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The physicochemical properties and antitumor activity of cellulase-treated chitosan

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Abstract

Cellulase was used to partially hydrolyse *N*-acetylated chitosan. The hydrolysis process was monitored by gel permeation chromatography. Factors affecting the enzymatic hydrolysis of chitosan were studied. The degraded chitosans were characterized by X-ray diffraction, thermogravimetric analysis, differential thermal analysis, Fourier transform infrared and carbon-13 magnetic resonance spectra. The results showed that the enzymatic hydrolysis was by endo-action, and the total acetylation degree of chitosan did not change after degradation. The decrease of molecular weight led to transformation of crystal structure, alteration of thermostability and increase of water-solubility, but the chemical structures of residues were not modified. Most reducing end-residues of produced oligomers were GlcNAc units. This water-soluble product inhibited the growth of sarcoma180 tumor cells in mice with maximum inhibitory rates of 50% by intraperitoneal injection and 31% by oral administration.

Keywords: Chitosan; Cellulase; Enzymatic hydrolysis; Antitumor activity

1. Introduction

Chitin is the second most abundant biopolymer on earth after cellulose, available largely in the exoskeletons of invertebrates and the cell walls of fungi (Kumar, 2000). Disposal of shellfish has been a challenge for most of the shellfish-producing countries. Chitosan is the name used for low acetyl substituted forms of chitin. They are linear heteropolysaccharides composed of β-1,4-linked D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) with various compositions of these two monomers. These polysaccharides have received much attention for a wide range of unique applications in food, including bioconversion for the production of value-added food products, preservation of food from microbial deterioration, formation of biodegradable films, purification of water and clarification and deacidification of fruit juices (Shahidi, Arachchi, & Jeon, 1999). These functions have been revealed to be dependent, not only on their chemical structure but also their molecular size (Babiker, 2002; Qin, Du, Xiao, Liu, & Yu, 2002). With the emergence of potential biomedical effects such as antitumor activity (Seo, Pae, & Chung, 2000; Suzuki, Mikami, Okawa, Tokoro, Suzuki, & Suzuki, 1986; Tokoro, Tatewaki, Suzuki, Mikami, Suzuki, & Suzuki, 1988), immuno-enhancing effects (Kobayashi, Watanabe, Suzuki, & Suzuki, 1990; Tokoro, Suzuki, Matsumoto, Mikami, Suzuki, & Suzuki, 1988), enhancing protective effects against infection with certain pathogens (Tokoro, Kobayashi, Tatewaki, Suzuki, & Okawa, 1989), for lower molecular weight water-soluble chitosan, the development of viable processes for the degradation of chitosan is attracting growing interest (Qin, Du, Xiao, & Gao, 2002). Enzymatic processes seem to be generally preferable to chemical reactions, since the hydrolysis course and product distribution are subject to more facile control, in spite of the faster rate of chemical reaction (Qin, Du, & Xiao, 2002). However, the high cost of specific enzymes, such as chitosanase and chitinase, inhibits their use on an industrial scale. Recently, several

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hydrolytic enzymes, such as lysozyme (Vårum, Holme, Izume, Stokke, & Smidsrød, 1996), pectinases (Shin-ya, Lee, Hinode, & Kajiuchi, 2001), hemicellulase and papain (Pantaleone, Yalpani, & Scollar, 1992) were found to catalyze the cleavage of the glycosidic linkage in chitosan. Nevertheless, there are few papers on the relation between the molecular weight and the physicochemical properties of obtained products, in detail, to date. An understanding of physicochemical properties is essential for better application.

This work is concerned with the degradation of chitosan using cheap, commercially available cellulase, which is widely produced in nature by many bacteria, fungi and plants. The method is suitable for scale-up manufacture of the low-molecular weight chitosan. Factors affecting the enzymic hydrolysis of chitosan are studied. The products, with different molecular weights, were comparatively investigated by GPC, FT-IR, ^{1}H NMR, TGA/DTA and chemical analyses. The products with low-molecular weight were used for assay of their antitumor activity against sarcoma 180.

2. Materials and methods

2.1. Materials

Chitosans, as initial material from shrimp shells, was obtained from Hubei Yufeng Biology Engineering Co., Ltd. (China). The UF membranes (OSOO1C11, OMEGA) with NMWL 100, 10, 3 and 1 kDa were purchased from PallFiltron Corporation (USA).

The crude cellusase, derived from *Trichoderma viride*, was a product of the Shanghai Institute of Physiology, Academia Sinica (China). The enzyme solution was the fraction of the culture supernatants that passed through a 100 kDa membrane but did not pass through 5 kDa one. Further purification was achieved by the following processs (Tan, Liu, Li, & Guo, 1992): solid ammonium sulfate was added to the enzyme solution, to 80% saturation; the solid enzyme was collected by centrifugation and redissolved in water. The solution was then loaded onto the Sephadex G-25 column and deionized water was used as the eluent. The eluate was monitored for protein by measurement of the absorbance at 280 nm. The main active fraction was concentrated by 1 kDa membrane, and collected as the purified cellulase solution with a content of 103 mg/ml).

2.2. Characterizations

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) were measured by GPC. The GPC equipment consisted of the connected column (TSK G5000-PW and TSK G3000-PW), TSP P100 pump and RI 150 refractive index detector. The flow rate was maintained at 1.0 ml/min. 0.2 M CH_3COOH/0.1 M CH_3COONa was used as the eluent. The standards used to calibrate the column were TOSOH pullulans. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

FT-IR spectra were recorded with KBr pellets on a Perkin-Elmer pectrum One B spectrophotometer.

^{1}H NMR spectra were recorded on a Varian Mercury 300 spectrometer. Water-soluble samples were dissolved in D_2O.

X-ray diffraction patterns of samples were measured by a Shimadzu XRD-6000 diffractrometer and used a CuK_α target at 40 kV and 30 mA at 23 °C.

TG and DTA of sample (5.0 mg) in stationary air were performed by a Differential Thermal Analyzer Model WCT-1 (Beijing Optical Instruments Factory, China) from 25 to 600°C at a heating rate of 10 °C/min.

2.3. Determination of the degree of deacetylation (DD)

The chitosan (0.3 g) was dissolved in a known excess of 0.1 M HCl acid (20 ml). From the titration of this solution with a 0.1 M NaOH solution, a curve with two inflexion points was obtained. The difference between the volumes at these two points corresponded to the acid consumed for the esterification of amine groups and allowed the determination of DDA of the chitosan. The titration was performed with a DELTA-320-S pH meter.

2.4. Batch experiments of enzymatic hydrolysis

Unless otherwise stated, the degradation process was carried out as follows: the chitosan powder (0.25 g) was introduced into a reactor containing liquor adjusted to the desired pH with acetic acid. The chitosan to liqor ratio 1:40 (w/w) was used. After the mixture was stirred for 1 h, the reactor was kept in a thermostatic water bath and enzyme solution (150 μl) was added. Details of the reaction conditions are given in the text. At various intervals, 0.5 ml of the reaction mixture was taken out. After heating in a water bath at 95 °C, filtrate was analyzed by GPC.

2.5. Preparation of samples by enzymatic hydrolysis

Chitosan (100 g) was completely dissolved in 2000 ml 2% acetic acid. After 3 h, the solution was neutralized to pH 5.5 and left overnight. The solution in the reaction vessel was placed in a water bath at 50 °C and 100 ml of enzyme solution was added in order to initiate reaction.

After 1 h, 300 ml of mixture were taken out and boiled for 10 min to denature the enzyme. The hydrolyzate was filtered, and the filtrate was neutralized with

10% NaOH to pH 9. The precipitate was washed thoroughly with distilled water and ethanol. The sample ES-S1 was collected after drying over phosphorus pentoxide in vacuo.

After 4, 12 and 24 h, UF-membrane of 10 kDa was used to remove the enzyme. The ultrafiltrates were concentrated to about one-twentieth of the original volume with a rotary evaporator under diminished pressure. The mixtures were neutralized to pH 9 and precipitated by adding ethanol. The precipitates were thoroughly washed with ethanol. The precipitates were collected after drying over phosphorus pentoxide in vacuo to give samples ES-S2, ES-S3 and ES-L4.

2.6. Estimation of water-solubility

The water-solubility of chitosan samples was estimated as follows. The weighed sample (0.5 g) was suspended in 10 ml distilled water at 25 °C for 2 h with constant mixing. Soluble chitosan was removed by centrifugation at 5000 rpm for 15 min. The precipitates were thoroughly washed with ethanol. The precipitates were collected and weighted after drying over phosphorus pentoxide in vacuo. The solubility of chitosan in water was determined by the percent of dissolved chitosan.

The pH-dependence of water-solubility of chitosan was evaluated from turbidity. The sample (100 mg) was dissolved in 1% AcOH (100 ml). Following stepwise addition of concentrated NaOH, the transmittance of the solution was recorded on a UV-1601 Shimadzu spectrophotometer using a quartz cell with an optical path length of 1 cm at 600 nm.

2.7. Assays of antitumor activities

At least 10 mice were used for each sample and dose. Mean values and S.D. were determined by standard methods (Suzuki et al., 1986). The significance of the difference was estimated by the standard student *t*-test.

Antitumor effects on sarcoma 180 were observed in normal femal Kunming mice or Balb/c mice. The test was reformed by observing the effect on the growth of the tumor, in ascite form of a dose of 0.2 ml (about 1.0×10^7/ml) implanted subcutaneously at the right groin. Twenty-four hours after the tumor implantation, the test sample dissolved in physiological saline was provided once a day by intraperitoneal injection (i.p.) or by oral administration (p.o.) for 10 days, while 0.9% saline was provided for the control group. The animals were sacrificed, and the tumors were dissected and weighed. The tumor growth inhibition ratio was calculated by using the formula: Inhibition rate (%) $= 100 \times (C - T)/C$, where C is the average tumor weight of the control group and T is the tumor weight of the treated sample group. The spleen index was calculated by using the formula: Spleen index = [spleen weight ÷ final bodyweight] × 100.

3. Results and discussion

3.1. Reduction in M_w

Samples were taken at intervals during the enzymic hydrolysis. Fig. 1 shows the GPC profiles of chitosan with 85% of degree of deacetylation (DD) and its degraded products. The chitosan was hydrolyzed with cellulase in acetic acid solution of pH 5.5 at 50 °C. The shift toward higher elution volumes, as a consequence of the degradation, could be observed for all samples. Obviously, the extent of degradation was increased by prolonging the duration. These profiles give information on the degradation process, indicating that the degradation of the backbone mainly occurred in a random fashion. A rapid decrease in the viscosity of chitosan solution was seen in 30 min.

Fig. 2 shows the effect of the pH of the medium on the rate of degradation, which corresponds to the M_w of the resulting chitosan. The cellulase preparation displayed an optimum pH of 6.0. Actually, a chitosan solution of

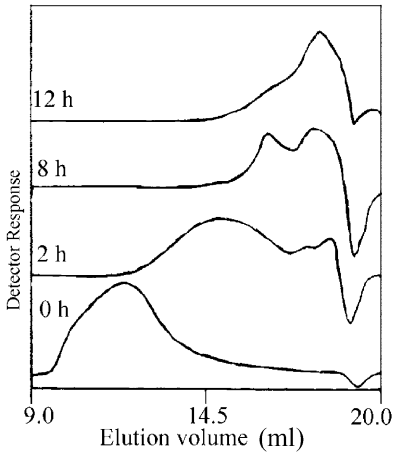

Fig. 1. GPC profiles of enzymatic products at different reaction periods.

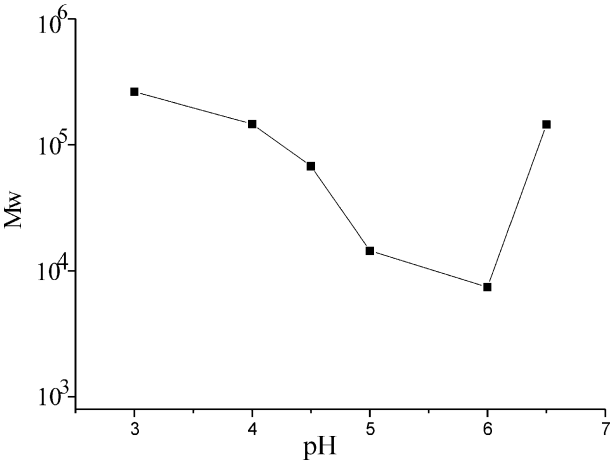

Fig. 2. pH dependence of the enzymatic hydrolysis of chitosan.

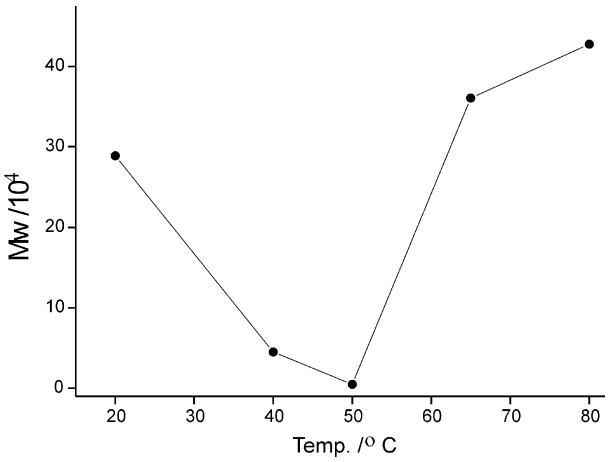

Fig. 3. Effect of temperature on the enzymatic hydrolysis of chitosan.

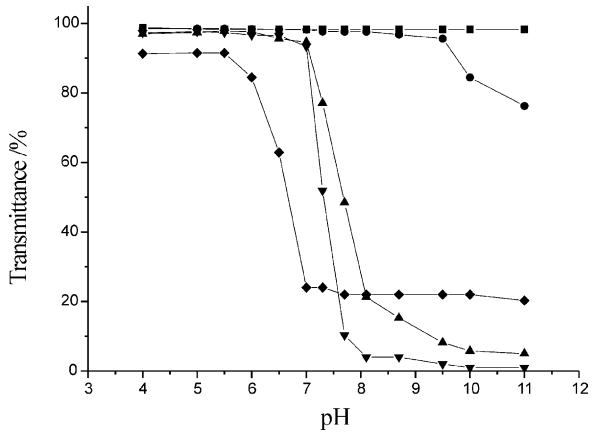

Fig. 4. pH dependence of water-solubility of chitosan with different molecular weights. (◆) SCH85, (▼) ES-S1, (▲) ES-S2, (●) ES-S3, (■) ES-L4.

pH 5–5.5 was adopted. It is difficult to make the initial chitosan dissolve completely at pH 6 on a mass scale. Above pH 6, the degradation is for a heterogeneous reaction. In general, the homogeneous reaction was more rapid than the heterogeneous reaction. Nevertheless, strong acidity also inhibited the hydrolysis of chitosan.

Fig. 3 shows the temperature dependence of specific activity of the biocatalyst in the process of chitosan hydrolysis. The optimum temperature for hydrolyzing 2.5% chitosan solution with the enzyme was determined using the batch reactor at pH 5.5, and was found to be 50 °C. The enzyme was relatively stable in the temperature range below 60 °C, but was rapidly inactivated at higher temperatures.

The influences of DD on chitosan enzymatic susceptibility were also examined (Table 1). The DD plays an important role in the action of enzyme on the chitosan polymer (Yalpani & Pantaleone, 1994). Cellulase is glycanase and has different affinities for the β-1,4 bond, which depend on its position and also on its microenvironment. *N*-acetyl groups and their distribution cause a change in the affinity between enzyme and substrate. Chitosan with DD of 85% was the preferred substrate for this enzyme preparation. The bimodal molecular weight distribution of partially degraded sample in Fig. 1 also confirmed the influence of *N*-acetyl groups. The distribution of the acetyl groups in initial chitosan does not even occur in the commercially available chitosan, which is generally produced by heterogeneous *N*-deacetylation of chitin.

3.2. Solubility of degraded chitosan

The properties of partially degraded chitosan products and their water-solubilities are listed in Table 2. SCH85 is the purified product of initial chitosan with 85.5% DD by acetic acid and NaOH. The total DD of chitosan did not change after degradation. But the water solubility obviously depends on the molecular weight of chitosan. Lowering the molecular weight helps to improve solubility in water.

Fig. 4 depicts the pH dependence of transmittance of chitosan solution. Water-solubility of the sample with $M_w < 1.5\times10^3$ was higher and retained over a wide pH range, whereas the solubility of sample with $M_w < 10\times10^3$ was high at acidic pH but abruptly decreased at a pH a little over neutrality. It seems that the high solubility of low-molecular weight chitosan is attributed to the decrease of intermolecular interactions, such as van der Waals forces and hydrogen bonds; the lower the molecular weight, the lower the intermolecular attraction forces (Kubota, Tatsumoto, Sano, & Toya, 2000). When the chitosans were dissolved in aqueous acetic acid, their solubility at neutral pH

Table 1
The enzymatic hydrolysis of chitosan with different DD

Initial chitosan		M_w ($\times10^{-3}$) of chitosan after hydrolysis
DD (%)	M_w ($\times10^{-4}$)	
72.6	85.9	4.42
85.0	42.7	3.20
92.8	36.6	6.25
97.6	28.0	62.1

Table 2
Properties of degraded chitosan

Sample	$M_w\times10^{-3}$	M_w/M_n	DD (%)	Solubility (%)
SCH85	427	4.35	85.0	0
ES-S1	63	2.68	85.2	0
ES-S2	10.2	1.86	85.4	5
ES-S3	2.3	1.29	85.9	26
ES-L4	1.4	1.23	86.3	99

appeared to be higher than that in pure water. Samples ES-S2 and ES-S3 were not soluble in pure water, but they did not precipitate from the solution at pH 7.0.

3.3. FT-IR spectra

FT-IR spectroscopy has been shown to be a powerful tool for the study of the physicochemical properties of polysaccharides. Curves *a* and *b* in Fig. 5 show the IR spectra of initial chitosan SCH85 and produced chitooligomers ES-L4. The absorption bands at 1654.5 and 1598.9 cm^{-1} in SCH85 are, respectively, attributed to the amide I and N–H bending mode of $-NH_2$ (Pearson, Marchessault, & Liang, 1960). The spectrum of ES-L4 is similar to that of SCH85, but the amide I shifted to lower wave number, suggesting that carbonyl groups had more opportunity to form stronger hydrogen bonds in that the scission of polymer chains led to the increasing mobility of molecules.

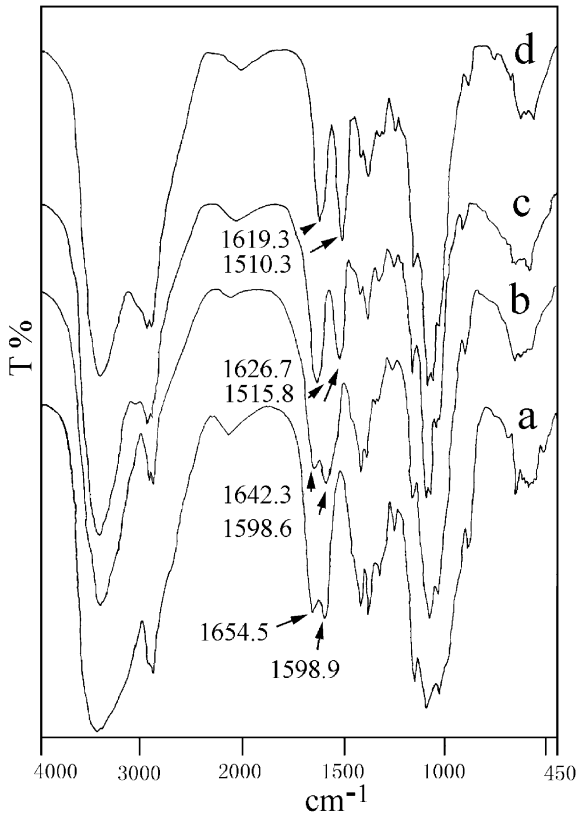

Fig. 5. FT-IR spectra of SCH85 (a), ES-L4 (b), hydrochloride salts of ES-L4 (c) and $(GlcN)_5$ (d).

Curves *c* and *d* in Fig. 5 show the IR spectra of the hydrochloride salts of ES-L4 and the standard sample chitopentaose $(GlcN)_5$. As can be seen, there is no significant difference between them. The characteristic peaks of $-NH_3^+$ vibration deformation appeared at 1619.3 and 1510.3 cm^{-1} for the hydrochloride salt of $(GlcN)_5$. In the spectrum of the hydrochloride salt of ES-L4, the two peaks of $-NH_3^+$ shifted to high wave number, which was attributed to the presence of amide I ($\nu_{C=O}$) and amide II (δ_{N-H}), respectively. The IR spectra suggested that there was no significant difference between the residues of chitosan before and after the enzymatic hydrolysis. These data coincided well with the data for determination of DD.

3.4. ^1H NMR spectra

To further confirm the structure of the degraded chitosan, the chitooligomer product ES-L4 was analyzed by ^1H NMR spectroscopy. Fig. 6 shows the ^1H NMR spectrum of ES-L4 measured in D_2O solution at ambient temperature. The resonance at 1.97 ppm is easily assigned to the three *N*-acetyl protons (Akiyama, Kawazu, & Kobayashi, 1995). The higher field peak at 4.4–4.6 ppm and the peak at 2.6–2.9 ppm correspond to H-1 and H-2 of GlcN (D-) units, respectively (Kobota et al., 2000). The resonance of H-3 of the D-units was at

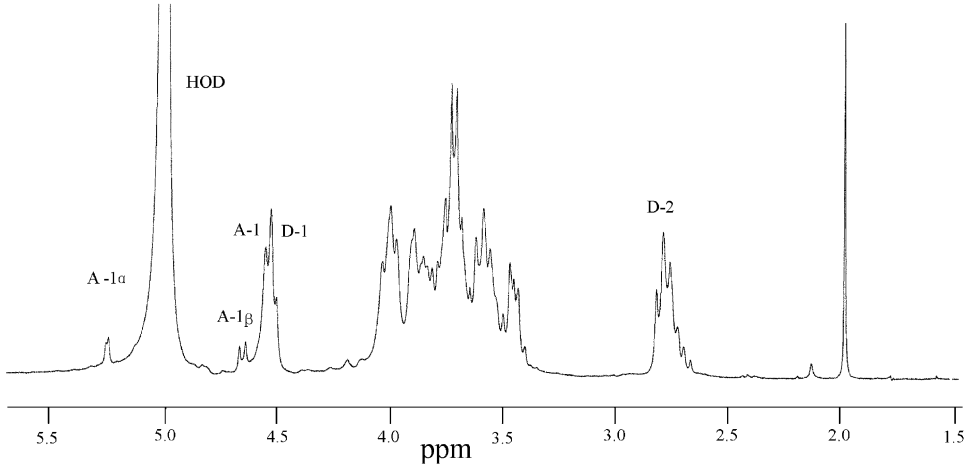

Fig. 6. The ^1H NMR spectrum of ES-L4 in D_2O.

3.54 ppm while the resonance of H-3 of GlcNAc (A-) units was at 3.45 ppm. The lower field peak at 4.4–4.6 ppm corresponds to H-1 of A-units. The weak signals at 5.22 ppm were assigned to H-1(α) of A-units, while signals at 4.63 and 4.65 ppm were assigned to H-1(β) of A-units (Sugiyama, 2001). The result suggested that the reducing ends of produced oligomers were mainly GlcNAc residues. No attempts were made to identify other peaks, because the spectra of unfractionated partially *N*-deacetylated chitooligomers are very difficult to interpret. Nevertheless, all signals were the resonances of oligomers of chitin and chitosan, which coincided with the reported data.

3.5. X-ray analysis

Fig. 7 shows the X-ray diffraction patterns of the main fraction of resulting chitosan by the progress of degradation. The WAXD pattern of initial chitosan SCH85 exhibited its two characteristic peaks at $2\theta = 10.4$ and 20.4°, which coincided with the pattern of the "L-2 polymorph" of chitosan reported previously (Saitô & Tabeta, 1987). ES-S2 displayed three main peaks. A peak at $2\theta = 22°$ rose and the peaks at $2\theta = 10.2$ and 19.8° also increased. Such a pattern characterized a chitosan polymorph, which is referred to as the "tendon hydrate polymorph" (Belamie, Domard, & Giraud-Guille, 1997). The crystallinity increases in the order of SCH85 < ES-S1 < ES-S2. As a consequence, there seems to be an obvious correlation between the depolymerization and the rise in crystallinity due to recrystallization of short chain chitosan. On the other hand, the water-soluble sample ES-L4 mainly shows the "annealed" type pattern, which was identified by the three characteristic diffraction peaks at $2\theta = 15.2$, 21.1 and 23.8°. ES-S3 were found to be a mixture of the previously mentioned "tendon hydrate polymorph" and "annealed polymorph". The higher molecular weight chitosan in ES-S3 did not show complete conversion to the annealed polymorph because of the lower mobility of its polymer chain.

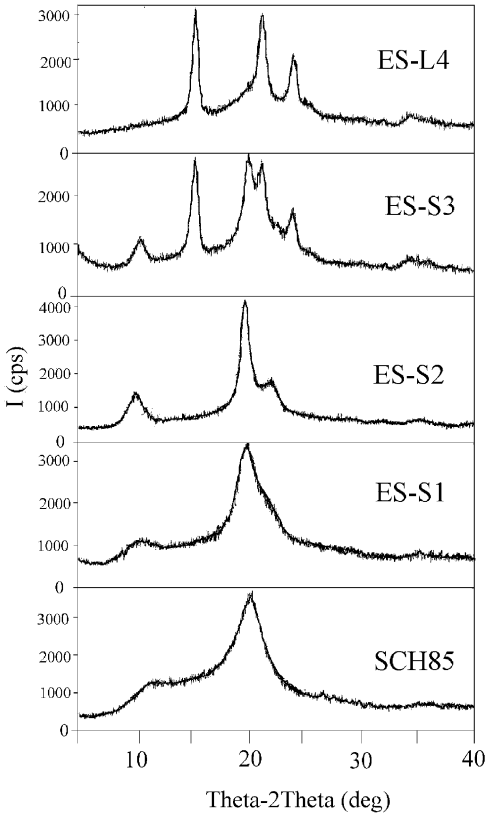

Fig. 7. X-ray diffraction patterns of initial chitosan and degraded chitosans.

3.6. Thermal analysis

Thermogravimetric curves for chitosan and its degraded products in static air are shown in Fig. 8. All samples had a weight loss before 140 °C as a result of absorbed moisture and bound water. As is known, polysaccharides usually have a strong affinity for water and hydration properties of these polysaccharides depend on the primary and supra-molecular structures (Kittur, Prashanth, Sankar, & Tharanathan, 2002). The water molecules are associated with hydrophilic amine groups and hydroxyl groups in chitosan. The temperature of maximum pyrolysis for initial SCH85 was at 290 °C, both ES-S1 and ES-S2 were at 268 °C, whereas ES-L4 with the lowest M_w was at 230 °C. Comparing the temperatures of onset of active pyrolysis, the ES-L4 was found to be the least thermally stable, starting to decompose before 180 °C. The decrease in the degree of polymerization (DP) could lead to a decrease in thermal stability. The thermostability was in the order SCH85 > ES-S1, ES-S2 > ES-L4. However, the crystal

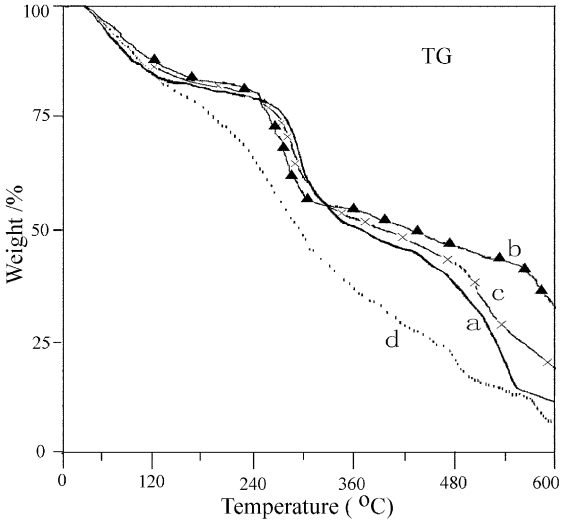

Fig. 8. TG curves of SCH86 (a), ES-S1 (b), ES-S2 (c) and ES-L4 (d).

structures also affect the thermostability (Prashanth, Kittur, & Tharanathan, 2002). Although DP of ES-S1 was higher than DP of ES-S2, ES-S2 was a little more stable than ES-S1 during active pyrolysis from 240 to 340 °C.

Fig. 9 shows the corresponding DTA curves of chitosan and its degraded products in static air. The first thermal event registered in all samples was endothermic slope curves (30–140 °C), which were associated with the evaporation of adsorbed and bound water. The degradation of chitosan should be composed of a set of concurrent and consecutive reactions. In the second range (200–350 °C), all samples were substantially decomposed due to the degradation of main chain and deacetylation (Kim & Lee, 1993; Qu, Wirsén, & Albertsson, 2000). The area of exothermic peak increased with the decrease of M_w. There should be an endothermic stage at the beginning (Tirkistani, 1998), which is mainly due to depolymersization and evaporation of degradation products. Then, there is an exotherm attributed to charring and oxidation of the products of thermal decomposition of chitosan. As is seen in curve *b*, an endotherm observed at around 230 °C is followed by an exotherm at around 272 °C. The endothermic and exothermic reactions could occur at the same time so that the expected endotherm for some samples might be overwhelmed by the following overlapping exotherm with partial compensation. The exotherm observed in the range 350–560 °C is likely to be due to further decomposition of degraded fragments and the slow oxidation of the char. The next exothermic peak around 581 °C is much sharper suggesting the combustion of char (Soares, Camino, & Levechik, 1995).

The pathway of thermal decomposition and the composition of the products are influenced by many physical and chemical factors, such as temperature, heating rate, type of atmosphere, size and packing density of sample powder, crystallinity, and impurities. The degradation process would seem at first to be so complex that a detailed explanation is too difficult (Kittur et al., 2002), and this should be further investigated. Nevertheless, these chitosan samples are thermostable below 120 °C and could undergo thermo-processing in food.

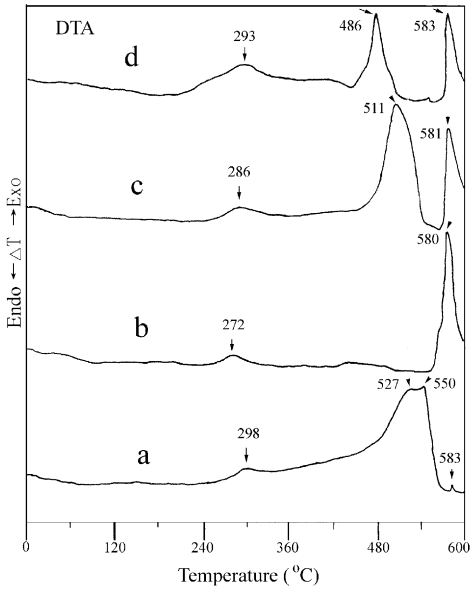

Fig. 9. DTA curves of SCH86 (a), ES-S1 (b), ES-S2 (c) and ES-L4 (d).

3.7. Antitumor activity

In medicine and the food industries, some applications of the native chitosan are limited by its high molecular weight, resulting in its low solubility in acid-free aqueous media. To be effectively absorbed in the human body, it is converted to the low-molecular weight chitosan.

The results in Table 3 show that the water-soluble chitosan, ES-L4, exhibited an antitumor effect against sarcoma 180 when administered by intraperitoneal injection. The inhibition rate reached 50.4% at a dose of 50 mg/kg/day, higher than 37.1% at a higher dose of 200 mg/kg/day. It is also interesting that the oral administration of the low-molecular weight chitosan was effective in decreasing the weight of the tumor, because the lentinan was reported to have no antitumor effects by oral administration (Lu, Yoshida, & Nakashima, 2000). Ftorafur, as the positive group, had high antitumor effect, but also had strong side effects, such as significant decrease of body weight and spleen weight.

The mechanisms for the antitumor activities of hexa-*N*-acetylchitohexaose and chitohexaose are assumed to be due to acceleration of the production of, and response to, interleukin-1 and interleukin-2 for maturation of splenic T-lymphocytes to killer T-cells (Tokoro et al., 1988). Seo et al. (2000) demonstrated that water-soluble chitooligomers could activate murine peritoneal

Table 3
Effect of low molecular weight chitosan on sacroma 180 solid tumor growth

Sample	Dose (mg/kg)	Tumor weight (g)	Inhibition (%)	Spleen index
Experiment I: 10 Kumin mice/each group by i.p.				
Control		1.58±0.68		–
ES-L4	50	0.78±0.34***	50.4	–
	200	0.99±0.21*	37.1	–
Experiment II: 11 Balb/c mice/each group by p.o.				
Control		1.08±0.49		1.12±0.17
Ftorafur	100	0.12±0.07***	88.9	0.52±0.17**
ES-L4	200	0.89±0.26	17.6	1.16±0.15
	400	0.74±0.30*	31.5	1.16±0.25
ES-S3	200	0.95±0.36	12.0	1.15±0.13
	400	0.82±0.42	24.1	1.06±0.14

* $P<0.05$.
*** $P<0.001$ (Student's *t*-test).

macrophases for tumor cell killing in the presence of IFN-γ. The effect of the acetyl groups, in the reducing end-residues, on antitumor activity will be further investigated.

4. Conclusion

Partially *N*-acetylated chitosan was hydrolysed with the cheap, commercially available and efficient cellulase. The enzymatic hydrolysis was by endo-action and mainly occurred in a random fashion. The optimum temperature was around 50 °C and the optimum pH was 5–5.5. The reduction in M_w of the resulting chitosan led to the transformation of the crystal structure and a decrease of thermostability. The total acetylation degree of chitosan was the same before and after degradation. IR and NMR spectra confirmed that the chemical structures of residues were not modified, and that most of reducing ends of the produced oligomers were GlcNAc units. This water-soluble product inhibited the growth of tumor cells in mice, with a maximum inhibitory rate of 50.4% by intraperitoneal injection and 31.5% by oral administration. These low-molecular weight chitosan samples have promising applications in food for human health.

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