Preparation and Characterisation of Food-Grade Chitosan from Housefly Larvae

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Abstract

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The preparation and characterisation of food-grade chitosan from housefly larvae are reported. A refinement procedure was developed to remove larval mouth hooks from the primary chitosan product, which greatly improved the quality of the final product and simplified the production procedures. Different factors affecting chitosan preparation were studied and an orthogonal experiment was designed to determine optimal preparation conditions. When prepared under optimal reaction conditions, the end product was snow-white in colour, had a high deacetylation percentage, good viscosity, and a low ash content. The end product was characterised by Fourier transform infrared spectral analysis, X-ray diffraction analysis, thermo-gravimetric analysis, and differential scanning calorimetry. Its physical and chemical properties and sanitary index were determined and compared to the relevant Chinese standards. The results show that the chitosan we produced under optimal conditions meets the Chinese Fishery Trade Standard SC/T3403-2004 for food-grade chitosan.

Keywords: *Musca domestica*; commercial applications; chitin; insect; derivates

Abbreviations

DDA – degree of deacetylation; PSM – prortion of the solution to material; RSM – ratio of the solution (ml) to material consider changing PSM to RSM, TGA – thermo-gravimetric analysis; DSC – differential scanning calorimetry; FTIR – Fourier transform infrared; XRD – X-ray diffraction

The housefly *Musca domestica* (Diptera: Muscidea) is commonly regarded as an important sanitary pest insect. However, due to its short life cycle, high fecundity, and efficient digestion of organic waste, it has also become a source of protein, chitin and chitosan (Onifade *et al.* 2001; Jing *et al.* 2007; Hao *et al.* 2008). Housefly larvae could be used as a high protein livestock feed and could thereby improve human nutrition by increasing the protein content of meat, milk, butter, and eggs (Boushy 1991). The simplicity and low cost of rearing housefly larvae on a commercial scale has made this activity very popular in China. The most feasible and easiest commercial utilisation of housefly larvae is to raise them on poultry manure and other organic wastes and then feed them fresh

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to poultry or other livestock. In addition to their value as a livestock feed, other valuable materials, such as protein or peptides, chitosan, phospholipid, and antibiotics have been extracted from housefly larvae (Bridges & Price 1970; Iaboni *et al.* 1998; Hou *et al.* 2007; Ai *et al.* 2008). Our preliminary observations indicate that dried housefly larvae contain approximately 55% protein, 9.1% crude chitin, and 8.8% fat (unpublished data). We were motivated to develop better techniques for rearing and utilising housefly larvae because we had previously reared lepidopteran insects on an artificial diet, much of which was wasted. Housefly larvae provide a potential means of converting this waste into useful biological products. A series of attempts were carried out to isolate and characterise protein, chitin, fat, and other materials from housefly larvae reared on the above-mentioned diet. This paper describes the results of the experiments undertaken to characterise chitosan extracted from the cuticle of these larvae.

Chitin (β-(1,4)-2-acetamido-2-deoxy-D-glucose) is the second most abundant natural carbohydrate polymer. Chitin and its derivative chitosan are thought to have more than two hundred uses in areas as diverse as agriculture, biomedicine, cosmetics, food, textiles, as well as the potential for use as chelating agent and in refining industrial effluents (Bahmani *et al.* 2000; Ravi Kumar 2000; Rinaudo 2006; Aranaz *et al.* 2009). Currently, the most available source of chitin is the exoskeletons of crustaceans, particularly shrimps and crabs (Minke & Blackwell 1978; Acosta *et al.* 1993). However, the relatively high calcium, wax, and pigment contents of shrimp and crab exoskeletons cause the extraction of chitosan from them to be relatively expensive. Compared to shrimps or crabs (Rødde *et al.* 2008; Youn *et al.* 2009), the cuticle of the housefly larvae is much easier to extract chitin from because it contains smaller amounts of crude protein, crude fat, and ash (our unpublished data). However, no one has yet developed a method to obtain high-grade chitosan from housefly larval cuticle. In this paper, we describe the results of the experiments aimed at optimising the extraction of chitosan from housefly larvae.

MATERIAL AND METHODS

Chitin extraction. Four-day old housefly larvae were isolated from waste feed, rinsed and boiled for 10–15 min, and subsequently macerated in a home juicer. The resultant granules of crude cuticle were strained from the mixture with a mesh sieve, extensively washed, and freeze-dried. Protein and lipid in the dry cuticle were removed by the treatment with 1 mol/l NaOH solution (Hengye Zhongyuan Chemical Co., Beijing, China) at 100°C for 3 hours. Crude chitin was obtained by rinsing the mixture with water until reaching neutral pH filtered with mesh sieve to remove water and then freeze-dried.

Chitosan preparation. Several single-factor experiments were conducted to evaluate various reaction conditions for the deacetylation of chitin. The factors were the granularity of the raw chitin, NaOH concentration, reaction temperature, proportion of solution to material (PSM), reaction time, and steeping time. Four grams of raw chitin were used in each experiment. The experiments were designed so that when one factor was tested at different levels, all the other factors were fixed. Other experimental constants included the diameter of the raw chitin granules (ca. 0.085 mm), NaOH concentration (50% w/v), reaction temperature (125°C), PSM (30 ml/g), reaction time (4 h), and steeping time (0.5 day). Based on these single-factor experiments, an orthogonal experimental design was applied to identify the optimal deacetylation conditions (Table 1). The data from the orthogonal experiment were analysed by general linear model analysis of variance with Duncan's multiple range test using SPSS software version 16.0 (SPSS Inc., Chicago, USA).

Removal of impurities from raw chitosan. To avoid the negative impact of the impurities on chitosan quality, a refinement procedure was developed for their removal from the deacetylated product. After deacetylation, chitosan was filtered and rinsed until neutral pH was achieved. Chitosan was then dissolved in acetic acid solution (1%) and the impurities were removed by filtration through nylon net with a mesh diameter of 0.050 mm. The

Table 1. Orthogonal experimental combination of different factors at various levels

Parameters	Levels		
	1	2	3
Proportion of solution to material (ml/g)	22.5	25.0	27.5
Reaction temperature (°C)	130	125	120
Reaction time (h)	4	6	8

pellucid filtrate was neutralised and precipitated with NaOH solution until reaching neutral pH. The precipitate was rinsed several times and then freeze-dried after which the yield, i.e. the amount of pure chitosan (% m/m) obtained from crude chitin, was calculated.

Determination of the degree of deacetylation, viscosity and ash content. The degree of deacetylation (DDA) was measured by the acid-base titration method (Domard & Rinaudo 1983) with modifications. In brief, chitosan (0.1 g) was dissolved in 30 ml HCl aqueous solution (0.1 mol/l) at room temperature with 5–6 drops of methyl orange added. The red chitosan solution was titrated with 0.1 mol/l NaOH solution until it turned orange. The DDA was calculated by the formula:

$$\text{DDA (\%)} = \frac{(C_1V_1 - C_2V_2)}{M \times 0.0994} \times 0.016$$

where:

C_1 – concentration of standard HCl aqueous solution (mol/l)

C_2 – standard NaOH solution (mol/l)

V_1 – volume of the standard HCl aqueous solution used to dissolve chitosan (ml)

V_2 – volume of standard NaOH solution consumed during titration (ml)

M – weight of chitosan (g)

The number 0.016 (g) is the equivalent weight of NH_2 group in 1 ml of standard 1 mol/l HCl aqueous solution, and 0.0994 is the proportion of NH_2 group by weight in chitosan.

One gram of the end product was dissolved in 1% acetic acid solution (100 ml) and its viscosity was measured by means of NDJ-1 rotation viscometer (Hengping Instrument Co., Shanghai, China) at 25°C. The ash content was determined gravimetrically after the incineration of the sample (0.5–1.0 g) in a muffle furnace at 500°C for at least 4 hours.

Chitosan FTIR analysis. Fourier transform infrared (FTIR) spectrum of the end product samples was measured in KBr pellets in the transmission mode in the range of 400–4000 cm^{-1} using a Bruker Equinox 55 spectrophotometer (Bruker Optik, Ettlingen, Germany).

X-ray diffraction analysis. The X-ray diffraction (XRD) experiment was performed in the range of 3–60° (2θ) using a PANalytical X'Pert PRO X-ray diffractometer (PANalytical Co., Almelo, the Netherlands).

Thermal analysis. Thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC) were carried out using Netzsch STA 499C thermal analyser (NETZSCH-Gerätebau GmbH. Selb, Germany) at a heating rate of 10°C/min under nitrogen atmosphere.

Determination of physical and chemical properties and sanitary indices. Some physical and chemical properties of the chitosan prepared under the determined optimal processing conditions were tested, including the amounts of water, ash, and heavy metals, such as lead (Pb) and arsenic (As), the DDA and viscosity, and bacteriological detections. Lead (Pb) content was determined by graphite furnace atomic absorption spectrometry according to the method of Oliveira *et al.* (2005) with slight modifications. Arsenic (As) was measured by hydride generation atomic fluorescence spectrometry according to the method of El-Hadri *et al.* (2007). Aerobic bacterial count was determined using the method described by Jarvis *et al.* (1977) and coliforms were enumerated by the method of Feldsine *et al.* (1994). Pathogens, including *Staphylococcus aureus* and *Salmonella*, were screened by the methods of Bennett *et al.* (1986) and June *et al.* (1995). The data were compared with those of Chinese Fishery Trade Standard SC/T3403-2004 (Ministry of Agriculture of the People's Republic of China 2005).

RESULTS AND DISCUSSION

Chitosan preparation

The effects of variation of different factors on the DDA, viscosity, yield, and ash content of the end product are shown in Figures 1 and 2. As shown in Figure 1, DDA generally increased with the granularity of the raw chitin, NaOH concentration, reaction temperature, PSM, reaction time, and steeping time, while viscosity was negatively correlated with these factors. NaOH concentrations of below 50% resulted in significantly reduced yields (Figure 2B). None of the factors examined had a significant effect on ash content (Figure 2). These results suggest that optimal conditions for the preparation of chitosan from housefly larvae were granularity of the raw chitin particles of about 0.85 mm, steeping time ≤ 1 day, reaction time of 4–8 h, reaction temperature of 120–130°C, and PSM of 20–30 ml/g.

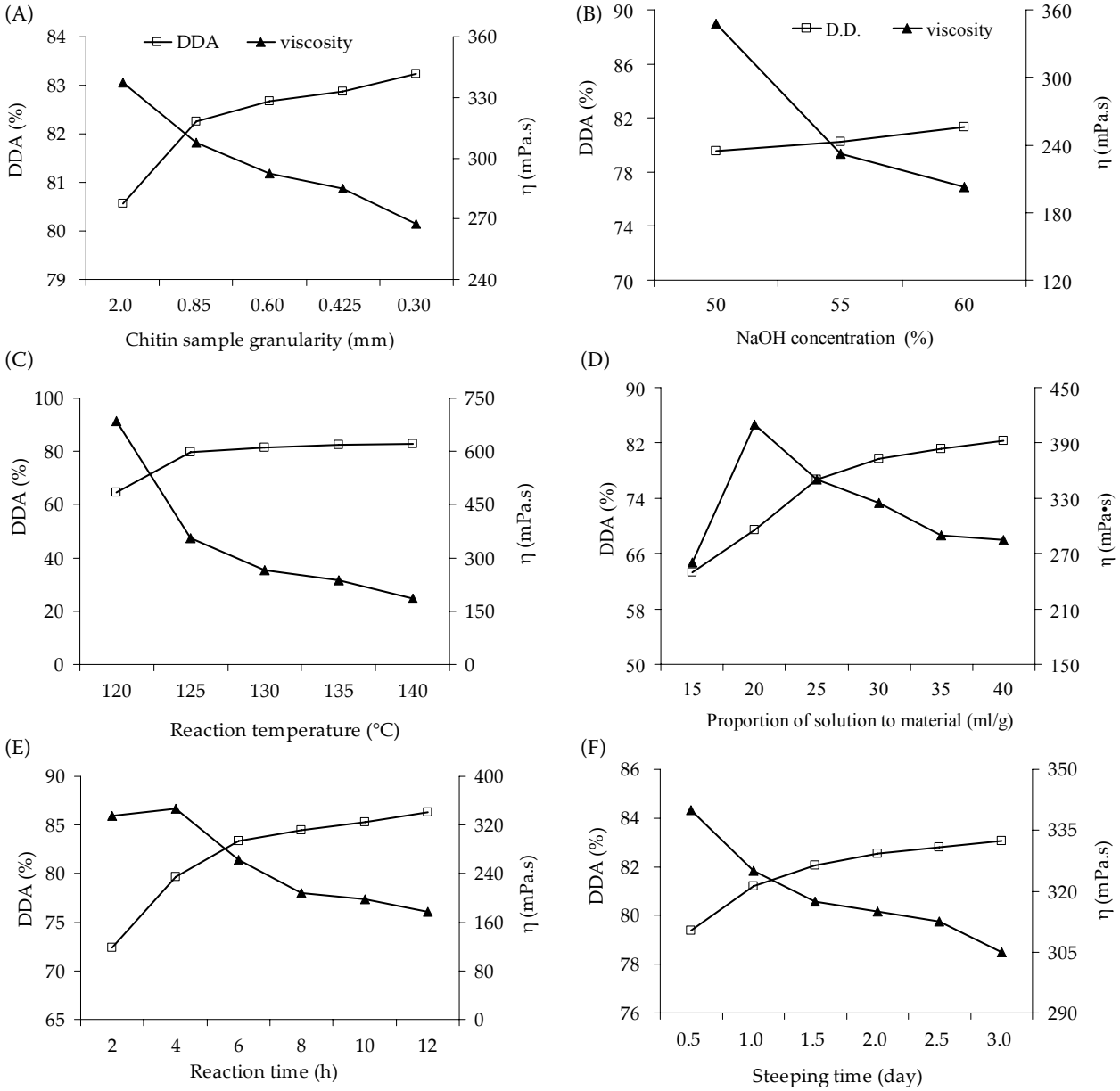

Figure 1. Effect of different factors on the degree of deacetylation (DDA) and viscosity of chitosan prepared from housefly larvae. The DDA (□) and viscosity (▲) were measured by the modified acid-base titration method and NDJ-1 viscosimetry, respectively, under different conditions, including chitin sample granularity (A), NaOH concentration (B), reaction temperature (C), proportion of solution to material (D), reaction time (E) and steeping time (F)

Different factors had different effects on the deacetylation reaction. Using exoskeleton particles that were too fine resulted in a great decrease of the yield and viscosity of the end product, while those that were too coarse reduced DDA (Figures 1A and 2A). Although NaOH concentrations below 50% reduced the yield by inhibiting the deacetylation reaction (Figure 2B), excessive NaOH reduced viscosity (Figure 1B). A low DDA and yield also occurred at high ash content at 120°C while high temperatures caused a sharp decrease in viscosity and yield (Figures 1C and 2C). Peak yields were obtained when PSM was 25 ml/g or 30 ml/g (Figure 2D). Although DDA increased with PSM, viscosity generally decreased (Figure 1D). A PSM of 15 ml/g seemed unsuitable because of inadequate soaking of the raw chitin resulting in a low DDA, viscosity, and a high ash content in the end product (Figures 1D and 2D). Prolongation of the reaction or steeping time improved DDA (Figures 1E and 1F), but excessive reaction or steeping time decreased the yield and viscosity (Figures 1E, 1F, 2E and 2F).

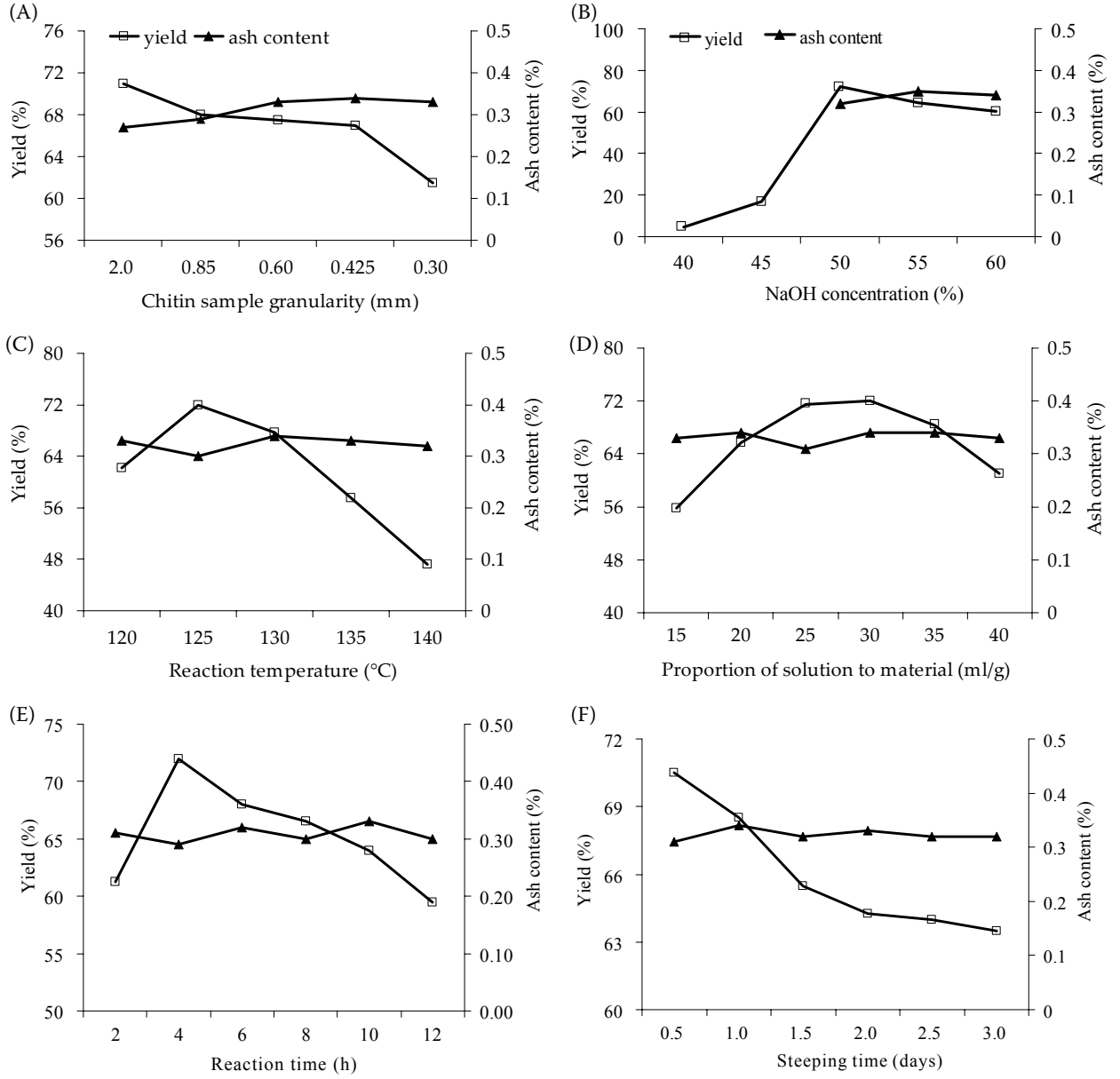

Figure 2. Effect of different factors on the yield and ash content of chitosan obtained from housefly larvae. The yield (□) and ash content (▲) were determined by standardised methods under different conditions, including chitin sample granularity (A), NaOH concentration (B), reaction temperature (C), proportion of solution to material (D), reaction time (E) and steeping time (F)

Optimal preparation conditions for chitosan

DDA, yield, viscosity, and ash content are the four main parameters influencing the preparation efficiency and chitosan quality. Our single-factor experiments (Figures 1 and 2) indicated that DDA and yield were the two key parameters that should be considered in the orthogonal experiment. Figure 3 shows the results of the orthogonal experiment on the effects of various levels of different factors on DDA and yield. PSM had no significant effect on DDA or yield. The reaction temperature and reaction time were the key factors affecting the DDA, while the yield was significantly affected only by the reaction temperature. Taking into account the production costs, these results suggest that optimal conditions for chitosan preparation are PSM of 22.5 ml/g, a reaction temperature of 125°C, and a reaction time of 6 hours.

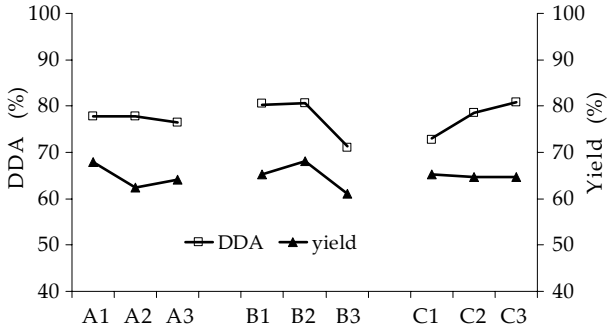

Figure 3. Results of an orthogonal experiment on the effect of varying proportion of solution to material (PSM), reaction temperature, and reaction time, on degree of deacetylation (DDA) and yield. Different letters in the same curve indicate a significant difference ($P < 0.05$), while the same letters in the same curve indicate no significant difference ($P > 0.05$)

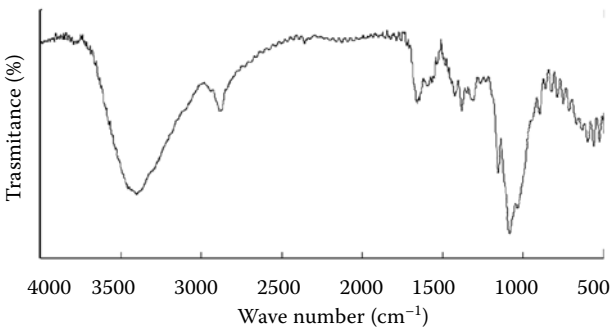

Figure 4. Fourier transform infrared (FTIR) spectrum of chitosan from housefly larvae produced under optimised preparation conditions (see text for explanation). The FTIR spectrum was measured in KBr pellets in the transmission mode in the range of 400–4000 cm^{-1} using an EQUINOX 55 spectrophotometer

Characteristics of the chitosan prepared

As shown in Figure 4, the FTIR spectrum of chitosan produced from housefly larvae was similar to that of chitosan made from the exoskeletons of shrimps, crabs, and other crustaceans; although there were some differences in minor peaks, the positions of the characteristic peaks and their intensities were nearly the same as those reported by other researchers (Duarte *et al*. 2002; Pawlak & Mucha 2003; Paulino *et al.* 2006; Abdou *et al.* 2008). The wide band at 3400 cm^{-1} corresponded to OH stretching vibrations of water and hydroxyls, and NH stretching vibrations of free amino groups. The band observed at 2881 cm^{-1} corresponded to CH stretching vibrations. The band at 1657 cm^{-1} corresponded to amide I band vibrations. The intensive band at 1597–1600 cm^{-1} corresponded to in-plane bending vibration of NH_2, a structural feature of chitosan and the occurrence of deacetylation. The band at 1355 cm^{-1} was attributed to the stretching of amide III vibration. The band at 1085 cm^{-1} could be attributed to the stretching of hydroxyl groups of C_3-OH, and the band at 1039 cm^{-1} could correspond to the stretching of hydroxyl groups of C_6-OH. The band at 898 cm^{-1} corresponded to the characteristic skeletal vibration of β-anomers.

Usually, both chitin and chitosan from the exoskeletons of shrimps and crabs have strong re-

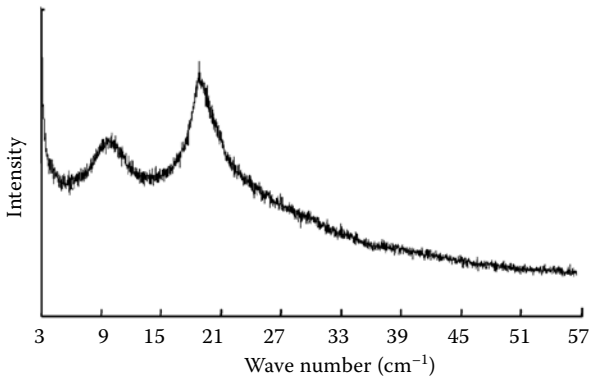

Figure 5. X-ray diffraction (XRD) patterns of chitosan from housefly larvae produced under the optimised preparation conditions (see text for explanation). The XRD experiment was performed in the range of 3–60° (2θ) using an X'Pert PRO X-ray diffractometer

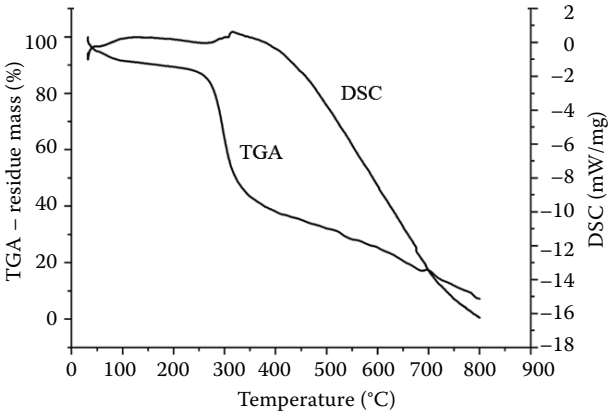

Figure 6. Curves of thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC) of chitosan obtained from housefly larvae prepared under the optimised preparation conditions (see text for explanation). TGA and DSC were carried out using Netzsch STA 499C TG-DSC integrated instruments at the heating rate of 10°C/min under a nitrogen atmosphere

Table 2. Comparison of the physical and chemical properties and sanitary indices of the prepared chitosan with that of Chinese Fishery Trade Standard SC/T3403-2004

Properties	Prepared chitosan	Industrial grade*	Food grade*
Color	white	white or buff	white or buff
Appearance	powder	powder or sheet	powder or sheet
Granularity (mm)	0.245	–	–
pH	6.96	6.5-8.5	6.5–8.5
DDA (%)	83.1	–	–
Viscosity (mPa·s)	347	–	–
Water (%)	2	≤ 12	≤ 10
Ash (%)	0.13	≤ 2.0	≤ 0.5
Undissolved particles (%)	0	≤ 1.0	≤ 1.0
Pb (mg/kg)	1.63	–	≤ 10
As (mg/kg)	0.41	–	≤ 0.5
Total bacterial count (cpu/g)	n.d.	–	≤1000
E. coli (MPN 100/g)	n.d.	–	–
Pathogens	n.d.	–	–

*Physical and chemical properties and sanitary indices of industrial grade and food grade chitosan as the Chinese Fishery Trade Standard SC/T3403-2004; n.d. – not detected

flections at 2θ around 9–10° and 2θ of 20–21° in their XRD spectrum (Abdou *et al.* 2008). However, the crystal peak near 10° in the chitosan obtained from the housefly larvae gradually disappeared and the crystal peak near 20° became wider and weaker (Figure 5). This suggests that the intramolecular hydrogen bonds in the end product had dramatically decreased after the deacetylation reaction, and that its molecular structure was in an amorphous state.

In thermograms, an upward DSC curve indicates an exothermic reaction and a downward curve an endothermic reaction. Two decomposition steps can be seen in the TGA curve with an endothermic reaction in the DSC curve (Figure 6). The first step occurred at about 32–100°C and corresponded to the evaporation of water. The second emerged around 270–320°C and can be attributed to the degradation of the chitosan sample.

Physical and chemical properties and sanitary indices of chitosan obtained from housefly larvae

Table 2 shows the results of the tests of the physical and chemical properties, as well as the sanitary indices of the chitosan produced under the above given optimal preparation conditions. The results indicate that water, ash, Pb, and As contents, as well as pH of the end product, met Chinese Fishery Trade Standard SC/T3403-2004 for food-grade chitosan. No pathogens were detected using the statutory examination methods of the above-mentioned Chinese national standards. Since China has currently only industrial and food grade trade standards for chitosan, we can not verify whither the end product is of sufficient quality for medical use until the respective standard is issued.

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