Carbohydrate Polymers 83 (2011) 1008–1010

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Preparation of chitosan from *Clanis bilineata* larvae skin using enzymatic methods

Shengjun Wu*

School of Food Engineering, Huaihai Institute of Technology, 59 Cangwu Road, Lianyungang 222005, China

ARTICLE INFO

Article history:
Received 21 July 2010
Received in revised form 4 August 2010
Accepted 13 August 2010
Available online 20 August 2010

Keywords:
Preparation
Flavourzyme
Clanis bilineata
Deacetylating

ABSTRACT

Chitosan was prepared from *Clanis bilineata* larvae skin by heating, protein removal through flavourzyme hydrolysis, salt removal with HCl, NaOH deacetylation, washing, and drying. The optimal hydrolyzing conditions were pH 6.5 and 50 °C. The optimal deacetylation conditions were 55% NaOH (w/w), 120 °C, and 4 h. The resulting product's protein content, degree of deacetylation, and chitosan yield were 0.16, 93.25, and 31.37% (w/w), respectively. FTIR spectra of the product showed the main component to be chitosan and indicated that this method presents a promising means for chitosan preparation.

1. Introduction

Chitosan, a polymer composed primarily of β-(1 → 4)-2-amino-2-deoxy-D-glucose (D-glucosamine) monomers, is derived by deacetylation of naturally occurring biopolymer chitin. Annually, ~10 ktons of *Clanis bilineata* (*C. bilineata*) larvae are consumed in China. During processing, ~2 ktons of larvae skin are produced and often randomly discarded, leading to environmental pollution. In the interest of improving the understanding of chitosan production from this source, we were interested here in investigating means for producing chitosan from *C. bilineata* larvae skin (CLS), with the goals of determining optimal conditions for protein removal by flavourzyme hydrolysis and for subsequent deacetylating agents and of examining the composition of the resulting product.

2. Materials and methods

2.1. Removal of protein from CLS

A 50 g lot of CLS, purchased from a local agricultural market, was protein denatured by heating at 90 °C for 30 min, cooled to room temperature (~25 °C), pulverized, and suspended in 1 L of distilled water. Aliquots of the suspension were adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, and 7 using 1 M HCl. 0.4 ASPU of flavourzyme (Novo Nordisk, Denmark, 1000 ASPU/g), which can hydrolyze protein into soluble peptide and amino acids, was added into Monod-type test tubes containing 10 mL of suspension and the reaction mixture shaken at 50 strokes/min by a Monod-type shaker (MD-100, Taitec Corporation, Koshigaya, Japan) at 35, 40, 45, 50, 55, and 60 °C for 8 h. Aliquots were taken from the reaction mixture through a steel filter to separate support particles and the reaction terminated by mixing with an equal volume of a 0.4 M trichloroacetic acid solution. The protein content of the particles was used for evaluating the degree of hydrolysis.

2.2. Deacetylating with NaOH

Protein-depleted CLS was soaked in 5% (v/v) HCl at room temperature for 12 h for salt removal and the product (SPCLS) deacetylated with added NaOH at 35, 40, 45, 50, 55, and 60% (w/w) at 90, 100, 110, 120, 130, and 140 °C in an oil bath for 1, 2, 3, 4, 5, and 6 h. The degree of deacetylation (DD) of SPCLS described below was used for evaluating deacetylation effectiveness. Deacetylated SPCLS was washed with distilled water to a neutral pH and dried to a constant weight.

2.3. Analytical methods

Ash, moisture, and protein content of the samples were determined by standard methods (Anon., 1984). The DD of SPCLS was measured by first derivative UV spectroscopic method (Muzzarelli, Rocchetti, Stanic, & Weckx, 1997) using a Shimadzu (option program/interface OPI-4) UV-Vis recording spectrophotometer UV 240 graphtcord (fast scan speed, 2 nm slit width, and 190–240 nm scanning range). Sample Fourier Transform infrared spectroscopy (FTIR) spectra were run as KBr pellets on a Nicolet Nexus FTIR 470 spectrophotometer from 500 to 4000 cm^{-1}.

* Corresponding author. Tel.: +86 518 8595427; fax: +86 518 8595428.
E-mail address: wushengjun008@sina.com.

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.08.033

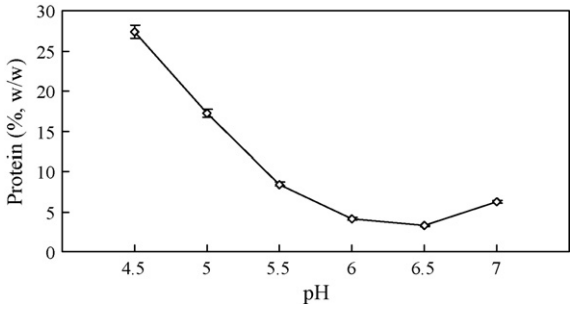

Fig. 1. Effect of pH on removing of the protein by hydrolyzing with flavourzyme (the results are from three replicate experiments).

3. Results and discussion

3.1. Effect of pH on protein removal by flavourzyme hydrolysis

As reaction mixture pH can influence flavourzyme activity and subsequently influence protein hydrolysis, it was useful to investigate the effects of pH values from 4.5 to 7 on flavourzyme activity (40 ASPU/g) at 45 °C for 8 h. The results showed that the minimum CLS protein content was achieved at pH 6.5 (Fig. 1). In other reports, optimal conditions for protein hydrolysis with flavourzyme indicated pH 6.0 (Nyam, Tan, Lai, Long, & Man, 2009; Shen, Wang, Wang, Wu, & Chen, 2008), 6.5 (Deng, Huo, & Xie, 2008), and 7.5 (Linder, Fanni, & Parmentier, 2005). The variety of reported optimal pHs may have been due to different flavourzyme sources and substrates.

3.2. Effect of temperature on protein removal by flavourzyme hydrolysis

As reaction mixture temperature plays an important role in flavourzyme activity, being decreased by too high or low temperatures, it was necessary to study the temperature effects from 35 to 60 °C on flavourzyme protein removal in reaction mixtures containing flavourzyme (40 ASPU/g) at pH 6.5 for 8 h. CLS protein content decreased with increase in temperature up to 50 °C due to enhanced activity of the enzyme. However, further increasing the temperature resulted in increase in CLS protein content. This is expected as too high temperature denatured the enzyme. The results showed that the minimum CLS protein content was achieved at 50 °C (Fig. 2), which differed from the optimal flavourzyme protein hydrolysis temperature reported elsewhere as 40 °C (Moreau, Nau, Piot, Guerin, & Brule, 1997), 45 °C (Deng et al., 2008), and 50 °C (Linder et al., 2005; Nyam et al., 2009; Shen et al., 2008). Again, the various reported optimal temperatures may have been due to different flavourzyme sources and the substrates.

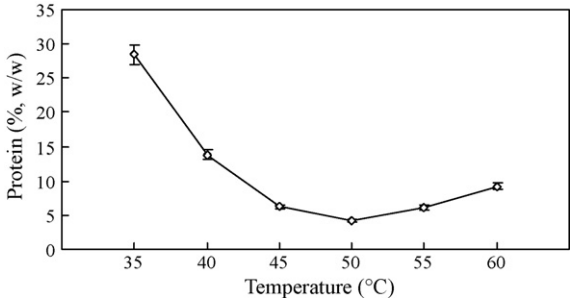

Fig. 2. Effect of temperature on protein removal by flavourzyme hydrolysis. Results from triplicate experiments.

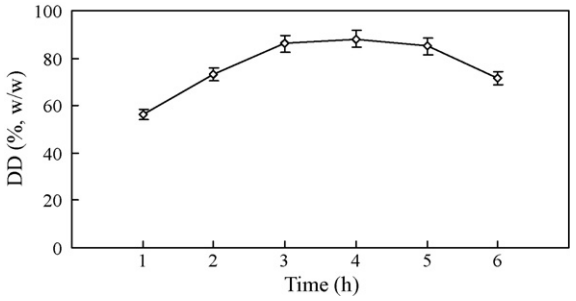

Fig. 3. Effect of time on degree of deacetylation. Results from triplicate experiments.

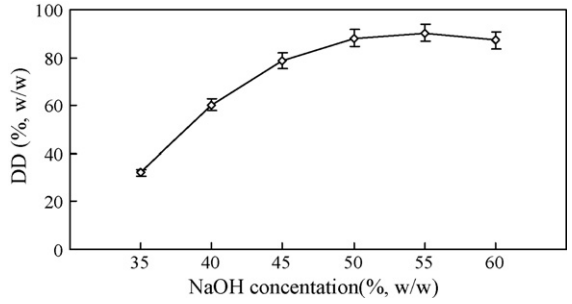

Fig. 4. Effect of NaOH concentration on degree of deacetylation. Results from triplicate experiments.

3.3. Effect of time on the DD

Time course studies of NaOH (50%, w/w) deacetylation at 110 °C for 6 h showed a sharp increase in DD within 3 h, a slower increase from 3 to 4 h, a decrease after 4 h, and a maximum DD after 4 h (Fig. 3). The decrease in DD after 4 h may have been due to destruction of the main chain of the polysaccharide. The optimum hydrolyzing time was judged to be 4 h.

3.4. Effect of the NaOH concentration on the DD

Examination of the effect of NaOH concentration from 35 to 60% (w/w) on deacetylation at 110 °C for 4 h showed an initial increase in DD and a decrease as NaOH increased, with a peak in DD at 55% (w/w) (Fig. 4). Here, the decrease in DD at >55% may have also been due to destruction of the polysaccharide main chain. Thus, the optimal NaOH concentration was 55% (w/w).

3.5. Effect of temperature on the DD

Examination of temperature effects on deacetylation by 55% NaOH (w/w) for 4 h showed a clear increase in DD with rising temperature to 110 °C, a slower increase from 110 to 120 °C, decreased

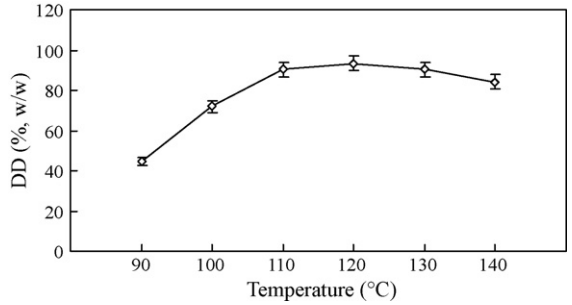

Fig. 5. Effect of temperature on degree of deacetylation. Results from triplicate experiments.

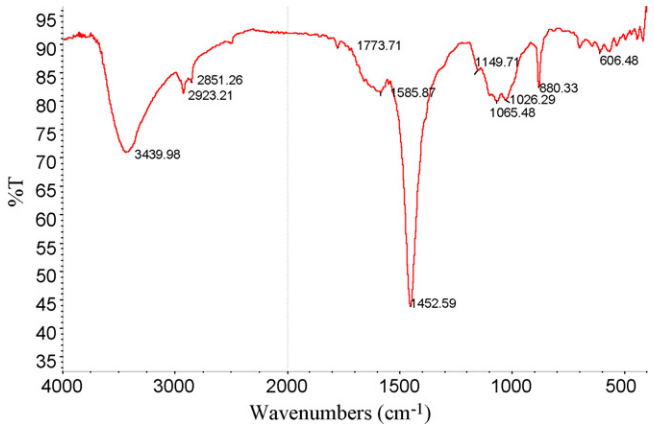

Fig. 6. An infrared absorption spectrum of a chitosan sample prepared from *Clanis bilineata* larvae skin.

DD beyond 120 °C, and a maximum DD at 120 °C (Fig. 5). Once again, the decreased DD beyond 120 °C may have originated from main chain polymer destruction at excessive temperatures. The optimal temperature was designated at 120 °C.

3.6. Characterization of the product

FTIR spectra of prepared samples showed absorption bands at 3439 and 2923 cm^{-1} (NH bond stretching), 1773 cm^{-1} (C=O bond stretching), 1585 cm^{-1} (NH vibrational mode), and 1452 cm^{-1} (CH bond angle vibration), indicating that the main component in these samples was chitosan (Fig. 6). The sample's ash, moisture, and protein contents were 0.81, 4.51, and 0.16% (%, w/w), respectively, and the product was a white, crisp, and water insoluble powder.

4. Conclusions

Flavourzyme treatment of CLS can remove most of the original protein. The optimum conditions for this result were flavourzyme at pH 6.5 and 50 °C. NaOH deacetylated the polymer effectively under the optimal conditions of 55% NaOH (w/w), 120 °C, and 4 h. FTIR spectra of the resulting samples indicated chitosan as the main product component. The protein content of the product and the chitosan yield were 0.16 and 31.37% (w/w), respectively.

References

Anon. (1984). *Handbook of Food Analysis (Parts II, III, IV)*. New Delhi: ISI., p. 18.
Deng, S. G., Huo, J. C., & Xie, C. (2008). Preparation by enzymolysis and bioactivity of iron complex of fish protein hydrolysate (Fe-FPH) from low value fish. *Chinese Journal of Oceanology and Limnology*, *26*, 300–306.
Linder, M., Fanni, J., & Parmentier, M. (2005). Proteolytic extraction of salmon oil and PUFA concentration by lipases. *Marine Biotechnology*, *7*, 70–76.
Moreau, S., Nau, F., Piot, M., Guerin, C., & Brule, G. (1997). Hydrolysis of hen egg white ovomucin. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, *205*, 329–334.
Muzzarelli, R. A. A., Rochetti, R., Stanic, V., & Weckx, M. (1997). Methods for the determination of the degree of acetylation of chitin and chitosan. In R. A. A. Muzzarelli, & M. G. Peter (Eds.), *Chitin handbook* (pp. 109–119). Italy: Atec.
Nyam, K. L., Tan, C. P., Lai, Q. M., Long, K. B., & Man, Y. C. (2009). Enzyme-assisted aqueous extraction of kalahari melon seed oil: optimization using response surface methodology. *Journal of the American Oil Chemists Society*, *86*, 1235–1240.
Shen, L. Q., Wang, X. Y., Wang, Z. Y., Wu, Y. F., & Chen, J. S. (2008). Studies on tea protein extraction using alkaline and enzyme methods. *Food Chemistry*, *107*, 929–938.