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Effects of freezing on the condensed state structure of chitin in alkaline solution☆

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ABSTRACT

Chitin powder was soaked in concentrated sodium hydroxide solution (45%, w/w), and freezed at −18/−35 °C for further modification (the ratio of chitin and sodium hydroxide solution was 1:10, w/v). The degree of deacetylation (DDA) was determined by acid–base titration while the structural changes of the regenerated chitin after alkali-freezing treatment were investigated by X-ray diffraction (XRD), infrared spectroscopy (IR) and differential scanning calorimetry (DSC). The results showed that the formation and growth of ice crystal during the slowly freezing process could break the intra- and inter-molecular hydrogen bonds, disorganize the ordered molecular structure, destruct the compact crystal structure and decline the crystallinity. However, such structural damage was unstable and reversible. Nevertheless, the study explained that the alkali-freezing treatment was beneficial for further modification of chitin, which could significantly promote its solubility and accelerate the chemical reaction rate, while it could also keep its main molecular chains completely.

1. Introduction

Chitin is an ideal linear heteropolymer primarily composed of β-(1→4)-linked 2-acetamino-2-deoxy-β-D-glucopyranosyl residues which is regarded as a high value-added natural polysaccharide. The chemical structure of chitin is very similar to cellulose, while the hydroxyl [–OH] on the C-2 position of cellulose is replaced by an N-acetylamino group [CH_3CONH–] on the chitin molecule. Chitin is usually extracted from shrimp and crab shells derived from offal product of the seafood processing industry, which is decalcified using diluted HCl and then deproteinized by 10% (w/w) sodium hydroxide solution (Lin, Lin, & Chen, 2009). Up to present, chitin is the only natural cationic basic polysaccharide, which is widely distributed in nature as the exoskeleton of marine invertebrates such as prawn, shrimp, crab, krill, lobster shells, molluscan organs, insects, and also found in the cell wall of fungi, mushrooms, yeasts, marine diatoms and algae. Besides, it is the second most abundant natural biopolymer on earth after cellulose, and the most important natural, renewable, high nitrogen content biomacromolecule other than protein. Each year, the amount of chitin biosynthesised by organisms on earth is about 10^{11} tons (Rege & Block, 1999; Tolaimate et al., 2000). Chitosan, composed of β-(1→4)-linked 2-amino-2-deoxy-β-D-glucopyranosyl residues, is one of the most important derivatives of chitin, which is usually prepared from chitin by partial N-deacetylation in concentration alkaline solution (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996). Due to the special chemical structure, chitin and chitosan have many unique properties, such as biorenewablity, biocompatibility, biodegradability, nontoxicity, biofunctional activities (including antibacterial, antioxidative, antitumor, antihypertensive, hypocholesterolemic activity and immuno-stimulating effect, etc.), film or fibre-forming property, heavy-metal ions chelating capacity, hydrophilicity, and a remarkable affinity for many proteins (Gamage & Shahidi, 2007; Rhazi et al., 2002; Rinaudo, 2006), which make them useful for a variety of applications in many fields. At present, chitin and chitosan have been widely applied in the fields of agriculture, textile production, environmental protection, water treatment, biotechnology industry, medicine, pharmaceuticals, dentistry, cosmetic/personal care, functional food, food processing, food packaging, especially as edible films and coatings and so on (Fan et al., 2009; Ravi Kumar, 2000).

However, due to the existence of strong intra- and inter-molecular hydrogen bonds, chitin has a highly ordered stereoregular structure. Therefore, chitin is insoluble in water, diluted acid, alkaline solution, and common organic solvents. But it can dissolve in highly concentrated acids, such as vitriolic acid, hydrochloric acid, phosphoric acid, nitric acid, anhydrous formic acid, amide/lithium chloride (like DMF/LiCl, DMAc/LiCl, and NMP/LiCl) systems (Ravi Kumar, 2000), and NaOH/urea systems (Hu et al., 2007). Nevertheless, these solvents can cause terrible problems

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such as the main molecular chain depolymerization simultaneously with dissolving because of the hydrolysis, the difficult removal of residual solvents, their toxicity, pollution and high cost (Rinaudo, 2006). Furthermore, its principal derivative, chitosan, which can be achieved by partial deacetylation and depolymerization of native chitin, is only soluble in some specific dilute aqueous acidic solution below pH 6.5 (Wang, Turhan, & Gunasekaran, 2004). Because chitosan can be converted into a soluble form $R–NH_3^+$, because of the presence of primary amino groups belonging to its backbone. But it is still insoluble in the neutral or basic range, in spite of high-reactivity amino groups substituting for some inert acetamino groups (Koide, 1998). The poor solubility of chitin/chitosan is the major problem that limits their applications. Recently, some interesting studies were engaged in damaging the rigid crystalline domains, and improving the solubility and chemical reactivity, which utilized the techniques such as chemical modification, high energy radiation, ultrasonic, alkali-freezing and micronization methods (Chau, Wang, & Wen, 2007; Cho, Jang, Park, & Ko, 2000; Ge & Luo, 2005; Liu, Bao, Du, Zhou, & Kennedy, 2006; Liu, Liu, Pan, & Wu, 2007).

The alkali-freezing treatment is one of the most popular methods, which has already been chosen as a convenient precursor for efficient modifications, because of its safety, efficient and minor damage to the main molecular chain. Researchers have observed that the micro-structure of carbohydrate was more or less destroyed by the existence and growth of ice crystal, and then the crystal structure was destructed, due to the mechanical energy produced during the frozen process (Feng, Liu, & Hu, 2004; Hu et al., 2007). However, it is still lack of systemic research on the freezing treatment of chitin. Therefore, to evaluate the effects of alkali-freezing treatment on the solid state structure of chitin is of great research and economic value. In frozen food industry, it is defined as slow freezing when the freezing temperature is above −18 to −23 °C and as rapid freezing when the temperature is below −30 °C. What's more, the common low-temperature of cold storage is usually kept at −35 °C, while that of most domestic refrigerator is usually −18 °C. In this study we chose two temperature (−18 and −35 °C) for further treatment allowing for the feasibility of industry and the properties of alkali solution (Liu, Li, et al., 2009). The effects of slow freezing and rapid freezing treatment were investigated on the condensed structure of chitin in concentrated alkaline solution, which was expected to accumulate theory and practical experience for extending this method utilization in carbohydrate research, as well as providing new research idea for further processing of chitin.

2. Materials and methods

2.1. Materials

Chitin powder derived from shrimp shells was purchased from Zhejiang Yuhuan Biochemical Co. (PRC). The commercial chitins were purified sequentially with 1 mol L^{-1} sodium hydroxide and 1 mol L^{-1} hydrochloric acid at ambient temperature, repeatedly. This was conducted to remove the trace residues of minerals and organic compounds loosely associated on the surface of the chitin particles. Then, they were washed and flushed with distilled water at room temperature, vacuum dried at 60 °C for 48 h and grounded with a laboratory scale hammer mill. The final product was screened to about 80 mesh powder, with a degree of deacetylation (DDA) of 21.11 ± 0.59%, as determined by alkalimetric estimation. The viscosity-average molecular weight was 1.20×10^6, measured in an Ubbelohde viscometer. All other commercially available solvents and reagents were analytical grade, without further purification. Double distilled water was used throughout unless otherwise specified.

2.2. Alkali-freezing treatment of chitin

Traditionally, chitosan is prepared from chitin by N-deacetylation in concentrated aqueous NaOH solution, with a mass concentration range from 40% to 60%. In this paper, 45% aqueous NaOH solution was used throughout. The ratio of chitin powder and NaOH solution was 1:10 (w/v). Chitin powder suspended in aqueous NaOH solution was allowed to keep at room temperature (about 25 °C) for 3 h under diminished pressure, and then placed at normal temperature and pressure for 5 h. This was designed to help the sodium hydroxide penetrate deeper into the internal of chitin particles. And then the mixture in the sealed containers were kept freezing separately at −18 and −35 °C for various time, ranging from 1 to 5 days, each condition in triplicate (Feng et al., 2004). The samples were thawed at room temperature, and neutralized using aqueous HCl (1 mol L^{-1}) in an ice-water bath carefully. Based upon visual observation, the apparent state was observed before neutralized. Then they were dispersed in 95% (v/v) aqueous ethanol (EtOH) under continuous stirring to a final ethanol concentration 70% (v/v), and kept at room temperature for 24 h. The precipitate was filtered and washed with 70% EtOH until the washings became no Cl^- remanet determined by 0.1 mol L^{-1} $AgNO_3$. Subsequently, the precipitate were dehydrated respectively with 95% and anhydrous ethanol, and then dried at 60 °C for 48 h under reduced pressure. Finally, the products were ground and named regenerated chitin.

2.3. Determination of solubility and the degree of deacetylation (DDA)

To estimate the solubility of chitin, 200 mg of dried samples completely dispersed into 20 mL aqueous HCl (0.1 mol L^{-1}) solution and vigorously stirred at 4 °C for 24 h, and then they were centrifuged at 10,000 rpm for 20 min at 4 °C (BC-010_TY2265, Beckman, USA). The insolubles were washed with deionized water to neutral at first and subsequently rinsed with 95% EtOH to dehydrate, then evaporated in vacuum at 80 °C for about 24 h and quantified. The acid–base titration method with a little modification was employed to determine the degree of deacetylation of the samples (Liu, Li, et al., 2009).

2.4. Characterization

Powder X-Ray diffraction (XRD) profiles of the chitin samples were measured with a D/Max diffractometer (D/Max-IIIA, Rigaku, Japan) under 20 °C with a graphite monochromator. The operating conditions were: 30 mA, 35 kV, DS/SS = 1°, RS = 0.3 mm with Cu $K\alpha_1$ radiation at λ = 1.54184 Å. The scanning rate was 4° min^{-1} and the diffraction angle (2θ) ranged from 4° to 60° with steps of 0.02°. The crystallinity index (CrI, %) was determined by the equation $CrI = (100(I_{110} - I_{am})/I_{110})$, where I_{110} is the maximum intensity at around 20°, and I_{am} is the intensity of amorphous diffraction at 16° (Hu et al., 2007; Zhang, Xue, Xue, Gao, & Zhang, 2005). All data were obtained using the MDI jade software package (jade 5.0, Materials Data Inc, Japan).

The FT-IR spectra of the samples were recorded with KBr pellets on an FT-IR spectrometer (NEXUS 470, Nicolet, USA) at ambient temperature (Liu, Li, et al., 2009). The intensity of the selected absorption bands were determined by the baseline method on the basis of the OMNIC software package of the instrument.

Differential scanning calorimetry (DSC) was performed in a Netzsch DSC 204 F1 Phoenix instrument (Germany). Indium metal (99.99%) has been used to calibrate the DSC modulus in relation to temperature and enthalpy. Accurately weighted 5.0 ± 0.1 mg samples were placed into a covered aluminum cups with a central pin hole, while an empty cup was used as reference. The samples were

Table 1
Thermal transitions of chitin, chitosan and regenerated chitin prepared by freezing at −18 and −35 °C for various time, respectively.

Sample		DDA	First run				Second run							
			Endotherm				Exotherm				Endotherm			
			T_o	T_p	T_e	ΔH	T_o	T_p	T_e	ΔH	T_o	T_p	T_e	ΔH
A	1d	24.29 ± 0.30	63.1	107.4	156.9	−274.0	271.2	293.2	320.9	35.2	297.8	343.3	374.0	−61.9
	2d	25.18 ± 0.52[a]	64.8	113.0	162.2	−280.8	269.2	292.0	319.8	33.9	298.2	336.7	372.2	−58.1
	3d	23.91 ± 0.32[a]	60.9	109.8	159.2	−232.3	274.4	297.0	324.1	37.7	301.9	359.0	388.3	−87.5
	4d	24.23 ± 0.77[a]	61.5	105.1	156.0	−257.0	273.2	294.8	324.1	32.3	299.2	340.2	378.9	−70.8
	5d	23.91 ± 1.41[a]	62.3	103.9	153.4	−253.9	273.1	294.3	324.5	36.8	298.8	350.2	385.1	−80.8
B	1d	23.03 ± 0.34	59.2	104.8	154.2	−285.3	277.1	296.0	318.0	33.4	298.6	337.6	373.5	−77.9
	2d	23.11 ± 0.69	61.5	109.5	157.5	−263.5	273.2	294.4	320.2	34.4	299.5	338.4	375.2	−65.8
	3d	24.12 ± 0.64	64.3	111.9	160.7	−255.2	275.4	297.1	324.7	28.1	300.7	354.9	388.1	−68.4
	4d	22.43 ± 0.74	61.2	106.3	158.0	−238.8	265.9	291.1	325.3	33.4	296.8	339.8	376.7	−62.9
	5d	23.65 ± 0.67	64.5	104.8	156.1	−256.9	274.3	295.1	322.2	36.5	300.4	349.4	386.2	−81.2
Chitin		21.11 ± 0.59	60.2	99.1	151.8	−144.3	–	–	–	–	305.8	400.7	419.8	−119.5
Chitosan		87.52 ± 0.37	62.1	102.4	148.7	−275.0	289.3	310.0	326.3	193.2	–	–	–	–

Notes: A, regenerated chitin samples that freezing at −18 °C; B, regenerated chitin samples that freezing at −35 °C; –, not detected. DDA, the degree of deacetylation, %, the values were presented as mean ± SD, $n = 3$; T_o, onset temperature; T_p, peak temperature; T_e, end temperature; ΔH, enthalpy change.
[a] Samples can swell in 0.1 mol L^{-1} HCl solution.

heated at constant heating rate of 20 °C min^{-1} from 30 up to 190 °C, and then maintained at the isothermal stage for 5 min. After cooling with liquid nitrogen, the samples were reweighted and heated from 30 up to 500 °C at a constant heating rate of 10 °C min^{-1} again. Data were collected and analyzed by the Proteus software package of the equipment. The reaction atmosphere was dry dynamic nitrogen while the flow of purge gas and protective gas was 20 and 60 mL min^{-1}, respectively (Guinesi & Cavalheiro, 2006; Kittur, Harish Prashanth, Udaya Sankar, & Tharanathan, 2002).

3. Results and discussion

3.1. N-deacetylation and solubility

Generally speaking, the heterogeneous N-deacetylation of chitin can be regarded as a typical solid-liquid phase reaction, which is followed the rules of multi-phase reaction. In a special range, the DDA increased nearly linearly with the increasing of reaction time and NaOH concentration, and followed the pseudo-first-order kinetics. In this study, by using the alkalimetry to estimate the DDA of chitin, the process of N-deacetylation can be monitored indirectly. And the effects of freezing temperature on the deacetylation of chitin mixed with concentrated NaOH were investigated. In the carbohydrates frozen processing, previous studies had shown that the ice crystal at first appeared in the particles' gaps of the sample during slow freezing, and they were distributed uneven. Therefore, the chemical structure was destroyed considerably. However, during the rapid freezing, the ice crystal can be dispersed evenly in the particles' gaps and formed a lot of small needle-like ice crystal. So the damage was weak (Feng et al., 2004; Hu et al., 2007). In the current research, the two temperatures −18 and −35 °C were chosen for further treatment, respectively.

After the freezing treatment, all samples were thawed and the apparent state of the chitin-alkali mixture was observed by eyes at first. It has been reported previously that chitin can be dissolved when an alkali chitin suspension with a polymer mass fraction of 0.01 and an alkali mass fraction of 0.1, which was mixed with plenty of crushed ice and stirred vigorously below 0 °C. It can also be dissolved into aqueous NaOH as the alkoxide form when the chitin-alkali mixture was stirred at room temperature and stored overnight at about −20 °C (Dong, Wu, Wang, & Wang, 2001; Tokura & Tamura, 2001). However, in the current study, it shows obviously that dissolution phenomena were not observed on all the samples frozen at two different temperatures. It was only observed that chitin powder can be infiltrated by NaOH when it was frozen at −18 °C, and the samples freezing at −35 °C have no significant changes. Furthermore, after 3 days freezing (−18 °C) treatment, and the thawed chitin-alkali mixture was centrifuged at 10,000 rpm for 10 min at 4 °C to remove the excess alkali solution, there was an interesting discovery that most of the precipitates can be dissolved in aqueous 10% NaOH solution and the solubility could reach to almost 80%. Nevertheless, the solution was not stable that it will become turbid when the temperature changes or when it is stirred.

Table 1 listed the DDA values of the regenerated chitin via alkali-freezing treatment at −18/−35 °C, respectively. It indicated the effects of alkali-freezing processing on the deacetylation reaction and solubility of chitin. After 5 days freezing treatment, the DDA of regenerated samples have no significant difference and there was no significant difference compared with the raw chitin material. It means that during alkali-freezing treatment the N-deacetylation reaction was very slow. This is similar to the popular method under usually conditions. Reaction temperature usually has a major effect on the rate of N-deacetylation, the reaction rate decreased with the temperature lowered. When a certain amount of regenerated samples dispersed in 0.1 mol L^{-1} HCl solution and mixed for a period of time, dissolution phenomena could not be observed. Samples frozen at −18 °C have slight swelling but samples frozen at −35 °C have no marked swelling. It indicates that in the slowly freezing processing the formation and growth of ice crystals could force the NaOH molecular penetrate into the internal of rigid chitin particles, and it could break the intra- and inter-molecular hydrogen bonds, destruct the compacted crystal structure of chitin. Furthermore, the damage on the entire molecular chain was slighter. It could preferably maintain the integrity of the chitin molecular structure. It means that chitin will be possible to be raw materials for further chemical modification.

3.2. Changes of crystal structure and hydrogen bonds

3.2.1. X-ray diffraction profiles of chitin

Many previous researches have been reported that crude chitin is an anhydrous crystalline polymorphic substance, which is classified into three different forms: α-, β- and γ-chitin. The α-chitin is the most abundant and also the most stable polymorphic form. It is primarily present in the cuticles of arthropods, the exoskeleton of crustaceans and the cell wall of some fungi and mushrooms, which is often associated with mineral deposition together to form a hard shell. In which, the adjacent chains are arranged in an antiparallel

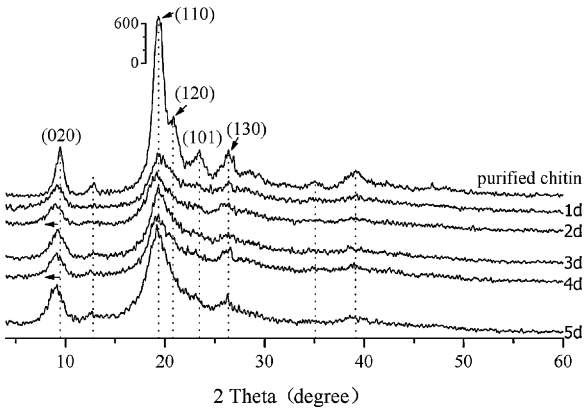

Fig. 1. XRD profiles of the crude chitin and regenerated chitin samples freezing at −18 °C in alkaline solution, respectively.

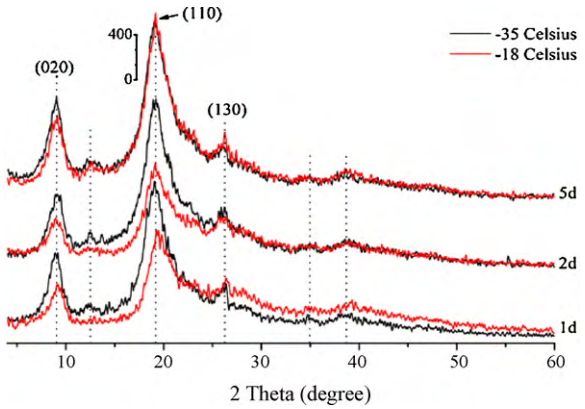

Fig. 2. XRD profiles of the regenerated chitin samples freezing at −35 °C compared with the samples freezing at −18 °C.

orientation, and the crystal structure is the most compact due to the existence of a large number of inter-chain hydrogen bonds. Compare to α-chitin, β-chitin is usually linked with collagen to offering many physiological functions, which is different from backstops composited of α-chitin. In nature, β-chitin occurs in squid pens, the extracellular spines of the euryhaline diatom, pogonophore tubes and many others. And in β-chitin the chains are arranged in a parallel manner with weak hydrogen bonding. Whereas the γ-chitin form has a mixture of antiparallel and parallel chains, and is very rare in nature. Therefore, the γ-form has not been subjected to the detailed analysis given to the α- and β-forms, and it has not yet been established that this is a true third structure. Additionally, the β- and γ-chitin forms can be irreversibly converted into the α-form (Al-Sawalmih, 2007; Cho et al., 2000; Franca, Lins, Freitas, & Straatsma, 2008; Noishiki et al., 2003).

Fig. 1 showed the wide angle XRD patterns of original chitin and regenerated chitin obtained from alkali-freezing treatment at −18 °C for various times. It illustrated clearly that the raw purified chitin exhibited five fairly sharp diffraction peaks at 9.44°, 19.28°, 20.80°, 23.70° and 26.40° in the 2θ range of 4–60°, which could be assigned to the diffraction planes (0 2 0), (1 1 0), (1 2 0), (1 0 1) and (1 3 0) of the orthorhombic crystal structure, respectively. Indeed, it indicated that the original chitin is α-form, being congruent with reported values (Cho et al., 2000; Liu et al., 2007; Zhang et al., 2005). The intensity of the five clear diffraction peaks were obviously weakened or to be concealed by various times' alkali-freezing treatment, especially the crystalline reflections at 20.80° and 23.70° corresponding to the (1 2 0) and (1 0 1) crystal planes were almost completely covered up. Furthermore, the sample (5d) with 5 days freezing treatment showed a very similar X-ray diffractogram to that of α-chitin, although the peaks were less intense and broader compared with raw α-chitin. Whereas the diffractograms of other samples (1d–4d) were considerably different from that of α-chitin. On the contrary, they were more similar to the diffractogram of β-chitin, which indicated that the crystal structure changed during alkali-freezing treatment. By peak fitting of the diffraction profiles in Fig. 1, the intensity of reflections and the crystalline index (CrI_{110}) were calculated out. It was found that the intensity of reflections at 19.28° diminished significantly compared with the raw chitin. Almost on the first or the second day, the intensity decreased to the minimum 35% and 34.62%, respectively. And then it was increasing slowly on the following days, and it recovered to 59.43% with 5 days' treatment in contrast with raw chitin. However, the changes of crystalline index were not significant by the comparison with the reflection intensity. The CrI_{110} of treated samples (1d and 2d) reduced from 99.15% for initial chitin to 83.16% and 82.30%, respectively. And it increased to 96.05% for 5 days' freezing treatment. The results showed that the alkali-freezing process could reduce the crystallinity of chitin during the first 2 days. If adopt this as a means of pre-treatment, it would be beneficial for further modification of chitin. Nevertheless, it is difficult and limited to improve further damage on the condensed structure of chitin by only increasing the alkali-freezing time.

It was worthwhile to note that the intensity of (0 2 0) reflection, decreasing linearly with the increase of DDA, which correlated with the crystallinity, which was suggested a possibility for XRD to determine DDA of macromolecular chitin (Zhang et al., 2005). Although in general terms, the intensity of (0 2 0) reflection was definitely reduced, it increased slowly after 2 days' treatment. The d-spacing of the (0 2 0) plane increased from 9.3685 Å for original chitin to 9.9580 Å for 2d sample, and then remained at about 9.7171 Å for other samples with a prolonged treatment. This was a further proof that the alkali-freezing treatment could reduce the regular crystalline structure and make the molecular chains arranged in loose with decreasing of crystallinity, which was conducive for further chemical modification. However, this was different from the reported values in the previous literature (Zhang et al., 2005) probably because the influence of alkali-freezing treatment on the DDA of chitin was insignificant. In this case, the effects of alkali-freezing treatment on the crystalline region were much larger than that of changes of DDA.

It was found that the X-ray diffractogram of samples (1d, 2d and 5d) frozen at −35 °C was very similar to the 5d sample that frozen at −18 °C, as shown in Fig. 2. And the intensity of reflections almost had no difference among all the three samples, but was larger than that frozen at −18 °C (1d and 2d samples). This illustrated that the temperature about −18 °C was more suitable for destructing the interaction force between the chitin molecules, whereas the lower temperature was not conducive to this place. The main reason was that there was a so called freezing concentration effect at approximately −18 °C, which was 1–2 °C below the freezing temperature of chitin-alkali mixture. Indeed, it would be found that it began to turn to ice crystal below −16 °C by measuring the approximate frozen temperature of the mixture (it cannot be regarded as physical concept "freezing point"). At this temperature, the motility of the small and medium-sized molecules in the ice crystals obviously was more violent than that at −35 °C. The ice grains were also larger than the latter, and the mechanical force induced by the repeated forming and recrystallizing of ice crystals during freezing at −18 °C was much larger. It could be more effective to undermine the crystal structure of chitin.

However, when freezing at −35 °C, the molecular motility could be greatly reduced and the formation of numerous ice crystals were smally and uniformly distributed, due to the mixed system

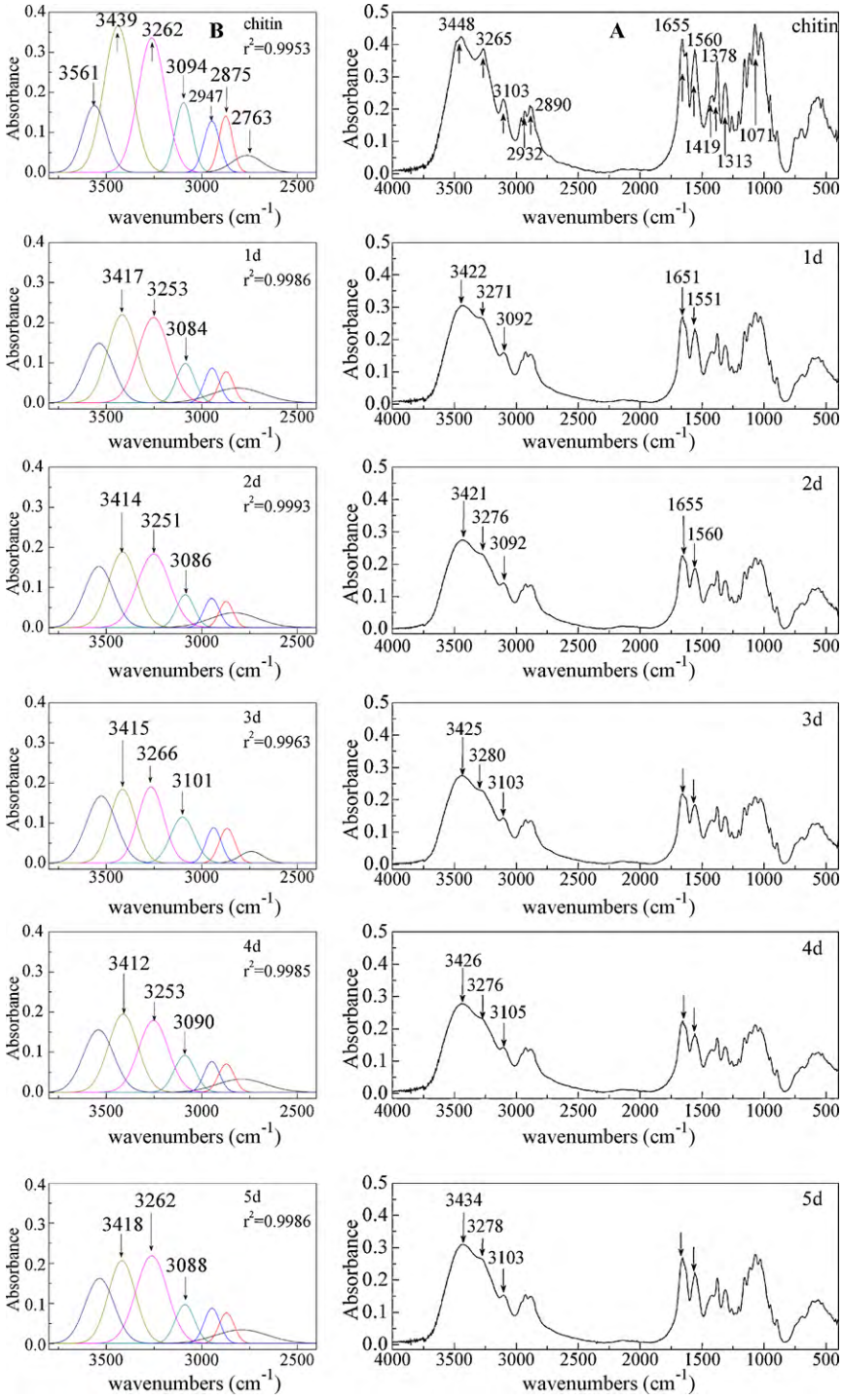

Fig. 3. FT-IR spectra (A) and curves fitting (B) of chitin and regenerated samples that freezing at −18 °C, respectively.

being completely under glassy state. Therefore, the destruction of the chitin crystalline structure was relatively limited, and which was consistent with many phenomena that was actually observed in frozen processing of carbohydrates. Furthermore, in the case of 5d sample frozen at −18 °C, the X-ray diffractogram almost completely coincided with the 5d sample that frozen at −35 °C. This confirmed that with a long time's treatment the strong motility of chitin molecules were forced to arrange very loosely, and was not in a steady state. They needed to afresh carry out self-assembly, such as to increase the entropy and make the chitin molecules return to a low energy state. Accordingly, to adapt to the new environmental phase state, it might have minor changes in some microcrystalline structure and the crystallinity would increase a little.

3.2.2. FT-IR spectra of chitin

Infrared spectroscopy is one of the most widely used methods to study hydrogen bonding and other interactions. To evaluate the changes of hydrogen bonds caused by alkali-freezing treatment, the FT-IR spectra of initial chitin and samples frozen at −18 and −35 °C were measured as shown in Figs. 3A and 4A, respectively. The major absorption wavenumbers and its ascription of chitin had been largely discussed in the previous literature. It could

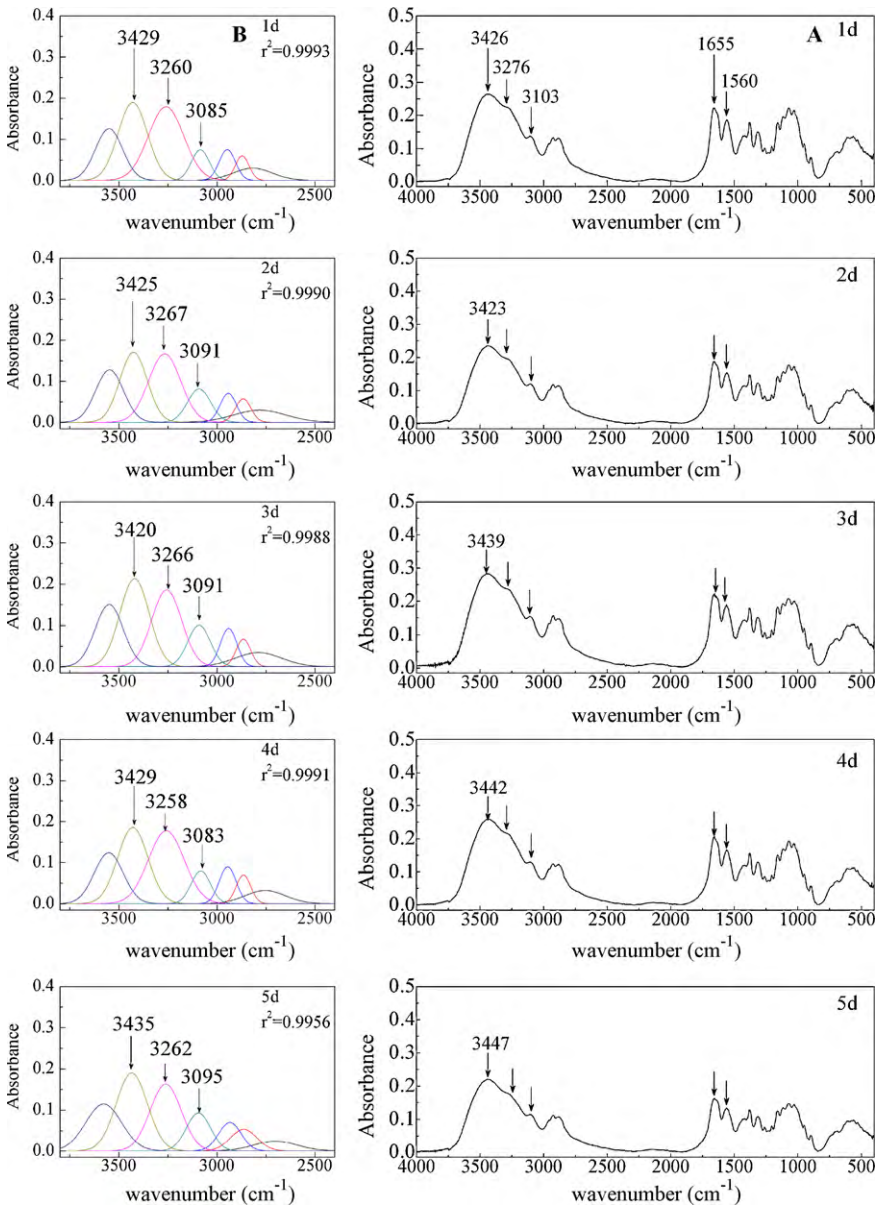

Fig. 4. FT-IR spectra (A) and curve fitting (B) of regenerated samples that freezing at −35 °C, respectively.

be seen that absorption band at 3448 cm^{-1} in chitin was referenced as OH stretching and the absorption of intra-hydrogen bonds (Hu et al., 2007), which became broader, weaker and shifted to a lower wavenumbers with increasing freezing treatment, whether it was dealt with in −18 or −35 °C. In addition, the difference was that the changes of samples frozen at −18 °C were much larger than that frozen at −35 °C. However, with an extension processing the shift would be slowly restituted again. Furthermore, the absorption peaks that appeared at about 3265 and 3103 cm^{-1} were attributed to the stretching vibration of N–H (amide III), which were reduced and almost concealed, indicating that the inter-molecular $C(2_1)NH\cdots O{=}C(7_3)$ and $C(6_1)OH\cdots HOC(6_2)$ hydrogen bonds were diminished, respectively. This result also implied that antiparallel arrangement of the molecular chains in α-chitin transformed to parallel arrangement with alkali-freezing treatment (Cho et al., 2000). Therefore, the results mentioned above suggested that the intra- and inter-hydrogen bonds were destroyed significantly by alkali-freezing with 1 or 2 days treatment at −18 °C. Additionally, the absorption bands at 1655, 1560 and 1313 cm^{-1} in initial chitin were considered as the contributions of amide I, II and III bands, respectively. The relative absorption intensities of them in the regenerated samples were weakened without moving, which suggested that the alkali-freezing treatment only demolished the rigid crystal structure but had not destroyed the main molecular chains. This was in conformity with the XRD results.

In general, due to the complicated and specific network of hydrogen bonds that was generated by the OH and NH groups, the FT-IR spectra of chitin was quite complex, and made the absorption bands in the wavenumbers range of 2300–3800 cm^{-1} were vulnerable to interference and not easy to distinguish each other. Thus, in order to assess the impact of alkali-freezing treatment more clearly, the diffractograms of Figs. 3A and 4A in the regions of 2300–3800 cm^{-1} were first normalized on the basis of constant total intensity, respectively. After normalization, the curves which mathematically best fitted to the original spectrum were obtained by the Gaussian function, with the software of PeakFit Suite (Version 4.12, SeaSolve Software Inc., USA), and then separated into each component peak of a symmetric nature associated

with $r^2 > 0.9900$, as shown in Figs. 3B and 4B, respectively. Indeed, they illustrated clearly that the absorption band at 3448 cm^{-1} in chitin could be separated into two single peaks, as 3439 and 3561 cm^{-1}, which were assigned to the inherent OH and free OH stretching, respectively. After alkali-freezing the band at 3439 cm^{-1} became broader and weaker with a shift to lower wavenumbers, but the band at 3561 cm^{-1} almost did not change. In contrast with −18 °C samples, the changes and shifts were smaller. Furthermore, the bands at 3262 and 3094 cm^{-1} in chitin also became weaker and broader after alkali-freezing. However, in the initial spectra of regenerated samples those absorption bands were almost concealed.

3.2.3. Thermal characteristics of chitin

Thermal methods, such as differential scanning calorimetry (DSC), differential thermal analysis (DTA) and thermogravimetry (TG) have emerged as powerful thermoanalytical techniques to monitor characteristic physical and chemical changes in biopolymers, as a function of temperature. Especially, DSC is one of the most attractive techniques, as it is sensitive, fast, user-friendly, low-priced and suitable for both solid and liquid samples, and both low and high temperature. It has been widely used in many fields of polymer research. For example, it is usually used to research the compatibility, weak phase transition and the molecular mobility in the crystalline and amorphous solid states of polymers (Guinesi & Cavalheiro, 2006). In fact, polysaccharides usually have a strong affinity for water, and in the solid state these macromolecules may have amorphous structures which can be easily hydrated. As was known, the hydration properties of these polysaccharides depended on the primary and supramolecular structures (Kittur et al., 2002). Moreover, the combined water may affect the observation of the real DSC thermograms. Therefore, in order to rule out the possibility of the effect, a two-step heating process was employed. The first run was performed by heating the samples from 30 up to 190 °C at a heating rate of 20 °C min^{-1} and then with an isothermal for 5 min. After cooling, the samples were immediately reweighed and heated from 30 up to 500 °C at a heating rate of 10 °C min^{-1}.

Indeed, the DSC curves for all samples in the first run have a wide endothermic peak centred between 99 and 113 °C with an onset at around 60 °C and an end at 148–162 °C. The values for the transition temperature and their associated enthalpies were given in Table 1. Whereas the peaks were not observed in the second run, and this result could be considered as the contribution of the vaporization of water that presented in the samples. It indicated that the samples were not truly anhydrous and some bound water was not completely removed during drying with reduced pressure. Table 1 showed obviously that not only the peak temperature and the end temperature but also the enthalpies of samples A and B, respectively, were higher than that of chitin and chitosan. Additionally, the values of samples A were even much larger. It might be possible to conclude that after alkali-freezing treatment there were more amorphous regions in the solid structure, which could combine more water than the initial samples.

Fig. 5 depicted the second heating scans of purified chitin, commercial chitosan (DDA = 87.52%) and regenerated chitin samples, respectively, after cooling the initially heated samples with liquid N_2. In the case of chitin, only one thermal event registered was a wide endothermic peak centred at around 400.7 °C with an onset and end at around 305.8 and 419.8 °C, respectively, which was primarily related to the decomposition of acetyl-glucosamine (GlcNAc) units and the evaporation of volatile low molecular products that formed during depolymerization. However, the thermal event registered in chitosan was a sharp exothermic peak between 289 and 326 °C and centred at around 310 °C, which was shifted to a lower temperature. Furthermore, it was similar to that of FTC

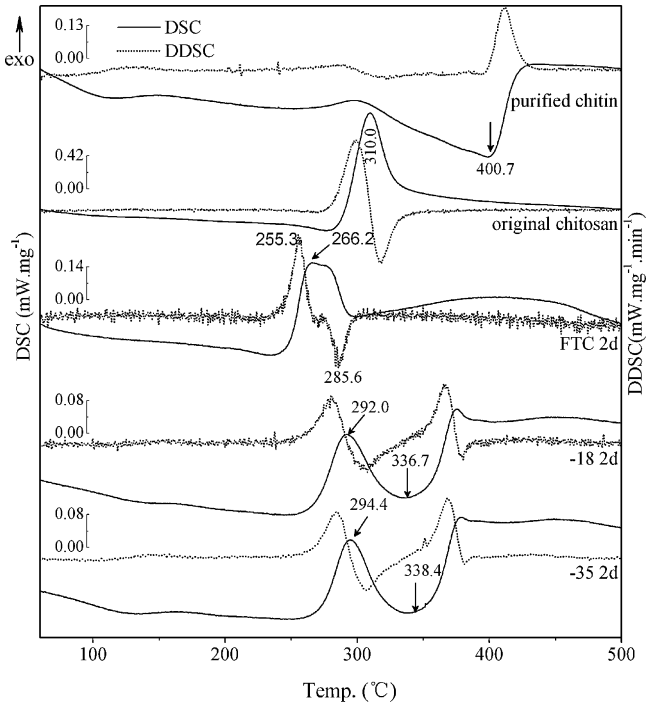

Fig. 5. The second run DSC (DDSC) thermograms of purified chitin, original chitosan and regenerated samples, respectively. *Note*: FTC 2d, regenerated chitin samples via 2 day's freezing-thawing cyclic treatment (Liu, Chen, et al., 2009). −18 2d/−35 2d, regenerated chitin samples only freezing at −18/−35 °C for 2 days, respectively.

2d sample, mainly ascribing to the decomposition of glucosamine (GlcN) units, and it was very different from the event of chitin due to the fewer GlcNAc units that existed in chitosan. Close examination of the thermograms in Fig. 5 revealed that the thermal event of regenerated chitin was neither similar to chitin and chitosan, nor to FTC 2d sample. The detailed analysis data were listed in Table 1. It could be seen clearly that there were two peaks which were not completely separated in the second run DSC curves for samples that via alkali-freezing at −18 and −35 °C. The first observed thermal event was a wide exothermic peak centred around 294 °C, which could be ascribed to the recrystallization of the molecular chains, suggesting that the damage of the crystalline structure was unstable and reversible in which new crystalline structure could be formed by prolonged heating. The second thermal behavior was similar to that in the case of chitin, but the difference was that the peak temperature shifted to a lower temperature in this case. It indicated that the thermal stable was lower after alkali-freezing, however, still higher than chitosan.

To sum up, the results might be possible to presume that the damage of crystalline structure was not very thoroughly induced by alkali-freezing treatment. However, this damage was reversible which could form a new crystal after heating. But even so, alkali-freezing processing could still significantly reduce the compact crystal structure, decrease the stability, and depress the decomposition temperature of chitin molecules, and so on.

4. Conclusions

Many analysis techniques, such as XRD, FT-IR and DSC were employed to investigate the structural changes of chitin that induced by alkali-freezing treatment. The detailed data showed that the formation, recrystallization and growth of ice crystals could significantly slack the intra- and inter-molecular hydrogen bonds, and destroy the highly rigid molecular structure, which can make the crystallinity declined, and it was conducive to further structural

modification. Additionally, the structural damage during slowly freezing was more dreadful than that during rapid freezing. They could force the chitin molecules to transform from steady state to metastable state, and rapid freezing even make the sample in a more labile metastable state. Based on the results, it was obvious that alkali-freezing treatment only destroyed the crystalline structure, but did not damnify the skeletal structure of the molecular chain, which was contributing immensely to maintain the inherent biological activity of chitin. Although the decrease of crystallinity induced by alkali-freezing treatment was limited and that was partially reversed. But it is certain that alkali-freezing treatment could be used as an effective and controlled pre-treatment method of chemical modification for chitin to enhance the reactivity.

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