ELSEVIER

Polymer 41 (2000) 2463–2469

polymer

On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin

A. Tolaimate[a, b, c], J. Desbrières[b,*], M. Rhazi[a, c], A. Alagui[c], M. Vincendon[d], P. Vottero[d]

[a]*Laboratoire des Macromolécules Naturelles (LMN), Ecole Normale Supérieure, BP S41, Marrakech, Morocco*
[b]*CERMAV, CNRS, Joseph Fourier University, BP 53, 38041 Grenoble Cedex 9, France*
[c]*Laboratoire de Chimie Organique Appliquée, Unité Synthèse Organique et Structurale, Faculté des Sciences, Semlalia, BP S15, Marrakech, Morocco*
[d]*Laboratoire de Reconnaissance Ionique, Service de chimie Inorganique et Biologique, Département de Recherches Fondamentales sur la Matière Condensée, CEA-Grenoble, 17, rue des Martyrs, 38054 Grenoble Cedex 9, France*

Received 1 January 1999; received in revised form 23 April 1999; accepted 25 May 1999

Abstract

Chitin was extracted from squid pens and the used conditions allow to obtain a completely *N*-acetylated β-chitin with a molecular weight large enough for the obtention of chitosans of high molecular weight. Deacetylation, leading to the obtention of chitosan, was performed according to two processes (Kurita and Broussignac conditions) and the physicochemical characteristics (degree of acetylation and molecular weight) of the obtained chitosans were compared. The influence of the reaction conditions (temperature, repetition of alkaline steps, etc.) is discussed in relation with the physicochemical characteristics of the chitosan. This will allow one to choose the best process for preparing chitosan so that it is suitable for its end use. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Squid chitin; Deacetylation process; Chitosan

1. Introduction

Chitin, a homopolymer of β-(1 → 4)-linked *N*-acetyl-D-glucosamine, is one of the most abundant, easily obtained, and renewable natural polymers, second only to cellulose. It is commonly found in the exoskeletons or cuticles of many invertebrates [1] and in the cell walls of most fungi and some algae [2]. Polymers isolated from crab and shrimp shells have been studied extensively owing to their easy accessibility. These chitins have the α-crystallographic structure where the main chains arrange in an anti-parallel fashion with strong intermolecular hydrogen bonding [3]. Chitin may also be obtained from squid pens [4], and so presents the β-crystallographic structure where chitin chains arrange in a parallel fashion with relatively weak intermolecular forces [5]. So far, only limited attention has been paid to β-chitin, and its chemistry has been scarcely exploited, primarily because of low accessibility. However, squid pens have recently been obtained in considerable amounts and will become increasingly common as another potentially important chitin source [6].

β-Chitin is considered to be of interest owing to some specific properties. It shows higher solubility and swelling than α-chitin [7,8] due to much weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains. Thus, β-chitin may be anticipated to exhibit better solubility properties in a variety of solvents and a higher reactivity than α-chitin [6]. Recently, β-chitin turned out first to actually show higher reactivity than α-chitin during deacetylation [4] and second to degrade efficiently during acetolysis [9] and in numerous other chemical reactions [10]. Chitosan is produced by thermochemical alkaline deacetylation of chitin. It is a biopolymer with unique properties favorable for a broad variety of industrial and biomedical applications [11–14]. Chitosan is characterized by its degree of *N*-acetylation (DA) and this degree influences not only its physicochemical characteristics [10,15–19] but also its biodegradability [20–22] and immunological activity [23]. It is worth noting that chitosan derived from β-chitin shows higher reactivity than that derived from α-chitin in *N*-phthaloylation [24].

In this work, conditions for β-chitin extraction from squid pens will be presented and different deacetylation processes will be compared. The physicochemical properties of

* Corresponding author. Address: CERMAV, CNRS, Joseph Fourier University, BP 53, 38041 Greenoble Cedex 9, France. Tel: +33-4-7603-7624; fax: +33-4-7654-7203.
E-mail address: desbrier@cermav.cnrs.fr (J. Desbrières)

PII: S0032-3861(99)00400-0

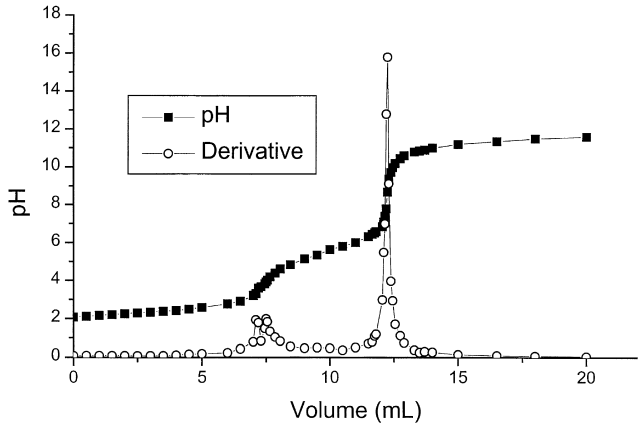


Fig. 1. Determination of the degree of acetylation (DA) from potentiometric titration of chitosan prepared using Kurita process for 3 × 3 h (pH measurement, derivative).

obtained chitosans will be discussed allowing the choice of adequate experimental conditions for desired properties.

2. Experimental

2.1. Isolation of squid chitin

The squid pens, from *Loligo vulgaris* species, were washed with water, dried and cut in small pieces (sieved from 2 to 5 mm).

The demineralization was carried out by washing twice at room temperature with 0.55 M hydrochloric acid for 2 h each time. It is followed by acidimetric titration and the end of the reaction is indicated by the persistence of the acidity of the medium. It was observed that the emission of carbonic acid gas was much important in case of shrimp than squid.

The deproteination was performed using alkaline treatments with 0.3 M sodium hydroxide solutions at 80–85°C. This treatment was repeated twice for 1 h each. The absence of proteins was indicated by the absence of color of the medium at the end of the second treatment. Then washings were carried out up to neutrality and drying occurred.

2.2. Deacetylation of the squid chitin

Two processes were compared. The first one was presented by Broussignac [25] using as deacetylation reagent a mixture of solid potassium hydroxide (50%, w/w), 96° ethanol (25%, w/w) and monoethyleneglycol (25%, w/w) which is nearly an anhydrous reaction medium. To prepare this mixture, the two solvents were first mixed and then solid potassium hydroxide was added in small portions under stirring. The dissolution is exothermic and the temperature of the mixture may increase up to 90°C during this step. One of the advantages of this reagent is that it can be used in both glass and stainless steel reactors. Chitin was then added to the reagent and temperature was increased up to the desired value. Alcohol was distilled and returned back in the reactor. The treatment was continued for the desired length of time, and after filtration, chitosan was washed with water until neutral pH reaction. It was then dried at room temperature in an air stream.

The second process was that of Kurita [4]. A suspension of chitin in an aqueous 40% by weight sodium hydroxide solution was heated at high temperature (80°C for example) under a nitrogen stream with stirring. After the desired time the solid was filtered, washed with water until neutral pH, then with methanol, and finally with acetone and dried.

2.3. Physicochemical characteristics of chitin and chitosan

The water content of the samples was determined using a 92-12 thermogravimetric analyzer from Setaram, France.

The potentiometric determination of the degree of acetylation was carried out following the method given by Broussignac [25] and Muzzarelli [26]. The chitosan was dissolved in a known excess of acid. From the titration of this solution with a 0.1 M sodium hydroxide solution a curve with two inflexion points was obtained. The difference of the volumes of these two points was corresponding to the acid consumed for the salification of amine groups and allows the determination of the degree of acetylation (DA) of the chitosan. The titration was performed with a pH meter Minisis 6000 from Radiometer, France. Fig. 1 shows an example of the curve and its treatment.

DA was also calculated from ^{1}H NMR considered to be the most sensitive technique using an AC300 Bruker spectrometer [27]. The samples were dissolved at a concentration of 10 mg/ml in D_2O in the presence of HCl (pH 4) and freeze-dried three times to exchange labile protons for deuterium atoms. The spectra were performed at 353 K. The DA value was determined from the integral of $-CH_3$ signal at 1.97 ppm compared with the integral of H-1 protons considered as internal standard. An example of the spectrum is given in Fig. 2. The DA values determined from NMR and titration were in very good adequation.

Solid-state CP/MAS ^{13}C NMR spectra were obtained on a Bruker MSL spectrometer operating at 50.3 MHz. A sample of 200 mg was placed in a zirconia rotor spinning at the magic angle at 3.3 kHz. The contact time and the recycle delay were set at 1 ms and 3 s, respectively. These conditions were tested on the solid *N*-acetyl-D-glucosamine in order to check the exact ratio of 1/6, between the *methyl*-C atom of the *N*-acetyl group and the six C atoms of the D-glucopyranosyl ring.

The viscosity measurements were performed using a Ubbelohde capillary viscometer ($\phi = 0.5$ mm) at 25 ± 0.1°C. The solvent was 0.3 M acetic acid/0.2 M sodium acetate and the average viscometric molecular weight was calculated from the viscosity law [28]:

$$[\eta] = 0.078 M_v^{0.76}.$$

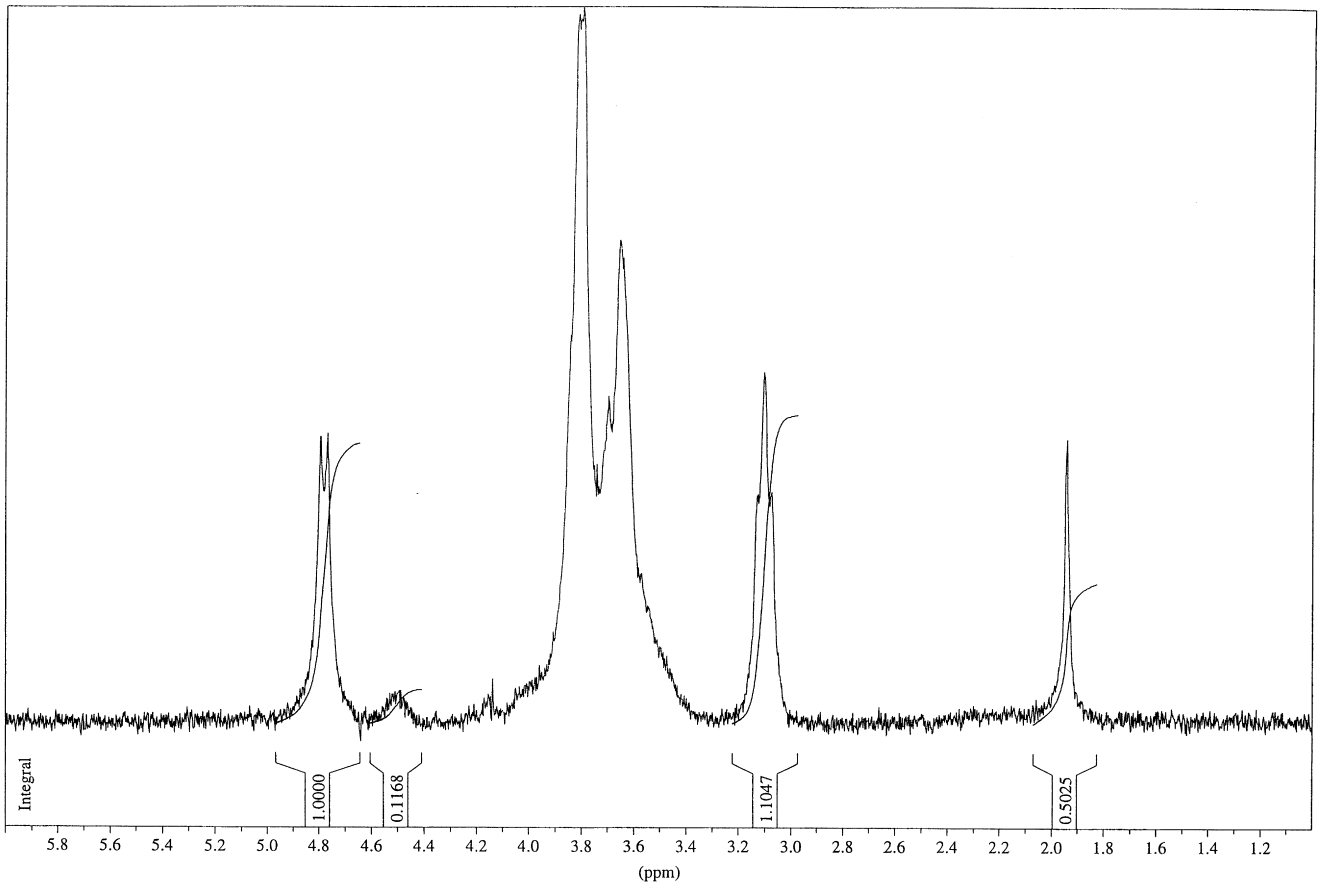


Fig. 2. ^{1}H NMR spectrum of chitosan (D_2O, pH = 4).

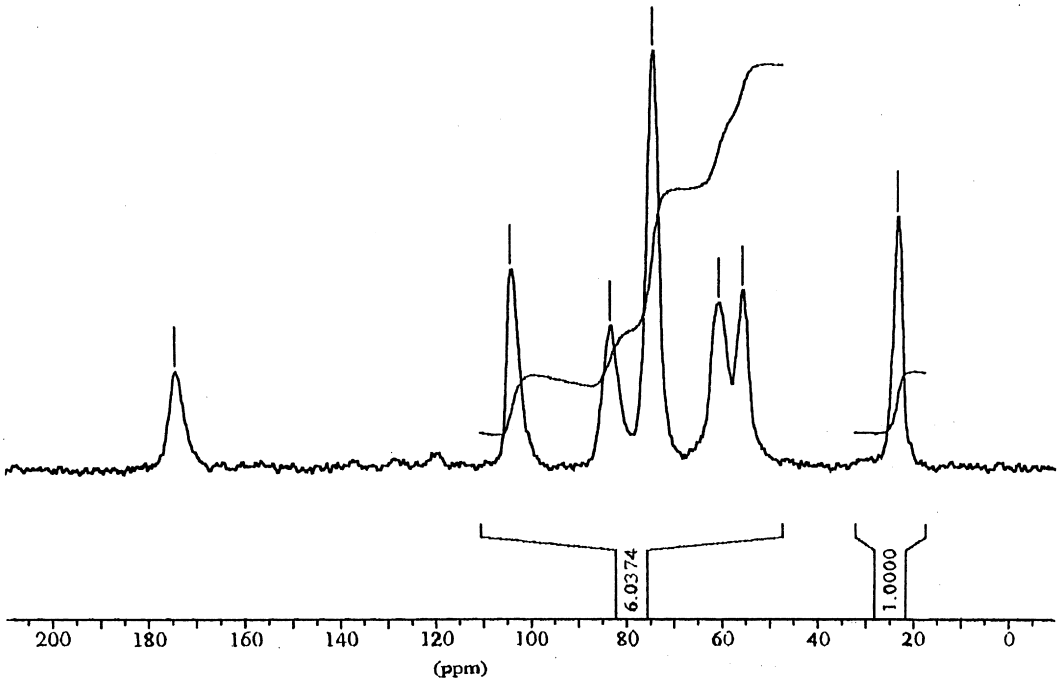


Fig. 3. Solid-state high resolution ^{13}C NMR spectrum of chitin extracted from squid pens.

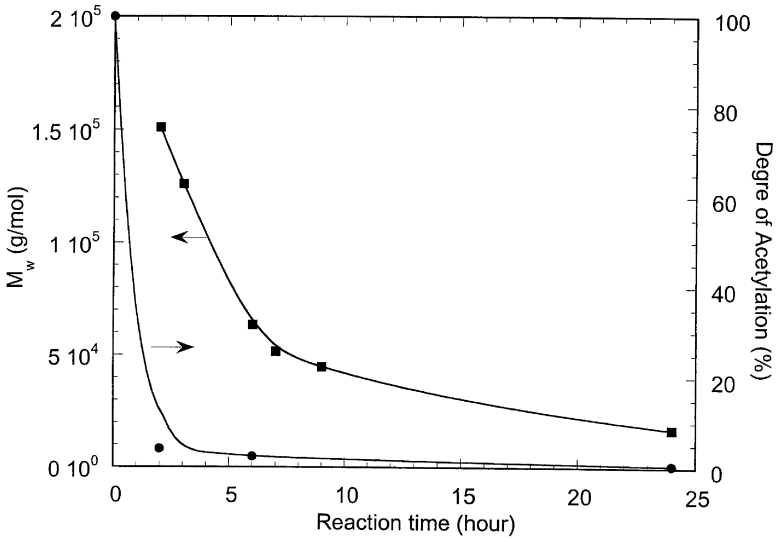


Fig. 4. Kinetics of the deacetylation reaction using the Broussignac process [KOH (50%, w/w) in 96° ethanol (25%, w/w) and monoethyleneglycol (25%, w/w), $T = 120$°C].

3. Results and discussion

3.1. Isolation of chitin from squid pens

β-Chitin was isolated by treating *L. vulgaris* pens. The degree of acetylation of the chitin was obtained from ^{13}C NMR in solid state (Fig. 3). From the integral of the peak at 23.1 ppm, characteristic of $-CH_3$, compared to those of the peaks in the 55–104 ppm region it may be concluded (from the average of three values) that chitin is completely acetylated contrarily to the data reported by Hackman and Goldberg [29]. When treating *Ommastrephes* pens, the degree of acetylation of isolated squid chitin was at least 0.9 [4]. The modifications we have done compared to Broussignac's process (0.55 M HCl in place of 1.4 and 0.3 M NaOH at 80–85°C compared with 1.2 M at 90°C) were carried out in order to preserve a structure as close as possible to original chitin and to avoid deacetylation and depolymerization as it will be demonstrated. It may be pointed out that the extracted chitin, after demineralization and deproteination, was white and clear. As a consequence the mild oxidizing treatment (H_2O_2/HCl in a ratio 9/1), to remove pigment traces responsible for the color of the chitin, was not necessary as reported for other shellfishes.

The chitin content was about 40% of the original weight of the dried pens. This content is high as compared to that of chitin we have found from shrimps, crabs or other shellfishes (10% from crabs, or 32% from crayfishes).

3.2. Deacetylation behavior of squid chitin

Regeneration of amino functions from acetamidodeoxy carbohydrates can be performed in acid and basic conditions [30], but unfavorable steric effects frequently hinder the reaction [31]. Despite numerous attempts, *N*-acetyl groups could not be removed by acid reagents without inducing hydrolysis of the polysaccharide backbone. In the presence of alkali, polysaccharide chains were found to undergo degradation because of the high concentration of reagents and prolonged reaction times required to obtain a complete deacetylation. The low reactivity of chitin against the deacetylation reaction was ascribed to the trans-arrangement of acetamido groups in the monomeric unit with respect to the hydroxyl group OH-3 [32].

Several alkaline methods have been proposed, most of them involving the use of sodium or potassium hydroxide solutions as well as a mixture of anhydrous hydrazine and hydrazine sulfate [33]. More recently, new methods have been reported, such as intermittent water washing [34], the use of a water-miscible organic solvent [35], high temperature [36], flash treatments [37] or enzymatic *N*-deacetylation [38]. In our study, the deacetylation reaction was performed under heterogeneous conditions contrarily to a pseudo-homogeneous process [39] in which chitosan is first dissolved in an acid solution before the addition of the alkaline solution. This leads to sparsely as well as non-uniformly accessible block copolymers of *N*-acetyl-D-glucosamine and D-glucosamine residues, whose physicochemical properties appeared quite different with respect to those of chitosan randomly deacetylated in homogeneous conditions [40]. The kinetics of homogeneous alkaline *N*-deacetylation of chitin was reported to be a pseudo-first-order reaction [41]. Similar results were obtained for heterogeneous deacetylation at 150°C [36] and optimal conditions for *N*-deacetylation were discussed [42]. All these experiments were carried out on α-chitin principally isolated from shrimps.

We have compared different deacetylation processes on β-chitin to allow the determination of experimental conditions to reach adequate molecular weights and

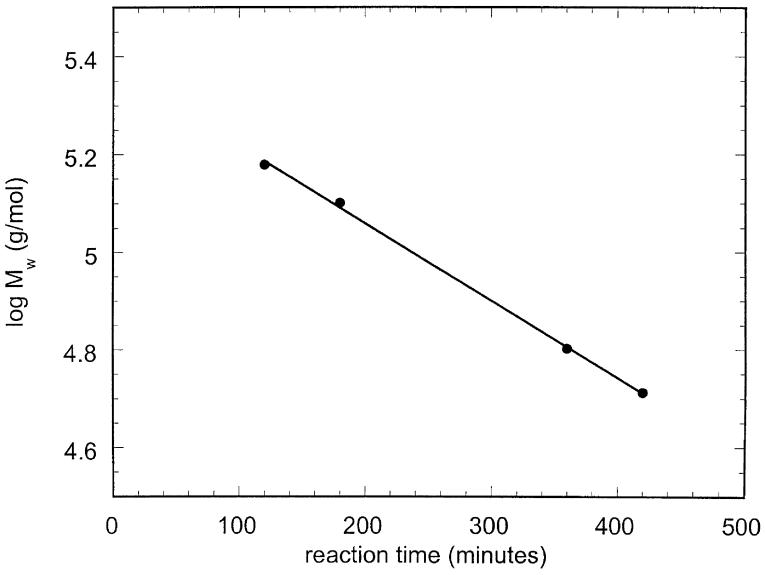
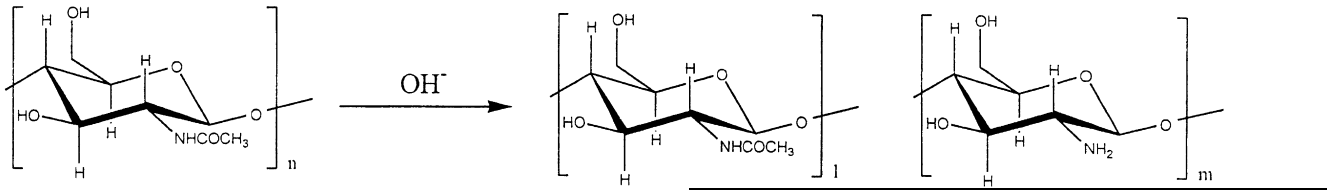


Fig. 5. Plot of the average viscosimetric molecular weight versus the deacetylation time (KOH (50%, w/w) in 96° ethanol (25%, w/w) and monoethyleneglycol (25%, w/w), $T = 120$°C).

DA for required properties. The reaction scheme is the following:



The mechanism is not well described. Kurita [40] has suggested that heterogeneous *N*-deacetylation takes place preferentially in the amorphous region of chitin, then proceeds from the edge to the inside of the crystalline region. Chang [42] has assumed that it may be controlled both by reaction and diffusion.

First, the Broussignac process has been studied to determine the kinetics of the reaction (Fig. 4). After 2 h the degree of acetylation is already lower than 4% and the yield of chitosan is 72%, compared to initial chitin. Increasing the reaction time does not increase significantly the deacetylation but lowers the molecular weight. The molecular degradation could be described by a pseudo-first-order kinetics (Fig. 5). The degradation rate constant is approximately equal to 1.58×10^{-3} min^{-1}, in the same order of magnitude of the data obtained by Chang on shrimp chitin [42]. By decreasing the temperature from 120 down to 95°C, keeping the same composition of the reaction medium, a degree of acetylation of 30% (compared to 4%) and an average viscometric molecular weight of 490,000 g/mol (compared to 150,000) were obtained after 2 h, confirming the role of the temperature. Moreover, taking this value into account it may be assumed that the original chitin was not depolymerized in a large extent during the extraction process. When the reaction is carried out in a nitrogen atmosphere, the molecular weight is not modified which is

Table 1
Influence of the number of repeated steps on the physicochemical characteristics of obtained chitosan and comparison with the continuous process (Broussignac process: KOH (50%, w/w) in 96° ethanol (25%, w/w) and monoethyleneglycol (25%, w/w), $T = 120$°C)

Reaction time (h)	Degree of acetylation (DA, %)	M_v (g/mol)
2	4	151,000
1 + 1	5	290,000
3		126,000
1 + 1 + 1	5	248,000
6	2.5	63,000
2 + 2 + 2	1.5	85,000

Table 2
Influence of the number of repeated 3 h steps on the physicochemical characteristics of chitosan (Kurita process: aqueous 40% NaOH, $T = 80$°C, under nitrogen atmosphere)

Number of steps	Degree of acetylation (DA, %)	Molecular weight (M_v, g/mol)
1	25	
2	3	595,000
3	1	500,000

Table 3
Influence of the process on the physicochemical characteristics of chitosan (Kurita process: aqueous 40% NaOH at 80°C, Broussignac process: KOH (40%, w/w) in 96° ethanol (30%, w/w) and monoethyleneglycol (30%, w/w), $T = 80$°C; nd: not determined)

Reaction time	Degree of acetylation (DA, %)		Average molecular weight (M_v, g/mol)	
	Kurita process	Broussignac process	Kurita process	Broussignac process
3 h	25	30	nd	nd
3 h × 2	3	26	595,000	nd
3 h × 3	1	26	450,000	430,000
3 h × 5		17		320,000

coherent with the fact that the breaking of bonds is not an oxidative process and thus the presence or not of dioxygen has no influence.

A discoloration of shrimp chitin was observed, and to avoid this phenomenon repeated alkaline treatments were suggested [34,43]. We have tested this technique on Broussignac's process, and the evolution of the deacetylation was examined as a function of the number of alkaline treatments (Table 1). The comparison of continuous treatments and repeated alkaline treatments shows that in the two cases deacetylation is identical, while the molecular weight is less affected when repeated treatments were carried out.

Kurita et al. [4] have determined the best experimental conditions for deacetylation of the squid chitin, using 40% NaOH at 80°C during 3 h step and considering the evolution of the degree of acetylation. Nevertheless, no information was given on the molecular weight values. The results of this process, carried out on squid chitin, are given in Table 2. One can notice an important value of the molecular weight ($M_v = 500,000$ g/mol) for the completely deacetylated sample in a nitrogen atmosphere.

We have compared the two processes to determine the best conditions allowing the adequate physicochemical characteristics for desired applications such as complexing or flocculating agents (Table 3). When the same temperature is applied in the case of both processes (80°C) the molecular weight is about equivalent but a better deacetylation is obtained by using the Kurita process. Moreover it was observed that, after two repeated alkaline steps, the final acetylation degree is obtained; the increase in the number of reaction steps only leads to depolymerization. To obtain the same degree of acetylation with the Broussignac process it is necessary to increase the temperature up to 120°C. In these conditions an important depolymerization is observed (molecular weight of 126,000 g/mol instead of more than 600,000, Tables 1 and 3).

Chang [42] has found that, using the Kurita process for shrimp chitin (α-chitin), the maximal degree of deacetylation was obtained in 26 ml of 60% (w/w) NaOH solution at 107°C per gram of chitin.

4. Conclusions

We have determined the conditions to prepare chitin, from squids, which is completely acetylated without affecting a lot the original molecular weight. Furthermore, we have compared deacetylation processes in order to be able to define the conditions for reaching the adequate physicochemical characteristics (molecular weight and degree of acetylation) for desired properties (metal complexation, floculating agent, etc.). Kurita's process is useable to obtain chitosans with high molecular weights and within a large range of deacetylation degrees. The Broussignac process may be carried out to obtain quickly chitosan with low degrees of acetylation and low molecular weights. The Kurita process is slower than the Broussignac one in terms of deacetylation, but is less detrimental to the molecular weight. The decrease of the temperature is detrimental to the degree of acetylation but allows to reach high molecular weights as 600,000 g/mol. Finally the interest of repeated alkaline steps, compared to a continuous process, is demonstrated.

References

[1] Jeuniaux C. Bull Soc Zool Fr 1982;107:363.
[2] Ruiz-Herrera J. Proc Int Conf Chitin Chitosan 1978:11.
[3] Minke M, Blackwell J. J Mol Biol 1978;120:167.
[4] Kurita K, Tomita K, Tada T, Ishii S, Nishimura SI, Shimoda K. J Polym Sci, Part A 1993;31:485.
[5] Gardner KH, Blackwell J. Biopolymers 1975;14:1581.
[6] Kurita K, Ishii S, Tomita K, Nishimura SI, Shimoda K. J Polym Sci, Part A 1994;32:1027.
[7] Lee VFP. PhD dissertation, University of Washington, Seattle, 1974.
[8] Austin PE, Castle JE, Albisetti CJ. In: Skjak-Braek G, Anthonsen T, Sandford P, editors. Chitin and chitosan. Essex: Elsevier, 1989. p. 749.
[9] Kurita K, Tomita K, Ishii S, Nishimura SI, Shimoda K. J Polym Sci, Part A 1993;31:2393.
[10] Sannan T, Kurita K, Iwakura Y. Makromol Chem 1976;177:3589.
[11] Rha CK, Rodriguez-Sanchez D, Kienzle-Sterzer C. In: Colwell RR, Pariser ER, Sinskey AJ, editors. Biotechnology of marine polysaccharides. Washington, DC: Hemosphere, 1984. p. 283.
[12] Knorr D. In: Colwell RR, Pariser ER, Sinskey AJ, editors. Biotechnology of marine polysaccharides. Washington, DC: Hemosphere, 1984. p. 313.
[13] Hirano H. In: Skjak-Braek G, Anthonsen T, Sandford P, editors. Chitin and chitosan. Essex: Elsevier, 1989. p. 37.
[14] Muzzarelli RAA. Carbohydr Polym 1996;29:309.
[15] Wang W, Bo S, Li S, Qin W. Int J Biol Macromol 1991;13:281.
[16] Errington N, Harding SE, Varum KM, Illum L. Int J Biol Macromol 1993;15:113.
[17] Rinaudo M, Milas M, Le Dung P. Int J Biol Macromol 1993;15:281.
[18] Varum KM, Ottoy MH, Smidsrod O. Carbohydr Polym 1994;25:65.
[19] Zydowicz N, Vachoud L, Domard A. In: Domard A, Jeuniaux C,

Muzzarelli R, Roberts E, editors. Advances in chitin science, vol I. Lyon: J. Andre, 1996. p. 262.
[20] Shigemasa Y, Saito K, Sashiwa H, Saimoto H. Int J Biol Macromol 1994;16:43.
[21] Hutadilok N, Mochimasu T, Hisamori H, Hayashi K, Tachibana H, Ishii S, Hirano H. Carbohydr Res 1995;268:143.
[22] Nordtveit RJ, Varum KM, Smidsrod O. Carbohydr Polym 1996;29:163.
[23] Peluso G, Petillo O, Ranieri M, Santin M, Ambrosio L, Calabro D, Avallone B, Balsamo G. Biomaterials 1994;15:1215.
[24] Kurita K, Tomita K, Tada T, Nishimura SI, Ishii S. Polym Bull 1993;30:429.
[25] Broussignac P. Chimie et Industrie- Génie Chimique 1968;99:1241.
[26] Muzzarelli RAA. Chitin. Oxford: Pergamon Press, 1977. p. 105.
[27] Rinaudo M, Le Dung P, Gey C, Milas M. Int J Biol Macromol 1992;14:121.
[28] Rinaudo M, Milas M, Le Dung P. Int J Biol Macromol 1993;15:281.
[29] Hackman RH, Goldberg G. Aust J Biol Sci 1965;18:935.
[30] Hanessian S. Methods Carbohydr Chem 1972;6:208.
[31] Thompson A, Wolfrom ML. Methods Carbohydr Chem 1963;2:215.
[32] Muzzarelli RAA. Chitin. Oxford: Pergamon Press, 1977. p. 94.
[33] Dmitriev BA, Knirel YA, Kochetkov NK. Carbohydr Res 1975;40:365.
[34] Mima S, Miya M, Iwamoto R, Yoshikawa S. J Appl Polym Sci 1983;28:1909.
[35] Batista I, Roberts GAF. Makromol Chem 1990;191:429.
[36] Castelli A, Bergamasco L, Beltrame PL, Focher B. In: Domard A, Jeuniaux C, Muzzarelli R, Roberts G, editors. Advances in chitin science, vol I. Lyon: J. Andre, 1996. p. 198.
[37] Focher B, Beltrame PL, Naggi A, Torri G. Carbohydr Polym 1990;12:405.
[38] Martinou M, Kafetzopoulos D, Bouriotis V. Carbohydr Res 1995;273:235.
[39] Rinaudo M, Le Dung P, Milas M. French Patent 9302182, 1993.
[40] Kurita K, Sannan T, Iwakura Y. Makromol Chem 1977;178:3197.
[41] Sannan T, Kurita K, Iwakura Y. Polym J 1977;9:649.
[42] Chang KLB, Tsai G, Lee J, Fu WR. Carbohydr Res 1997;303:327.
[43] Domard A, Rinaudo M. Int J Biol Macromol 1983;5:49.