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Carbohydrate Polymers 52 (2003) 119–123

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Heterogeneous *N*-deacetylation of squid chitin in alkaline solution

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Received 4 July 2002; revised 11 September 2002; accepted 11 September 2002

Abstract

Chitin was extracted from squid pens and its heterogeneous alkaline deacetylation was performed using various conditions. The reaction followed the pseudo-first-order kinetics during an initial period. The influences of alkaline concentration, temperature, time and chitin to solution ratio on the *N*-deacetylation were investigated. The degree of deacetylation (DD) increased mainly with increasing temperature, NaOH concentration and time. The effect of the chitin to solution ratio was insignificant. In the temperature range of 40–100 °C, the apparent rate constant and the activation energy of the reaction ranged from 1.0×10^{-3} to 2.4×10^{-2} min^{-1} and from 5.4 to 11.9 kcal/mol, respectively. The linear regression analysis was performed to predict the optimum conditions for 90% DD chitosan product.

Keywords: Pseudo-first-order kinetics; *N*-deacetylation; Squid chitin

1. Introduction

Chitin is an ideally (1 → 4)-linked polysaccharide composed of 2-acetamido-2-deoxy-β-D-glucopyranosyl residues, and chitosan is the ideally fully *N*-deacetylated product of chitin. Both chitin and chitosan have been widely investigated for the last two decades on their industrial and biomedical applications (Hirano, 1996). Chitosan is usually prepared from chitin, and chitin has been found in a wide range of natural sources (crustaceans, fungi, insects, some algae, etc.) (Tolaimate et al., 2000). However, chitosan is only manufactured from crustaceans (crab, krill and crayfish) primarily because large amount of the crustacean exoskeleton is available as a by-product of food processing. Squid pens removed from the squid during processing are also regarded as waste. They have recently been obtained in considerable amounts and will become increasingly common as another potentially important chitin source. Normally, in the preparation of chitin from the crustacean sources, the exoskeleton must be treated with acid to remove calcium but squid pens are very low in calcium so the acid extraction step is not necessarily required. This change in the chitin extraction procedure should reduce the cost and may reduce acid-hydrolysis of the chitin during the process. Thus, chitin extracted from squid pens could be better quality than chitin extracted from other sources. In addition, chitin from squid pens is β-chitin which has more open structure (parallel chain alignment) than α-chitin (antiparallel chain alignment) found in crustacean exoskeletons (Shepherd, Reader, & Falshaw, 1997). It, therefore, shows higher solubility and swelling than α-chitin, due to much weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains. β-chitin also shows higher reactivity than α-chitin during deacetylation (Tolaimate et al., 2000). However, the deacetylation of squid chitin has not been widely studied, unlike the deacetylations of chitin isolated from crab and shrimp shells have been studied extensively owing to their wide availability. The kinetics of homogeneous alkaline deacetylation of α-chitin was reported to be a pseudo-first-order reaction (Sannan, Kurita, & Iwakura, 1977). Similar results were obtained for heterogeneous deacetylation at 150 °C (Castelli, Bergamasco, Beltrame, & Focher, 1996). However, Chang, Tsai, Lee, and Fu (1997) has reported that under different concentrations of alkali heterogeneous deacetylation of shrimp chitin appeared to be more complicated than a pseudo-first-order reaction. It might be controlled by a higher-order reaction and a diffusion-controlled reaction (Chang et al., 1997).

In this study, we applied the experimental study for response surface design to examine the heterogeneous alkaline deacetylation process of squid chitin. This will allow one to choose the best condition for preparing squid

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PII: S0144-8617(02)00300-4

chitosan suitable for its end uses. The kinetics of the process was also investigated.

2. Experimental

2.1. Isolation of squid chitin

The squid pens, *Loligo formosana* Sasaki, were washed with water, cut into small pieces (size 2 × 2 cm^2) and dried. The demineralization was carried out by soaking with 0.1 M hydrochloric acid (10 ml/g of squid pens) for overnight at room temperature. The deproteination was performed using an alkaline treatment with 3 M sodium hydroxide solutions (10 ml/g of squid pens) at 80–85 °C for 3 h. Then several washes were carried out up to neutrality and drying occurred. The resulting chitin (21% degree of deacetylation (DD)) contained 0.9% protein, no detectable ash and calcium carbonate.

2.2. N-deacetylation

An experimental design was used to establish the conditions for deacetylating chitin. The experiment covered the range of 10–20 ml of solution per gram of chitin, 20–60% NaOH, and 40–100 °C of temperature. Samples were heated in round bottom flasks under reflux in a silicone oil bath maintained at the desired temperature. The sample was removed for analysis after 15, 30, 45, 60 and 120 min of deacetylation.

2.3. Determination of degree of deacetylation

^{13}C CP/MAS NMR spectra acquired using the optimized pulse delay (4 s) and contact time (1 ms) were recorded at 75 MHz on a Bruker DPX-300 spectrometer with spinning rate of 5 kHz. Chemical shifts were quoted in ppm from adamantane. The DD of the samples was calculated from the ratio of the intensities of signals of methyl and anomeric carbons, using computer software obtained with the spectrometer (Struszczyk, Loth, & Peter, 1997).

2.4. Determination of molecular weight of chitosan

Gel permeation chromatography (GPC) was performed using PL-GPC 110 system connected to RI detector (Polymer Lab. Ltd, UK). Chitosan solutions (2 mg/ml) were applied to the column (Ultrahydrogel, Waters, USA) and eluted at 30 °C at a flow rate of 0.6 ml/min with 0.5 M acetate buffer (pH 4). Pullulan polysaccharides (MW 738-1,660,000) were used as reference materials to calibrate the system.

2.5. Statistical analysis

Results were analyzed using the regression analysis for response surface design. Full quadratic model was used to explore the relationship. The experimental variables were solid/liquid ratio, time, temperature and NaOH concentration. The response variable was DD. Selected model was evaluated by F test for regression model, t-test for the regression coefficients and coefficient of determination (R^2).

3. Results and discussion

3.1. Alkaline N-deacetylation of chitin

Effects of chitin to solution ratio, NaOH concentration, temperature and reaction time on DD were investigated. Each deacetylation condition was carried out in triplicates. By using NMR for determination of DD, the process of *N*-deacetylation can be monitored. Table 1 and Fig. 1 show the change of DD in several conditions (some data not shown). There were no differences in DD between chitin/solution ratio of 1/20 and 1/10 (data not shown). At low concentration of NaOH (i.e. 20%), there were no significant changes in DD even though temperature and time of the reaction were increased up to 100 °C and 120 min, respectively. On the other hand, at higher concentrations (i.e. 40–60% NaOH), all deacetylation proceeded in the same pattern. The DD rapidly increased as a function of NaOH concentration, temperature and time until it reached the maximum and leveled off.

3.2. Optimal condition for N-deacetylation

In order to investigate the optimum condition of *N*-deacetylation, DD of samples obtained from various conditions were statistically analyzed using linear

Table 1
Degree of *N*-deacetylation of squid chitin/chitosan obtained from various conditions

Chitin/solution ratio	NaOH concentration (%)	Temperature (°C)	Time (min)	%DD (average)
1:20	20	100	15	21.6
			120	22.8
1:10	40	40	15	21.6
			120	43.3
		60	15	34.8
			60	52.4
			120	68.0
		80	15	56.7
			60	70.9
			120	84.0
		100	60	73.6
			120	88.7
	60	40	120	27.5
		60	120	70.7
		80	45	84.2
			60	90.2
			120	94.7
		100	30	94.0
			60	97.3

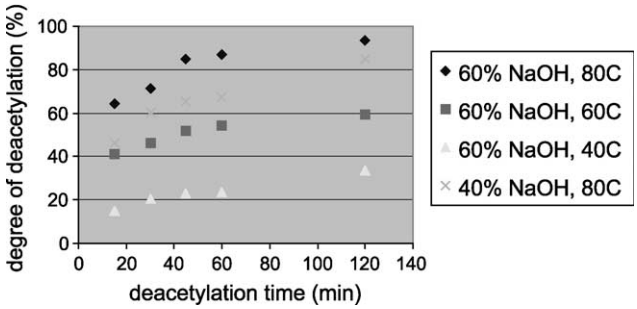

Fig. 1. Time course of alkaline *N*-deacetylation of squid pen chitin under various conditions.

regression analysis. The results are shown in Table 2. It revealed that NaOH concentration, reaction temperature, reaction time, interaction of temperature and NaOH concentration and interaction of time and NaOH concentration played a dominant role on influencing the DD (p-value <0.05). The quadratic term of temperature and time ($Temp^2$, $time^2$) also showed significant effects on the DD (p-value <0.05). The effect of chitin to solution ratio was insignificant (p-value >0.05, data not shown). It is well corresponded with the results of Chang et al. (1997) that the deacetylation of shrimp chitin increased mainly with increasing temperature or NaOH concentration and the effect of the chitin to solution ratio was insignificant. Response surface for the change in DD as a function of NaOH concentration and temperature, and that of NaOH concentration and time are shown in Fig. 2(a) and (b), respectively. The best fit regression Eq. (1) for the optimum deacetylation within the experiment range was obtained from the statistical analysis. According to this equation, the optimum conditions for the product of 90% DD as shown in Table 3 were predicted to occur.

$$\%DD = -14.9 - 0.90\times NaOH + 0.99\times Temp + 0.61 \times time - 7.5\times 10^{-3}\times Temp^2 - 1.37\times 10^{-3}\times time^2 + 2.15\times 10^{-2}\times NaOH\times Temp - 4.42 \times 10^{-3}\times NaOH\times time \quad (1)$$

Table 2
Regression analysis between the degree of *N*-deacetylation and the alkaline *N*-deacetylation variables

Parameter	Parameter estimate	T for Ho parameter	p-value
Intercept	−14.9	−1.87	0.063
%NaOH	−0.90	−6.76	0.000
Temperature	0.99	6.40	0.000
Time	0.61	8.75	0.000
$(Temp)^2$	-7.50×10^{-3}	−8.13	0.000
$(Time)^2$	-1.37×10^{-3}	−4.09	0.000
NaOH × Temp	2.15×10^{-2}	13.01	0.000
NaOH × time	-4.42×10^{-3}	−4.34	0.000
R^2 (adj)	95.0%		

where DD is the degree of deacetylation (%); NaOH, NaOH concentration (%); Temp, reaction temperature (°C); time, reaction time (min).

3.3. Kinetics of N-deacetylation

At the same alkaline concentration, the deacetylation process followed the pseudo-first-order kinetics (Table 4) at the initial period. Then it leveled off after 1 h of reaction as shown in Fig. 1. When the DD leveled off, the kinetics behavior changed. In case of shrimp chitin (Chang et al., 1997), the deacetylation leveled off within 2 h which took longer time than that of squid chitin. In addition, the maximum DD for only single alkaline treatment of shrimp chitin was about 75–85% whereas that of squid chitin reached nearly 100%. Squid chitin has been proved to show much higher reactivity than shrimp chitin in deacetylation because of the loose arrangement of chitin molecules. From Table 4, the results showed that temperature had dramatically significant influences on rate of reaction compared with NaOH concentration. Rate of reaction increased as a function of temperature with an exception at 100 °C due to the reach of maximum DD. For most conditions, semi-logarithmic plots between the amount of *N*-acetyl-D-glucosamine residues and the deacetylation time had correlation coefficients (R^2) well above 0.9. Nevertheless, some irregular patterns were observed in the time-course data. First of all, the *N*-deacetylation at 20% NaOH was the slowest process among all the conditions as shown in Table 1. At low concentration (20%), reaction did not follow the pseudo-first-order kinetics since there was no change in DD when temperature and time increased. It also appeared that more concentrated sodium hydroxide solution (60%) was necessary to achieve substantial deacetylation within 1 h at 80 °C. When 40% NaOH solution was used to deacetylate chitin at 80 °C or below, the degree of DD remained relatively low (<90%), even after 2 h of the reaction. For those conditions using high NaOH concentration (60%) and high temperature (80 °C), the deacetylation proceeded extremely fast. The deacetylation rate constant depended on temperature. At $T \leq 60$ °C, increasing of NaOH concentration did not affect the increasing of the rate constant. The rate constant also increased about 1.5–2.0 times with a 1.5-fold increase of NaOH concentration at $T \geq 80$ °C. In other words, concentration of alkaline had significant influence on the degree and rate of deacetylation at higher temperatures.

From the apparent rate constants for 40 and 60% NaOH, the activation energy (Table 5) was found to be approximately 10 and 5 kcal/mol, respectively. The results indicated that the reaction at higher concentration proceeded easier than that at lower concentration. Once again, the ratio of alkaline solution to chitin did not influence the activation energy. In comparison to heterogeneous *N*-deacetylation of shrimp chitin reported

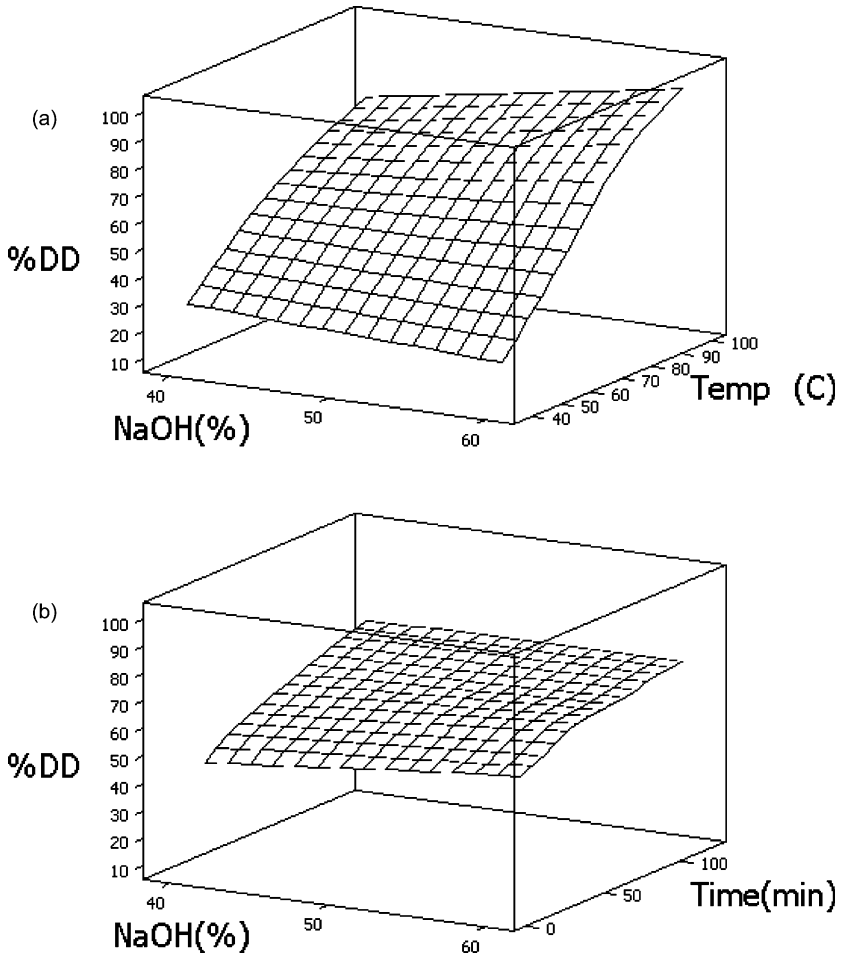

Fig. 2. Effects of (a) NaOH concentration and temperature; (b) NaOH concentration and time on the degree of *N*-deacetylation of squid chitin (1:20, chitin:solution).

by Castelli et al. (1996) (35% NaOH, 1:25 of chitin to solution ratio, at 80 and 120 °C, E_a: 8.5–13.8 kcal/mol), it was confirmed again that deacetylation of squid chitin occurred easier than that of shrimp chitin.

3.4. Molecular weight

Chitosan properties depend on two fundamental parameters: the DD and degree of polymerization. It is well known that chitin degradation can occur during

Table 3
Predicted optimum conditions for obtaining chitosan with 90% DD

NaOH concentration (%)	Temperature (°C)	Time (min)
40	80	158.1
	100	108.7
50	80	142.0
	90	99.7
	100	60.0
60	80	125.8
	90	53.1
	100	18.5

Table 4
Apparent first-order reaction rate constant of *N*-deacetylation

Condition	Temperature (°C)	Rate constant ($k \times 10^3$ min^{-1})	R^2
NaOH 40%			
chitin/solution ratio			
1:10	40	3.0	0.9953
	60	6.6	0.9909
	80	9.8	0.9926
	100	13.5	0.9970
1:20	40	2.3	0.9249
	60	7.2	0.9939
	80	11.5	0.9833
	100	8.8	0.9738
NaOH 60%			
chitin/solution ratio			
1:10	40	1.0	0.9503
	60	6.4	0.9587
	80	18.7	0.9045
	100	20.1	0.8553
1:20	40	2.2	0.9667
	60	5.8	0.9913
	80	24.4	0.9479
	100	22.2	0.9815

Table 5
Activation energy of *N*-deacetylation between 40 and 100 °C

Condition	E_a (kcal/mol)	E_a (kJ/mol)
NaOH 40%, chitin/solution ratio 1:10	11.90	49.80
NaOH 40%, chitin/solution ratio 1:20	9.83	41.13
NaOH 60%, chitin/solution ratio 1:10	5.74	24.04
NaOH 60%, chitin/solution ratio 1:20	5.39	22.56

Table 6
Molecular weight of chitosan obtained from various *N*-deacetylation conditions

Condition	MW × 10^{-5}
40% NaOH, 80 °C, 120 min	8.74
40% NaOH, 100 °C, 60 min	4.58
60% NaOH, 80 °C, 60 min	10.9
60% NaOH, 80 °C, 120 min	8.87
60% NaOH, 100 °C, 60 min	4.53
60% NaOH, 100 °C, 120 min	3.22

N-deacetylation. Under drastic conditions, the molecular weight of obtained chitosan decreased from 1.09×10^6 to 3.22×10^5 as shown in Table 6. Molecular weight (MW) also decreased about 20–30% with a 2-fold increase in reaction time. Temperature played a significant role in degradation. At a constant concentration and time, a 1.25-fold increase in temperature resulted in about 60% decrease in chitosan MW. Alkaline concentration did not affect the MW of the obtained chitosan.

3.5. Mechanism of N-deacetylation

Under different concentrations of alkali, heterogeneous *N*-deacetylation of squid chitin appeared to be more complicated than a pseudo-first-order reaction unlike that of shrimp chitin. The mechanism is not well described. Concentration of alkali and temperature had significant influences on the degree of *N*-deacetylation and the *N*-deacetylation rate constant. Chang et al. (1997) suggested that these might be due to the following reasons. Firstly, the morphological effect could attribute to the dependence of deacetylation on the concentration. Results from Kurita, Sannan, and Iwakura (1977) also supported that the heterogeneous *N*-deacetylation took place preferentially in the amorphous region of chitin, then proceeded from the edge to the inside of the crystalline region. The second reason is concerned about the equilibrium of the deacetylation reaction and the degradation of chitosan. The amount of *N*-acetyl-D-glucosamine residues kept decreasing during deacetylation whereas D-glucosamine residues continued to increase. The degradation occurred simultaneously with the deacetylation may interfere and compete the deacetylation resulting in the dependence of *N*-acetylation on the concentration and temperature, and the leveling off the DD. Finally, the mechanism of the heterogeneous *N*-deacetylation may be controlled by both reaction and diffusion.

4. Conclusions

Heterogeneous *N*-deacetylation of squid chitin followed pseudo-first-order kinetics at the initial period and leveled off after 1 h. Rate constants of the reaction dependent on alkaline concentration, temperature and time ranged from 1×10^{-3} to 2.4×10^{-2} min^{-1}. The activation energy in the experimental range was 5–10 kcal/mol. Various optimum conditions for obtaining 90% DD chitosan were predicted from the best fit regression equation.

Acknowledgements

The authors would like to thank Ms Temsiri Wangtaveesab for GPC analysis.

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