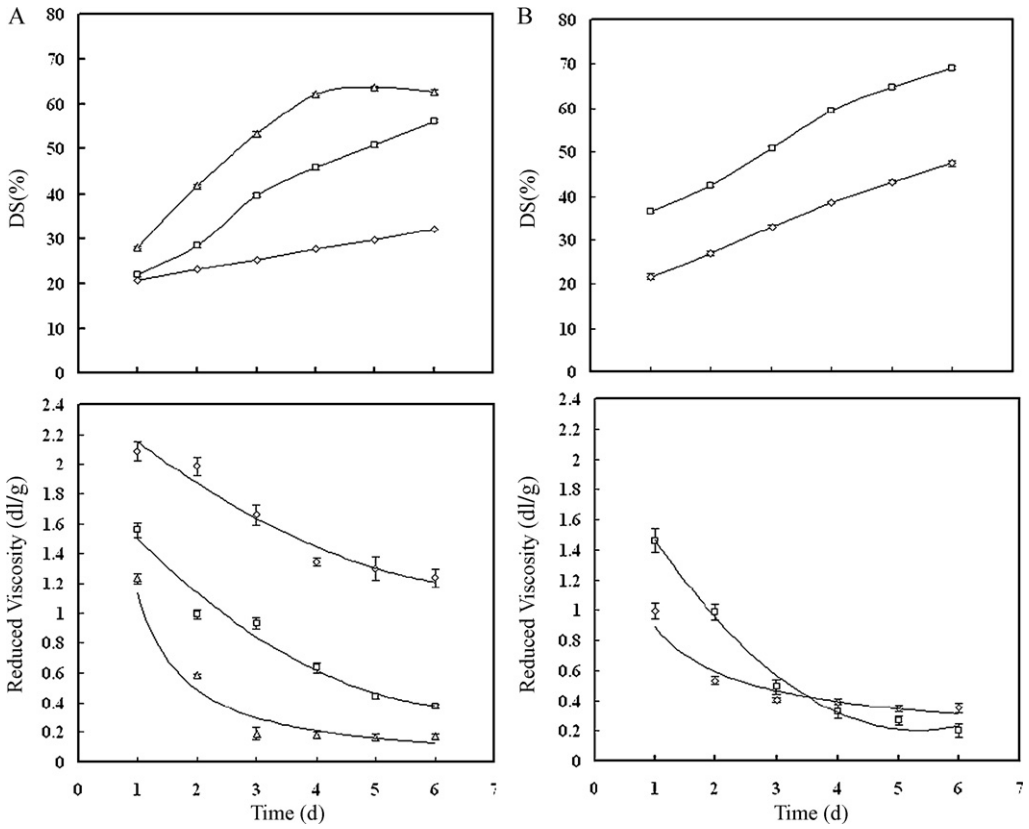

Fig. 4. Effect of Maillard reaction on degree of deacetylation of water soluble Schiff base typed chitosan–glucose/fructose derivatives for 6 days. (A) Reaction with 1.0 eq. ($-NH_2$) glucose at 50 °C (Glc50, ◊) or at 60 °C (Glc60, □) or at 70 °C (Glc70, △). (B) Reaction with 1.0 eq. ($-NH_2$) D-fructose at 70 °C (D-fru70, ◊) or with 3.0 eq. ($-NH_2$) D-fructose at 70 °C (D-fru70, □). The error bars indicate the standard derivation.

CH_3COOH/0.1 mol/L CH_3COONa at 30 °C. As depicted in Fig. 4(A), the reductive viscosity decreased with increasing reaction temperature and time, and leveled off on the third day at 70 °C, in terms accompanying with a increased degree of substitution.

Generally, the DS of the derivatives increased as the amount of D-fructose increased, but accompanying with a decreased reduced viscosity (Yang et al., 2002). As depicted in Fig. 4(B), the reductive viscosity of Schiff base typed chitosan derivative reacting with 3.0 eq. ($-NH_2$) D-fructose was higher than that reacting with 1.0 eq. ($-NH_2$) D-fructose until the third day, which was partly the same with the results published (Yang et al., 2002).

3.5. *Effect of reducing reaction on solubility of Schiff base typed chitosan derivatives*

0.0168 mol/L D-fructose was mixed with 0.0056 mol/L chitosan (90% DD) solution and reacted at 70 °C for 1–6 days. Every day each sample was reduced by 10.0 eq. ($-NH_2$) sodium borohydride at room temperature for 24 h and were dialyzed (membrane tubing, molecular weight cutoff 12 000–14 000, Spectrum Laboratories, Savannah, GA, USA) against distilled water for 4–6 days and then freeze-dried and weighed. Surface functionalization of the lyophilized Schiff base type of chitosan derivatives with aldoses or ketoses via a heterogeneous system in water resulted in a soft and losser chitosan.

Results of the solubility, and the pH stability of water-soluble N-alkylated chitosan with 3.0 eq. ($-NH_2$) D-fructose are shown in Fig. 5. Gel formation happened during the reducing of some samples related to various periods of Maillard reaction. Solubility and pH stability of Schiff base-typed chitosan–fructose derivatives increased with relatively longer reaction time, reaching a maximum on the forth day. While the solubility and pH stability of N-alkylated chitosan–fructose derivatives almost leveled off at pH 7 from the first day. Apparently, comparing to the derivatives before reducing, the solubility and pH stability of N-alkylation chitosan–fructose was much more lower, which haven't mentioned elsewhere.

The solubility of chitosan derivatives affected by inter- and intra-molecular hydrogen bond can also been distinguished from

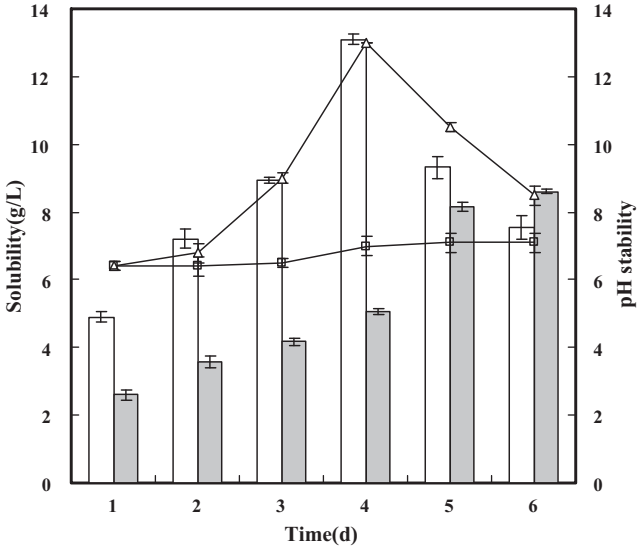

Fig. 5. Solubility (bars) and pH stability (circles) of chitosan derivatives. Schiff base typed chitosan–fructose reacting with 0.0056 mol/L D-fructose at 70 °C (D-fru70, open bars, △), N-alkyated typed derivative reacting with 0.0056 mol/L D-fructose (D-fru70, filled bars, □). The error bars indicate the standard derivation.

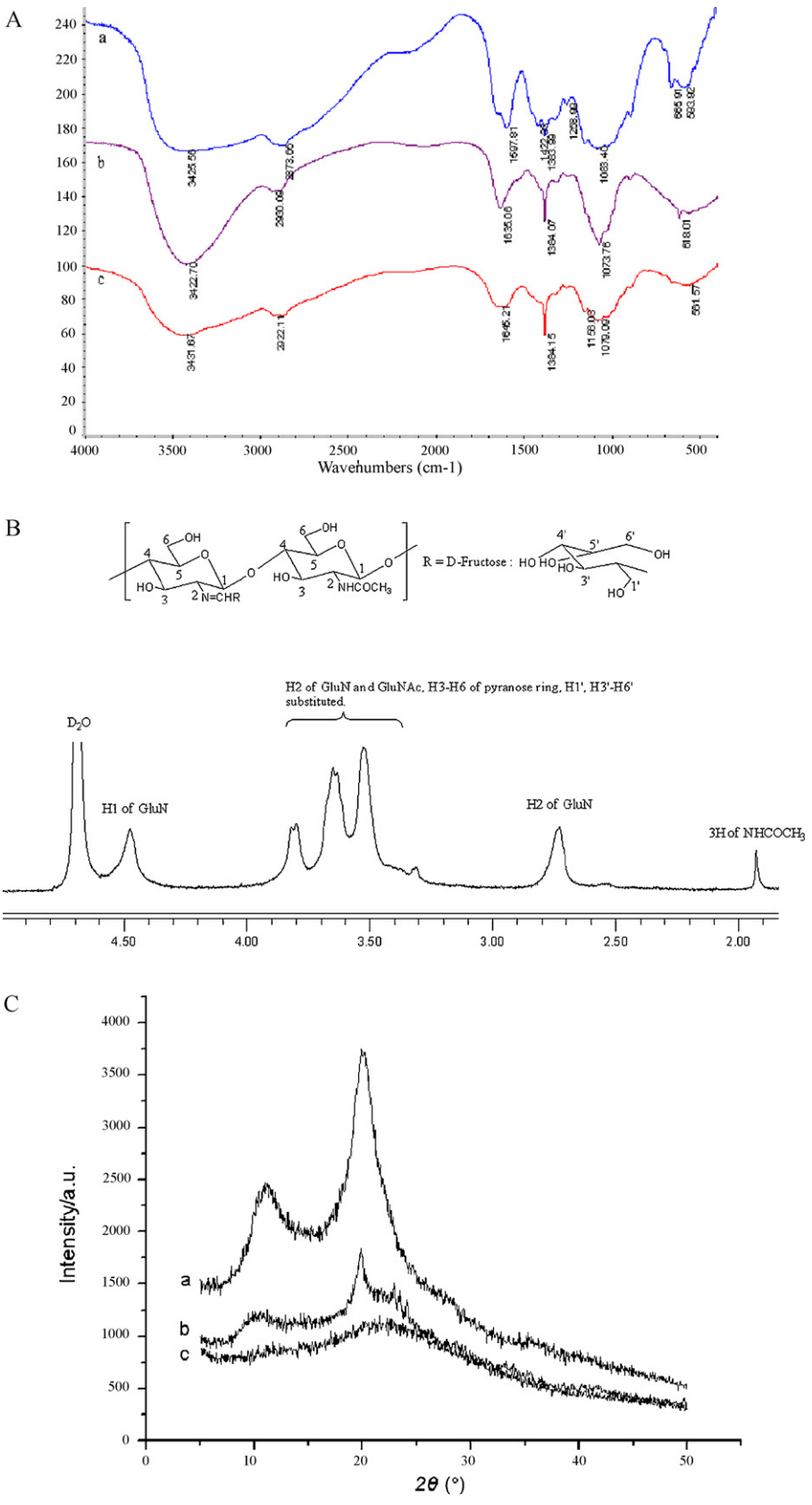

Fig. 6. (A) FTIR spectra of chitosan and chitosan derivatives. Chitosan (a), Schiff base typed chitosan–fructose derivative reacting with 0.0168 mol/L D-fructose at 70 °C for 4 days (b), N-alkylated chitosan–fructose derivative reacting with 0.0168 mol/L D-fructose at 70 °C for 4 days and reduced by 10 eq. $NaBH_4$ (c). (B) ^1H NMR spectra of Schiff base typed chitosan–fructose derivative reacting with 0.0168 mol/L D-fructose at 70 °C for 4 days. (C) WAXD patterns of (a) chitosan, (b) N-alkylated chitosan–fructose derivative reacting with 0.0168 mol/L D-fructose at 70 °C for 4 days and reduced by 10 eq. $NaBH_4$ and (c) Schiff base typed chitosan–fructose derivative reacting with 0.0168 mol/L D-fructose at 70 °C for 4 days.

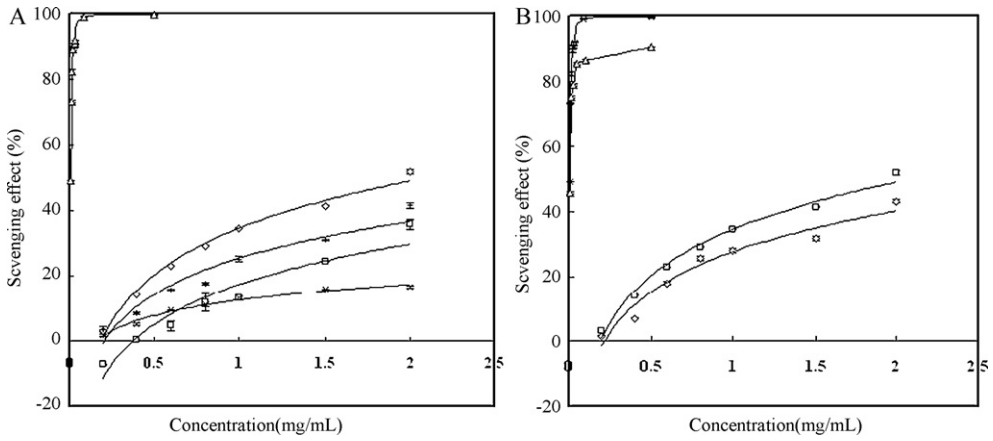

Fig. 7. Scavenging effect of chitosan derivatives on DPPH radical. Reaction with 0.0168 mol/L D-fructose at 70 °C on the forth day. (A) Ascorbic acid (△), Shiff base typed derivative (D-fru70 2d, +; D-fru70 4d, ◊), N-aklyated derivative (D-fru70 4r, □), and chitosan (×) in 0.6% AcOH. (B) Ascorbic acid (×), and Shiff base typed derivative (D-fru70 4d, □) in 0.6% AcOH; Ascorbic acid (△), and Shiff base typed derivative (D-fru70 4d, ◊) in pure water. The error bars indicate the standard derivation.

Fig. 6(C). Schiff base-typed chitosan derivative was amorphous, while N-alkylation chitosan derivative still had inter- or intra-molecular H-bond. The solubility of chitosan and N-substitute chitosan derivatives here in acidic region would be caused by the protonation of amino group changing form $-NH_2$ to $-NH_3^+$ (Hitoshi & Yoshihiro, 1999). The results indicated that N-alkylation chitosan derivative need more concentration of H^+ (lower pH value) to break down the intermolecular H-bond and to dissolve in aqueous solution, which also explained the reason why Schiff base-typed chitosan derivative had better pH stability. Actually, the pH stability of Schiff base-typed chitosan–fructose derivatives here was partly accorded with Chung's results (Chung et al., 2006), because they did not mention that the pH stability would decrease. The pH stability seemed to have the same trend with solubility. Also, the yields after reducing were much lower than the derivatives before reducing (data not shown).

3.6. Characterization of chitosan derivatives

Fig. 6(A) showed the FT-IR spectra of chitosan and Schiff base typed chitosan–fructose derivatives with different DS. The characteristic absorption bands around 3430 cm^{-1} and 2930 cm^{-1} attributed to –NH, –OH and –CH– stretching vibration, as well as inter- and extra-molecular hydrogen bonding of chitosan molecules. The characteristic peaks at around 1645, 1598 and 1323 cm^{-1} were assigned to amide I, amine II and amide III absorption bands of chitosan, respectively. The absorption band at 1156 cm^{-1} was the asymmetric stretching of the C–O–C bridge. Bands at 1073 and 1033 cm^{-1} were assigned to the skeletal vibration of C–O stretching (Brugnerotto et al., 2001). It was worth noting that when fructose was added to the chitosan, the peaks at around 1597 cm^{-1}, 1156 cm^{-1}, and 897 cm^{-1} disappeared or decreased, while the peak at 1635 cm^{-1} represented C=N appeared in Schiff base typed chitosan–fructose derivatives. Compared with Schiff based typed chitosan–fructose derivative, the peak at around 1635 cm^{-1} decreased while the peak at around 1645 cm^{-1} assigned to amide I in N-alkylated chitosan–fructose derivatives. The results suggested that the fructose had attached to chitosan.

The ^1H NMR spectroscopic technique was used to determine the chemical structures of chitosan and its derivatives. Compared to acid-soluble chitosan, Schiff base typed chitosan–fructose derivative can dissolve in H_2O and D_2O. Fig. 6(B) exhibited the ^1H NMR spectra of Schiff base typed chitosan–fructose. The characteristic of the ^1H NMR pattern of chitosan, i.e., the multiplet at δ 4.5, δ 3.7–3.3 ppm due to H_1, H_3, H_4, H_5, H_6, and two singlets at δ 2.8 and 1.9 ppm due to the H_2 proton of the GlcN and N-acetyl protons of GlcNAc, respectively. The ^1H NMR spectrum of Schiff base typed chitosan–frucotse exhibited the multiplet at δ 3.9–3.3 ppm due to H_1', H_3', H_4', H_5', and H_6'.

The solubility of chitosan is molecular-dependent that with the molecular higher than 5000 cannot dissolve in water because of the strong intermolecular hydrogen bonding (Lu, Song, Cao, Chen, & Yao, 2003). The X-ray diffraction spectra of chitosan, Schiff base typed chitosan–fructose derivative, and N-alkylated chitosan–fructose derivative (Fig. 6(C)) show that chitosan exhibits two reflection fall at $2\theta = 5^\circ$, $2\theta = 20^\circ$. It is reported that the reflection fall at $2\theta = 5^\circ$ was assigned to crystal form I and the strongest reflection appears at $2\theta = 20^\circ$ which corresponds to crystal forms II (Samuels, 1981). However, the XRD spectrum of N-alkylated chitosan–fructose derivative has much smaller peaks at around $2\theta = 5^\circ$ and 20°, and the spectrum of Schiff base typed chitosan–fructose derivative has only broad peak at around $2\theta = 20^\circ$, which indicates that crystal forms have been destroyed in N-alkylated chitosan–fructose derivative and even destroyed more in Schiff base typed chitosan–fructose derivative macromolecules. This result suggests that the intramolecular hydrogen bonding in both derivatives have been greatly decreased after chemical modification in comparison with that of chitosan. As a result, the solubility of the Schiff base typed chitosan–fructose derivative and N-alkylated chitosan–fructose derivative is better than chitosan.

3.7. Scavenging effect of Schiff base typed and N-aklyated chitosan derivatives on DPPH radical

DPPH possesses a proton free radical with a characteristic absorption, decreasing significantly on exposure to proton radical scavengers. Therefore relatively stable DPPH radical has been widely used to test the ability of compounds as free radical scavengers or hydrogen donors in order to evaluate the antioxidant activity. Fig. 7(A) depicts the percentage DPPH free radical scavenging activity of ascorbic acid, chitosan and chitosan derivatives in 0.6% AcOH ($p < 0.05$). Obviously, ascorbic acid showed a plateau of scavenging abilities of 90.1–99.5% at 0.03–0.5 mg/mL. However, at 2.0 mg/mL chitosan scavenged DPPH radicals by 16.6%, which indicated that chitosan was not a good scavenger. The similar results are also reported by other researchers (Kanatt, Chander, & Sharma, 2008). The derivative of N-alkylation of chitosan–fructose on the forth day, Schiff base typed chitosan–fructose on the second and

forth day are endowed with a scavenging ability of 35.8%, 51.9% and 41.6% at 2.0 mg/mL, respectively. The results of scavenging ability of ascorbic acid and Schiff base typed chitosan–fructose in pure water and 0.6% AcOH are shown in Fig. 7(B), where the latter one is better than the former one. That is because the charge properties of substituting groups may affect the antioxidant activity of chitosan and its derivatives (Guo, Xing, Liu, Zhong, & Li, 2008). Apparently, the scavenging ability is enhanced after N-alkylation of D-fructose and enhanced even higher by formation of Schiff base at the concentration above 0.2 mg/mL. Furthermore, the scavenging effect on DPPH increased with the increasing DS, which is contrary to the results published (Lin & Chou, 2004).

The antioxidant activity of N-alkylation of chitosan–fructose and Schiff base typed chitosan–fructose derivatives mainly related to the content of active hydroxyl and amino groups in the polymer chains (Feng, Du, Li, Hu, & Kennedy, 2008). The fact Maillard reaction can significantly elevate antioxidative effects of proteins was reported (Nakamura, Kato, & Kobayashi, 1992), and the structure of –C=N exists higher activity of reacting with free radical than –C–N. In fact, the antioxidant activity of chitosan derivatives increased with decreasing of the DD caused by the increasing primary amino groups (Yen, Yang, & Mau, 2008) and with the decreasing of molecular weight causing the inter- and intra-molecular hydrogen bonds partly destroyed (Chien, Sheu, Huang, & Su, 2007; Xing et al., 2008). In this research the increasing DS of the derivatives caused the decreasing $-NH_2$ and the increasing –OH and –N=C (Schiff base-typed chitosan derivatives) or –NH–C (N-alkylation of chitosan derivatives). The fact that the scavenging effect of N-alkylation chitosan derivative at concentration lower than 0.5 mg/mL was negative indicated that the $-NH_2$ and –C=N had better antioxidant ability than –NH–C, because the scavenging effect of chitosan and Schiff base-typed chitosan derivatives at any concentration was positive.

Overall, the results indicate that chitosan–fructose derivatives possess hydrogen donation ability, so they have the potency to react with DPPH radicals, which is also the reason why acid solution enhances the scavenging ability. Although more active amino groups could donate more hydrogen to react with DPPH radical and thus have high scavenging ability (Sun et al., 2008), apparently, the results shows that the attachment of N-alkylation of D-fructose and Schiff base on chitosan enhances the ability of scavenging DPPH radicals. The antioxidant efficiency of chitosan–fructose derivatives is concentration-dependant. The higher concentration is, the higher scavenging ability they possess.

4. Conclusion

The Maillard reaction with reducing sugars is a facile way of improving the solubility of chitosan, and all the derivatives were partially or totally soluble at neutral or basic pH in water. The optimal solubility and yield of chitosan derivatives depended on the reaction temperature, reaction time, the type and amount of saccharide used. Rheological properties of the Schiff base typed chitosan derivatives were affected by the Maillard reaction extent, reaction time, and especially the reaction temperature. Considering the solubility and the ability of scavenging DPPH radical, the Schiff base typed chitosan-saccharide derivatives were better than the derivatives after reducing. Based on the results of yield, solubility, and pH stability, in this study the most potentially water-soluble chitosan was the Schiff base typed chitosan–fructose derivative which also exhibited higher ability of scavenging DPPH radical compared with chitosan or N-alkylation chitosan derivative. In a words, Schiff base typed chitosan–fructose derivative produced through Maillard reaction is a promising facile and versatile commercial product.

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