Available online at www.sciencedirect.com
SCIENCE DIRECT®
Polymer 44 (2003) 7939–7952
polymer
www.elsevier.com/locate/polymer

Contribution to the preparation of chitins and chitosans with controlled physico-chemical properties

A. Tolaimate[a], J. Desbrieres[b,*], M. Rhazi[a], A. Alagui[c]

[a]*Laboratoire des Macromolécules Naturelles (L.M.N.), Ecole Normale Supérieure, BP 2400 Marrakech, Morocco*
[b]*Centre de Recherches sur les Macromolécules Végétales (C.E.R.M.A.V.), C.N.R.S., associated with 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 2390 Marrakech, Morocco*

Received 26 February 2003; received in revised form 8 September 2003; accepted 6 October 2003

Abstract

In this paper, from many new examples, our approach on the preparation of chitins and chitosans with controlled physico-chemical characteristics was presented. The chitosan samples were prepared from α-chitin from crustacean shells and β-chitin from squid pens. The chitin deacetylation was carried out according to two methods using, respectively, as alkaline agent, the aqueous sodium hydroxide solution and anhydrous potassium hydroxide. The role of the source and of the process on the *N*-deacetylation reactions is confirmed. The effect of the addition of sodium borohydride or thiophenol within the reaction medium was studied. One of the parameters conditioning the physico-chemical characteristics of chitosan being in relation with the nature and the quality of original chitin, the role of this parameter was examined and the isolation process was discussed to put in evidence its advantages compared to other processes quoted in the literature by relying on new results.

Keywords: Chitin; Physico-chemical characteristics; *N*-Deacetylation

1. Introduction

Chitin is one of the most abundant natural polysaccharides produced by many living organisms; it is usually found as a component of crustacean shells. This polymer consists of a linear chain of (1 → 4) linked 2-acetamido-2-deoxy-β-D-glucopyranose units (Fig. 1a). But this polymer is not soluble in usual solvents and for its use, chemical modifications are performed [1]. The most common derivative prepared is the chitosan, coming from the partial deacetylation of chitin [2,3]. When the degree of acetylation (DA, Fig. 1b) is lower than 0.5, chitosan becomes soluble in acidic aqueous solutions and it behaves as a cationic polyelectrolyte.

Chitin was produced by biosynthesis [4–6] and its natural production is inexhaustible; arthropods, by themselves count more than 10^6 species from the 1.2×10^6 of total species compiled for all the animal kingdom, constitute permanent and large biomass sources. Although it is present within numerous taxonomic groups [7,8], chitin, prepared in laboratories or industrially produced, is usually isolated from exoskeletons of crustaceous and more particularly from shrimps and crabs; in these cases α-chitin was produced [9]. However, when β-chitin has to be prepared, essentially squid pens are used as a polysaccharide source [10,11]. α-Chitin is the most common form while β-chitin shows higher reactivity [11–14] and higher affinity towards solvents [15,16].

Potential and usual applications of chitin, chitosan and other chitin derivatives are estimated to be more than 200 [17]. The wide range of applications concerning cosmetics, agriculture, food, biomedical, textile or refining of industrial effluents [18–24] come from physico-chemical and biological properties of chitosan. Especially its ability, as cationic polyelectrolyte, to fix anionic species, large compatibility with living tissues and its properties to be biodegradable [25–27], permeable to oxygen and also stimulating effect on immunity system are exploited [28]. Nevertheless so that each of these uses kept one's promise,

* Corresponding author. Tel.: +33-4-76-03-76-24; fax: +33-4-76-54-72-03.
E-mail address: jacques.desbrieres@cermav.cnrs.fr (J. Desbrieres).

0032-3861/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymer.2003.10.025

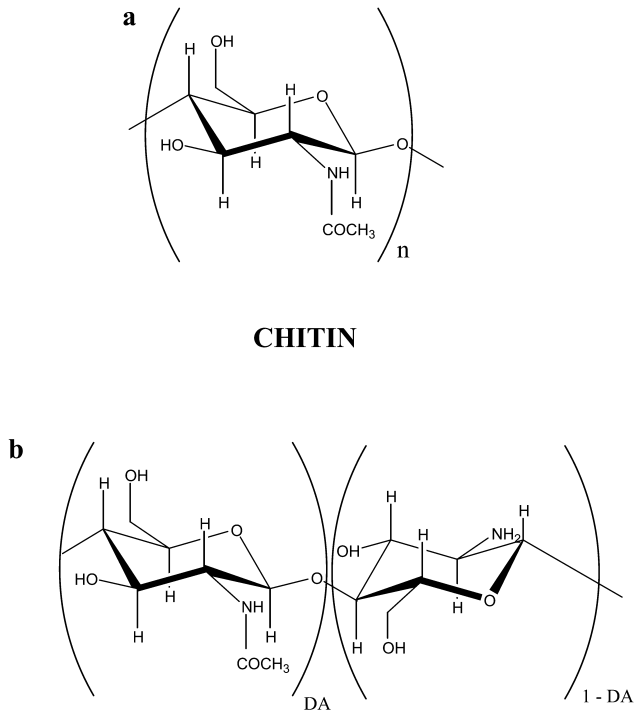

Fig. 1. Chemical structure of chitin (a) and chitosan (b). DA is the degree of acetylation.

it is necessary to control the different parameters which influence the chitosan characteristics so as to produce it according to the characteristics desired by companies. In many cases the production of highly deacetylated chitosan, with very low degree of depolymerization is desirable. Moreover, DA influences not only its physico-chemical characteristics [14,29–32] but also its biodegradability [25–27] and immunological activity [28].

In this paper, the role of some parameters which influence on properties of chitin and chitosan is studied. Our investigations show that the proper adjustment of the process conditions will make possible to produce chitins very similar to their native form, and to prepare chitosans with well-controlled characteristics.

2. Experimental [33,34]

2.1. Isolation of chitin

The raw material was obtained in solid form without any treatment from the different sources coming from Moroccan seaside (Table 1). It was washed with water, desiccated at room temperature and cut into small pieces (sieved from 2 to 5 mm). When dry, it was kept at atmospheric air as long as wanted.

Table 1
Chitin sea sources, conditions of chitin isolation and its characteristics

Source of chitin	Acid bath 0.55 M HCl number	Alkaline bath 0.3 M NaOH number	H_2O_2 treatment	Chitin content (%)	Type of chitin	Taxonomy
Barnacle (*Lepas anatifera*)	2	4	Yes	07	α	Class *Crustacea*, subclass *Cirripedia*
Marbled crab (*Grapsus marmoratus*)	3	3	Yes	10	α	Class *Crustacea*, subclass *Malacostraca*, order *Decapoda*, Suborder *Reptantia*, section *Brachyura*
Red crab (*Portunus puber*)	5	3	Yes	10	α	
Spider crab (*Maia squinado*)	3	3	Yes	16	α	
Lobster (*Homarus vulgaris*)	3	3	Yes	17	α	Class *Crustacea*, subclass *Malacostraca*, order *Decapoda*, Suborder *Reptantia*, section *Macrura*
Locust lobster (*Scyllarus arctus*)	2	3	Yes	25	α	
Spiny lobster (*Palinurus vulgaris*)	3	7	Yes	32	α	
Crayfish (*Astacus fluviatilis*)	2	3	Yes	36	α	
Shrimp (*Palemon fabricius*)	3	3	Yes	22	α	Class *Crustacea*, subclass *Malacostraca*, order *Decapoda*, suborder *Natantia*
Squilla (*Squilla mantis*)	3	3	Yes	24	α	Class *Crustacea*, subclass *Malacostraca*, order *Stomatopoda*
Cuttlefish (*Sepia officinalis*)	3	3	No	20	β	Class *Cephalopoda*, order *decapoda*
Squid (*Loligo vulgaris*)	3	2	No	40	β	

Table 2
Comparison of chitin production conditions according to the major papers from literature and our work

Source	Deproteinization				Demineralization			References
	NaOH concentration (M)	Temperature (°C)	Number of baths	Treatment duration (h)	HCl concentration (M)	Temperature (°C)	Treatment duration (h)	
Crab	0.5	65	1	2	1.57	Room	5	Muzzarelli et al. [38]
Shrimp	0.125	100	1	0.5	1.25	Room	1	Madhavan et al. [39]
	0.750	100	1	–				
Krill	0.875	90–95	1	2	0.6	Room	2	Anderson et al. [40]
Crab	1	80	1	3	1	Room	12	Mima et al. [41]
Crab	1	100	1	36	2	Room	48	Shimahara et al. [42]
Crab	1.25	85–90	3	24	1.37	Room	24	Broussignac [35]
Shrimp	1.25	100	1	0.5	1.57	20–22	1–3	Moorjani et al. [43]
Crab	2.5	Room	3	72	11	−20	4	Whistler et al. [44]
Lobster	1	100	5	60	2	Room	5, 48	Hakman et al. [45]
Crab	1	100	3	72	1	Room	–	Hakman et al. [46]
Squid	2	Room	2	One night	1	Room	One night	Kurita et al. [11]
	2	100		4				
12 Species of crustaceous and cephalopods	0.3	80–85	From 2 to 7 according the source	1 h for each bath	0.55	Room	15 mn to 1 h by bath repeated 2–5 times according the source	Tolaimate et al [33], Rhazi et al [34]

The mineral content of exoskeletons of crustaceous (determined using element analysis) is not the same for each source (see Table 3). Hence studied chitin sources do not need the same treatments. Demineralization was carried out at room temperature using 0.55 M hydrochloric acid baths. Each bath was performed with 100 ml of acid solution and 10 g of raw material. The number of baths and their duration (between 15 and 60 min) were dependent upon the source [34] (see Tables 1 and 2). Demineralization was followed by acidimetric titration: the evolution of pH towards neutrality means acid consumption and hence it is necessary to proceed other treatments; the end of the repeated series of baths was indicated by the persistence of acidity in the medium. It was observed that the emission of CO_2 gas was more or less important according to the studied species. It is, for example, strong in the case of spider crabs or shrimps, and weak in the case of squids. It also depends upon the mineral content (counterions of carbonate or phosphate ions) of the different species and penetration of the shells by hydrochloric acid. As an example, the shell of shrimps contains 18.17 wt% of calcium and 1.91 wt% of phosphorus, whereas squid pens contain only 1.06 wt% of calcium and 0.51 wt% of phosphorus. The larger mineral content causes increase of the gas emission. The demineralization efficiency is given in Table 4 (the mineral content being determined from element analysis).

Deproteinization was performed using alkaline treatments with 0.3 M sodium hydroxide solutions at 80–85 °C. This treatment was repeated several times during 1 h. The number of baths was given in Table 1. The absence of proteins was indicated by the absence of colour of the medium at the end of the last treatment (as confirmed by NMR spectra in which no peak but chitin ones is observed). Washings were then carried out up to neutrality, after which samples were dried. After these steps (demineralization and deproteinization) chitin was dried in an oven at 50 °C during 24 h.

At this stage, chitin isolated from squid pens is perfectly white unlike those isolated from crustaceous sources were lightly pink. Pigment traces responsible for this colour are removed using a mild oxidizing treatment (30 volumes H_2O_2/33% HCl in a volume ratio 9/1).

Chitin content was determined from weight measurements of the raw material and of the chitin obtained after acid and alkaline baths (and possibly the bleaching step). Purity of chitin was evaluated from ^{13}C solid state NMR spectra.

2.2. Preparation of chitosan

Two processes were studied. First, the standard process presented by Broussignac [35] was applied to the isolated chitin. During this process, a mixture of solid potassium hydroxide (50 w/w%), 96% ethanol (25 w/w%) and monoethylene glycol (25 w/w%) which is nearly an anhydrous reaction medium was used as deacetylation

Table 3
Mineral content (determined from element analysis) of sources of chitin taking into account the part of the organism submitted to the isolation process

Source	Ca (%)	Na (%)	P (%)	Mg (%)	K (%)	Total (%)
Lobster	30.54	0.42	0.90	2.03	0.10	33.99
Red crab	26.60	1.65	1.12	1.51	0.25	31.13
Spider crab	22.62	0.72	0.72	1.78	0.12	25.96
Pink shrimp	18.17	0.80	1.26	0.91	0.36	21.50
Squilla	15.34	0.25	2.22	1.03	0.054	18.89
Squid	01.06	0.028	0.57	0.035	0.015	1.70

reagent. 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 or stainless steel reactors. Chitin was then added progressively to the reagent and temperature was increased up to the desired value (detailed reaction conditions are given in Section 3). Alcohol was distilled and returned back to the reactor. The treatment was continued for the required duration, and after filtration chitosan was washed with water to neutral pH. It was then dried at room temperature in air stream. The reaction was carried out on 500 mg of chitin suspended in 30 ml of the mixed solvent.

Chitosan was also produced using the process of Kurita et al. [11]. A suspension of 500 mg of chitin in 30 ml of aqueous sodium hydroxide solution (50% w/v) was heated up to 80 °C, for example, under a nitrogen stream with stirring. After the desired time the solid was filtered off, washed with distilled water to neutral pH, then with methanol, and finally with acetone. Drying was than performed in an oven at 50 °C during 12 h. The reaction parameters of these processes (reaction conditions: one or multiple repeating steps, reaction time, temperature (80–120 °C), concentration and nature of alkaline reagent (aqueous NaOH or alcoholic KOH)) were varied.

$NaBH_4$ and thiophenol may be added (at [chitin]/[$NaBH_4$ or thiophenol] weight ratio equal to 1/1) during deacetylation process of chitin to prevent polymer degradation.

2.3. Physico-chemical characteristics of chitins and chitosans

The water content of the samples issued from the different reactions (extraction of chitin or deacetylation of chitin) was determined using a 92–12 thermogravimetric analyser from Setaram (France).

The potentiometric determination of the DA was carried out following the method given by Broussignac [35] and Muzzarelli [2]. The chitosan was dissolved in a known amount of acid (in excess). 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 NaOH solution volumes corresponding to these points corresponds to the acid consumed for the salification of amine groups and allows the determination of the DA of the chitosan. The titration was performed with a pH-meter Minisis 6000 from Radiometer (France). The DA was calculated from the relation:

$$\mathrm{DA} = \frac{1 - 161Q}{1 + 42Q},$$

where $Q = N\Delta v/m$, Δv is the volume of NaOH solution between the inflexion points (in l), N is the concentration of NaOH solution (in mol/l, in this paper equal to 0.1), and m is the dry weight of polymer sample (in g).

The DA was also calculated from ^{1}H NMR considered to be the most sensitive technique using an AC300 Bruker spectrometer [36]. The samples were dissolved at a concentration of 10 mg ml^{-1} in D_2O in presence of HCl (pH 4) and freeze-dried three times to exchange the 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 used as the internal standard. The DA values determined from NMR and titration were very close.

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 for quantitative analysis were tested on solid *N*-acetyl-D-glucosamine in order to check the exact ratio of 1/6, between the methyl carbon atom of the *N*-acetyl group and the six carbon 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 viscosity-average molecular weight of chitosans was calculated from the viscosity law [37]:

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

3. Results

3.1. Chitin isolation

One of the major problems related to the preparation of pure chitins keeping a structure as close as possible than the native form is to minimise the partial deacetylation and chain degradation caused by demineralization, deproteinization and bleaching applied during processing of the raw materials [3].

The isolation process we have finalised [33,34] is based on the use of small concentrations of acidic reagents (demineralization with 0.55 M hydrochloric acid) or basic ones (deproteinization with 0.3 M sodium hydroxide) and

Table 4
Demineralization step of the chitin isolation process: evolution of the mineral content determined by element analysis during successive acidic baths (0.55 M HCl)

Source		Ca (%)	Na (%)	P (%)	Mg (%)	K (%)	Total Content (%)
Lobster	Shells	30.54	0.42	0.90	2.03	0.10	33.99
	First bath	29.86	0.24	1.16	1.65	0.02	32.93
	Second bath	19.34	0.13	2.24	1.07	0.01	22.79
	Third bath	0.11	0.002	0.06	0.016	0.03	0.218
Spider crab	Shells	22.62	0.72	0.72	1.78	0.12	25.96
	First bath	20.50	0.41	1.10	1.49	0.46	23.96
	Second bath	0.083	<0.002	0.079	<0.002	0.002	<0.168
	Third bath	0.018	<0.002	0.08	<0.002	0.015	<0.117
Pink shrimp	Shells	18.17	0.80	1.26	0.91	0.36	21.50
	First bath	11.00	0.07	2.18	1.32	0.015	14.585
	Second bath	0.043	<0.002	0.16	0.018	0.003	0.226
	Third bath	0.035	0.003	0.15	0.001	0.001	0.185
Squilla	Shells	15.34	0.25	2.22	1.03	0.054	18.89
	First bath	10.13	0.069	3.6	0.017	0.59	14.4
	Second bath	0.0485	0.0185	0.064	0.002	0.012	0.145
	Third bath	0.006	<0.001	0.042	<0.001	<0.001	0.051
Squid	Pen	1.06	0.028	0.57	0.035	0.015	1.708
	First bath	0.005	<0.002	0.009	<0.002	0.0165	0.0345
	Second bath	0.0025	<0.002	0.005	<0.002	0.009	0.02

repeated baths every time the observation of the reaction medium shows that it was necessary (as given in Section 2). The choice of conditions which are very moderate compared with these used in other processes reported in literature [11, 35,38–46] (Table 2) considers in a first step the role of the source. Each species has a chitin which is associated to organic and inorganic matter. As an example, the dosage of major mineral elements present in the exoskeletons of the different chitin sources shows that the contents change with the species (Table 3) and hence processing conditions. It follows that it is not reasonable to apply the same treatment conditions (in terms of quantity of acid and duration of the purification) to sources with as large differences as squid pen (1.7%), squilla (18.9%) or lobster (34%). Nevertheless, that was the case in Kurita et al. work where chitin was isolated from squid pens or shrimp using the same reaction conditions [11]. One of the advantages of the isolation process we propose is to allow to adapt durations and quantities of purification agents to peculiarities of the source so as to they are neither excess nor insufficient because in these cases the quality of the extracted chitin will be affected.

The study of the evolution of the demineralization by titrating the residual minerals after each acidic bath shows for all samples that the proportion of eliminated minerals after the first bath is very small compared to that eliminated during following baths (Table 4). During the first bath, it may be assumed that the structure of the treated organism was modified to improve the accessibility of acid during further baths. The exhaustion of minerals becomes much easier after the first bath. This suggests strongly that the multi-stage process will be more efficient than the one

Table 5
Influence of the acid concentration and the treatment mode (successive steps or continuous process) on the efficiency of the demineralization step (example of spider crab, considered minerals: Ca, Na, K, P, Mg)

HCl concentration (M)	Treatment duration (h)	Residual mineral content (%)
	1 h 30 in three steps (15′ + 15′ + 1 h)	0.117
0.55	1.5	10.1
	3	8.48
	24	0.187
1.1	1.5	0.909
	3	0.331
	24	0.292
1.65	1.5	0.718
	3	0.593
	24	0.463

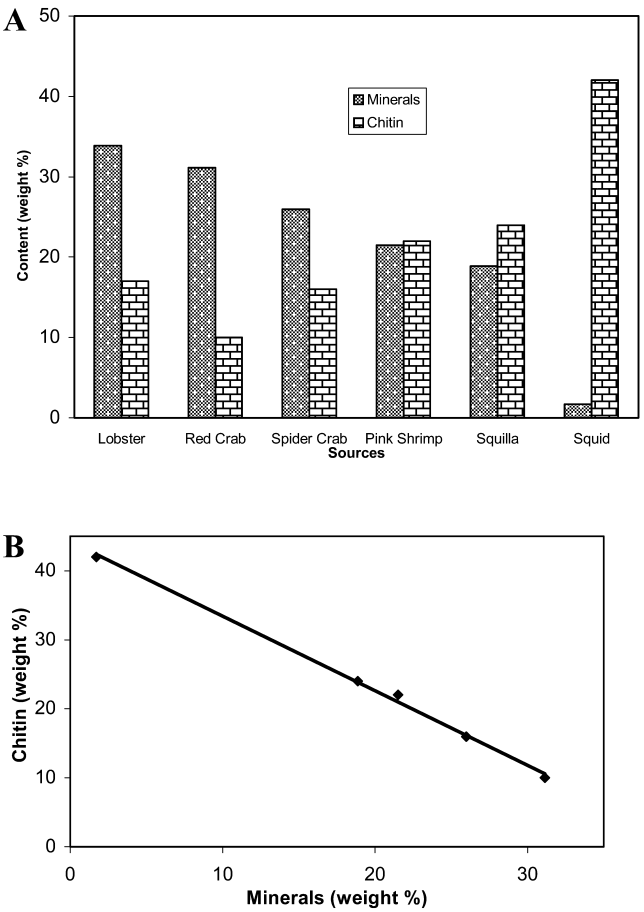

Fig. 2. Correlation between the chitin content and the mineral content (wt%). A. Relative contents in calcareous elements and in chitin from different sources. B. Variation of the chitin content as a function of mineral content.

carried out in one stage. Indeed, this assumption was confirmed by our results (Table 5), when the demineralization of spider crab shell was performed in the same concentration and time conditions according to a continuous treatment (1.5 h in one time instead of three steps). The elimination of minerals is distinctly smaller. By more concentrated acid solutions and larger reaction times, no improvement of the efficiency of the demineralization reaction was observed. The correlation between the harshness of acidic treatments and the efficiency of demineralization was not simple: the persistence of minerals during demineralization reactions would be related not only to concentration and duration effects (in relation with an optimal swelling of chitin) but also to the process according it is continuous or performed by stages. This observation may be enlarged to the deproteinization stage.

Table 6
Mineral content of chitins (standard chitin from SeaCure)

Chitin source	Ca (ppm)	Na (ppm)	P (ppm)	Mg (ppm)	K (ppm)	Total content (%)
Standard chitin	3900	810	1500	<10	<10	0.623
Pink shrimp	110	<20	<20	<20	36	0.020
Grey shrimp	150	<20	<20	<20	<20	0.019
Red crab	170	<20	<20	<20	<20	0.025
Spider crab	<20	<20	<20	<20	<60	0.014
Lobster	230	40	100	230	<20	0.062
Crayfish	1600	<20	<20	<20	<20	0.168
Squilla	64	<20	<20	<20	<20	0.014
Squid	<20	<20	<20	<20	90	0.017

Table 7
Determination of the degree of acetylation (DA) and the crystallographic morphism of chitin from different sources

Chitin source	Chitin form	DA (%)				
		From (RMN/CPMAS)	From microanalysis			
			C (%)	N (%)	N/C	DA (%)
Pink shrimp	α	100	44.74	6.36	0.142	100
Grey shrimp	α	100	44.82	6.42	0.143	100
Spider crab	α	96	43.95	6.55	0.149	91
Marbled crab	α	99	–	–	–	–
Red crab	α	97	43.86	6.50	0.148	94
Squilla	α	100	–	–	–	–
Anatife	α	100	–	–	–	–
Crayfish	α	100	45.00	6.53	0.145	100
Squid	β	100	–	– –	–	–

The application of this isolation process to various sources has allowed us to determine the chitin content (Table 1) and notice a possible correlation between this chitin content and hardness of exoskeletons from which it was isolated. This chitin content is all the more so small since the exoskeleton is hard. The hardness of shells being in a large part attributed to their content in calcareous elements, we have studied this correlation and the chitin content was demonstrated to be all the more so small since the calcium content was high (Fig. 2, the correlation coefficient being 0.9987).

The content of residual mineral elements of the different chitins isolated according to our process was very small. It is of the order of some tens of micrograms per gram (ppm) for most of prepared and analysed chitins, when it is around 1.5% for chitins prepared according other processes [47,48]. In the case of commercial chitin, considered as pure, the obtained content was 0.6%. This allows to conclude that the demineralization process we have performed is efficient and supplies chitin without residual minerals (Table 6). Moreover, RMN-CP MAS spectra demonstrate that prepared chitins are pure (no presence of peaks from organic compounds was detected) and keep a high DA (Fig. 3). In many cases it is close to 100% (Table 7) and confirmed by elemental analysis.

These results are very interesting because, at our knowledge, such values of DA were never obtained, the different papers on this subject give data between 80 and 90% [3,11,49]. The rare works to prepare a completely acetylated chitin come from Kurita et al. [50] and Hirano

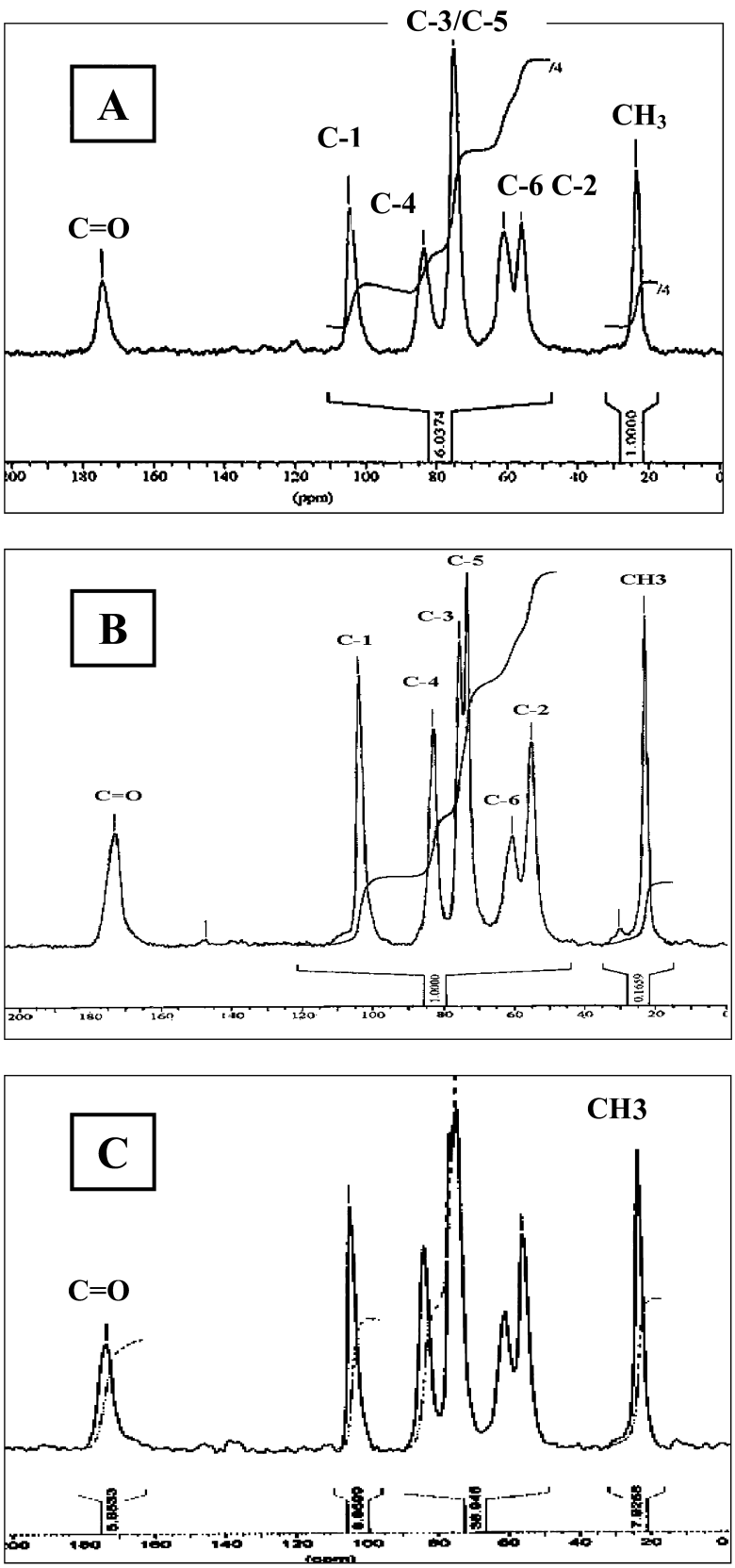

Fig. 3. Solid state ^{13}C NMR. A. β-Chitin isolated from squid pen. B. α-Chitin isolated from shrimp. C. α-Chitin isolated from squilla.

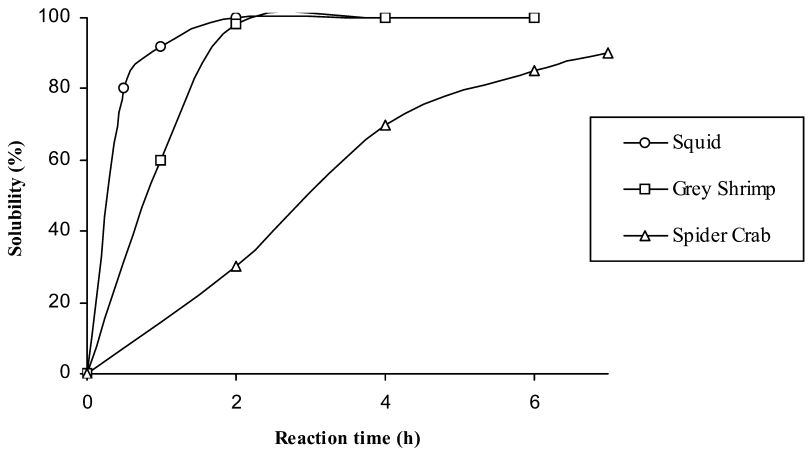

Fig. 4. Influence of the source on the solubility in the 5% acetic acid solution of chitosan from chitin deacetylation.

et al. [51]. But they prepare such chitins from *N*-acetylation reactions of chitosan and not from production processes. The chitin prepared from reacetylation of chitosan does not keep its original physical structure (nothing is indicated in these papers), and the average molar mass of this chitin was affected by the conditions used for chitin isolation and its deacetylation during preparation of chitosan. Moreover the *N*-acetylation of chitosan was accompanied by the esterification of alcohol groups. Their hydrolysis needs to use an alkaline treatment which causes a partial hydrolysis of *N*-acetyl functions which, even limited, reduces the possibility to produce chitosan completely reacetylated. To find a solution to the drawbacks observed during the *N*-acetylation of chitosan, Kurita et al. have attempted to process using the *N*-acetylation of chitin [12]. They conclude that this approach allows to succeed only when β-chitin was used. The *N*-acetylation of α-chitin, which is the most usual form, is not feasible in used reaction conditions.

From the comparison of our results with those obtained elsewhere it is clear that our process allows to prepare chitin which is highly acetylated, while being applicable to α-chitin or β-chitin, preserving original crystalline structure, providing degrees of acetylation as close as possible to 100% without modifying other characteristics as purity or molar mass. This is due to the fact that chitin isolated from

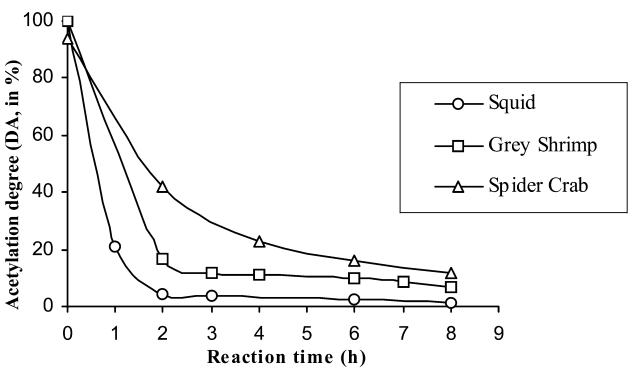

Fig. 5. Influence of the chitin source on the evolution of the deacetylation reaction performed according to the Broussignac process.

used sources is fully acetylated. But native chitin from other organisms may be not fully acetylated, and in this case the proposed process keeps its advantages because it does not modify the physico-chemical characteristics of original chitin (molecular weight and DA).

3.2. Preparation of chitosans with controlled characteristics

3.2.1. Role of the source

In a previous work, we have demonstrated the influence of the source on the viscosity-average molar mass of chitosans [34]. The source has also an influence on the evolution of the *N*-deacetylation reactions of chitins and the DA of resulting chitosans. When chitins from different sources are submitted to *N*-deacetylation using Broussignac process [35], the minimal duration necessary to obtain preparations soluble in 5% acetic acid depends upon the chitin source. A reaction time of 1 h is sufficient in the case of β-chitin from cuttlefish bone or squid pen. In the case of α-chitin the minimal time is around 2 h for chitin from grey shrimp and squilla and from 3 to 4 h for chitin from prawn, pink shrimp and crayfish. Regarding chitin from lobster, red crab or spider crab, a product which is well soluble in 5% acetic acid (AcOH) solution was obtained only after 6 or 7 h of treatment.

The results obtained in the case of α-chitin allow to notice that the minimal duration time necessary to reach a product soluble in 5% acetic acid solution is enhanced when the shell from the source organism is hard (Fig. 4).

We have studied the evolution of the deacetylation reaction during the first 8 h for chitins from squid pen, grey shrimp and spider crab. The evolution of the DA as a function of the reaction time (Fig. 5) shows a behaviour which differs, particularly during the first 2 h, depending on the source of chitin. For the same reaction time of 2 h, the deacetylation efficiency is around of 95% for the chitin from squid pens, 83.5% for grey shrimp and only 55% for chitin from spider crab.

Table 8
Influence of the deacetylation process on physico-chemical characteristics of obtained chitosan

Chitin source	Reaction time (h)	KOH 50%, 120 °C[a]		NaOH 50%, 120 °C[a]	
		DA (%)	M_v[b] (g/mol)	DA (%)	M_v[b] (g/mol)
Squilla	4	9.2	150,000	47	Insoluble
	10	5.7	126,000	4.2	23,000
Grey shrimp	4	10.5	127,000	51[c]	Insoluble
	6	9.9	112,000	39[c]	Insoluble
	7	8.8	111,000	28[c]	Insoluble
	12	6.1	92,600	3	45,000

[a] A mixture of solid potassium hydroxide (50 w/w%), 96% ethanol (25 w/w%) and monoethylene glycol (25 w/w%) or aqueous sodium hydroxide solution (50% by weight).
[b] The viscosity-average molar mass was determined at 25 °C using as solvent: 0.3 M AcOH/0.2 M AcONa.
[c] DA determined from acido-basic titration; the other values were determined from ^{1}H NMR.

3.2.2. Role of the N-deacetylation process

In our previous works [33,34,52], we have studied the preparation of chitosan using two processes, the Broussignac process with potassium hydroxide (KOH) in an anhydrous medium or the Kurita process with sodium hydroxide (NaOH) in aqueous medium. In case of β-chitin from squid pen, the *N*-deacetylation with sodium hydroxide in aqueous medium was performed faster and chitosan samples with low DA and high molecular weights were obtained at more moderate conditions (NaOH 40%, $T = 80$ °C) as compared to Broussignac process. However, when α-chitin from crustacean shells was used the opposite behaviour was observed: the Broussignac process presents the advantage to provide chitosan with better quality (higher molecular weight and smaller DA) in only one short stage (2 h for shrimp, 4 h for squilla and 7–8 h for spider crab) when the Kurita process does not allow to reach a large deacetylation (in the same concentration (50% KOH) and temperature (120 °C) conditions) without larger reaction time and a larger degradation of the polysaccharide chain (Table 8). These results are unexpected because in the literature the Kurita procedure was considered as the most common process and reaction conditions are clearly more moderate [39,49,53–59]. We have indeed examined a large number of combinations of these reaction conditions used in so-called typical processes using NaOH concentrations from 40 to 50%, temperatures from 80 to 100 °C and reaction times from 6 to 12 h with α-chitins totally acetylated from shrimp or squilla (as demonstrated previously [52], see also Table 8). The following of the reactions shows unambiguously that such conditions do not allow to reach well soluble product in 5% AcOH solution. The DA values determined from potentiometric titration for aliquots taken off between 6 and 10 h are generally larger than 30%. We have strong reasons to think that such conditions were applied to β-chitins or to the deacetylation of low acetylated chitosans but they cannot be generalised to α-chitins which are initially largely acetylated. When the deacetylation reaction in these two media was considered, the observed differences may be related to:

- the larger solubility of NaOH in aqueous media compared to KOH in ethanol/monoethylene glycol mixture. The dielectric constant of water was equal to 80.1 at 298 K and to 25.3 in ethanol or 41.4 in monoethylene glycol,
- the development of ionic charges which appear in the transition state of the deacetylation reaction of which first step would be a nucleophilic addition of amide function on carboxyl group. This development of charges was favoured by the high value of the dielectric constant of NaOH aqueous solution compared to anhydrous KOH one.

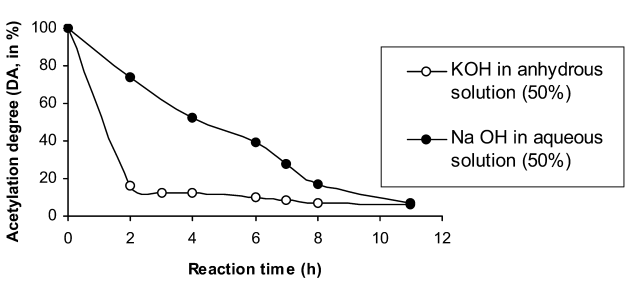

Fig. 6. Influence of the process on the evolution of deacetylation of α-chitin (from grey shrimp).

To put in evidence the behaviour of α-chitin towards sodium hydroxide we have followed the deacetylation reaction of α-chitin during the first 10 h comparing the two used processes. Results concerning the shrimp, for example, show that it is obvious that the deacetylation with aqueous NaOH was very slow for these reaction times (Fig. 6). A complete solubility in 5% AcOH solution and a DA of 16% were obtained after 2 h when anhydrous KOH was used and similar values are obtained after only 8 h with aqueous NaOH process. For higher reaction times using Kurita process, a high deacetylation was observed at the expense of the molecular weight of chitin and its quality. All the samples are coloured and molecular weight was five times smaller than those obtained with Broussignac process for similar reaction times. The reactional behaviour of α-chitin in NaOH aqueous media reveals two phases: a first one (during the first 10 h) during which the deacetylation is very slow and then a second one (for reaction times larger than 10 h) where deacetylation and degradation are very quick. A DA as low as obtained with the Broussignac process was obtained with Kurita process after only 11 h. It may be suggested that the large and quick deacetylation observed during the second phase (in NaOH aqueous media) is preceded by morphological modifications of α-chitin leading to amide groups being more accessible to sodium hydroxide than in the first phase. The induction of such

Table 9
Preparation of chitosan with controlled characteristics. Influence of process and of the addition of $NaBH_4$ on the degree of acetylation and the viscosity-average molar mass

Original chitin	Deacetylation process				Acetylation degree (DA; %)	Intrinsic viscosity ([η]; ml/g)	Viscosity-average molar mass (M_v; g/mol)
	NaOH (w/w%)	T (°C)	Duration (h)	Addition $NaBH_4$			
β Chitin, squid pen, DA: 100%	40	80	9	−	17	1127	298,000
	40	80	3 h × 3	−	1	1675	500,000
	40	80	3 h × 3	+	0	2027	644,000
	40	80	6	−	20	1368	384,000
	40	80	3 h × 2	−	3	1906	590,000
	40	80	3 h × 2	+	5	2917	1040,000
α Chitin, grey shrimp, DA: 100%	50	120	12	−	3	269	45,000
	50	120	6 h × 2	−	3.9	430	84,000
	50	120	6 h × 2	+	1.8	679	153,000
	50	120	4 h × 3	+	1	658	147,000
	50	120	3 h × 4	+	0	667	149,000
	50	120	3 h × 3	+	1	801	190,000

+ : presence of $NaBH_4$; − : absence of $NaBH_4$.

modifications would be quicker in the ethanol–glycol mixture than in water and would be related to a different swelling in these two different media.

3.2.3. Effect of the introduction of $NaBH_4$ and thiophenol in the reaction medium

The literature data [3,59] show that the addition of sodium borohydride ($NaBH_4$) to the reaction medium ensures a protective action against the chain degradation. It reduces, at the end of the polysaccharide chain, the terminal aldehyde in alditol group. In the goal to improve the molecular weight of prepared chitosans we have studied the use of $NaBH_4$ when it is combined to the positive effect of the multi-stage process as previously demonstrated [33]. When the deacetylation reaction was performed in aqueous NaOH solution, the chitosan samples obtained from α-chitin (shrimp) or β-chitin (squid pen) are distinguished from these prepared without $NaBH_4$ by their white colour and their largest molecular weights. The combined effects of the multi-stage process and the addition of $NaBH_4$ allow an improvement of the characteristics with a ratio which may reach 300% (Table 9). We have particularly observed a very good quality of chitosan samples: they are completely deacetylated (Fig. 7) and have very large molecular weights.

But when the deacetylation was carried out with alcoholic KOH solution, the addition of $NaBH_4$ does not allow to improve the molecular weight of the obtained chitosan. The observed effect leads to be negative, particularly for the DA (Table 10). We have thought about thiophenol and supposed that its addition substituting $NaBH_4$ should be more adapted to an anhydrous deacetylation. The thiophenol, although not being a reducing agent, was mentioned by Rinaudo et al. [60] as being efficient to minimise the polymer degradation during deacetylation reactions with aqueous NaOH. The different reactions we proceed have not demonstrated that the addition of thiophenol in anhydrous KOH solution does improve the molecular weight of chitosans after deacetylation reactions. On the contrary samples with lower molecular weights and larger degrees of acetylation were obtained (Table 11).

3.2.4. Influence of the quality of original chitin and extraction process

In the literature there is a tendency to focus particularly on the deacetylation process, which leads to suppose that a given process, applied to a given chitin, may be generalised for every chitin without changing the reaction conditions and give the same result (considering molecular weight and

Table 10
Effect of the addition of $NaBH_4$ in the reaction medium when the chitin deacetylation was carried out using KOH in anhydrous solution according Broussignac process

Chitin source	KOH 50%; $T = 120$ °C		Acetylation degree (DA, %)	Viscosity-average molar mass (M_v, g/mol)
	Duration (h)	$NaBH_4$		
Grey shrimp (α chitin)	6	−	9.9	112,000
	6	+	15.4	108,000
Squid (β chitin)	1 h × 3	−	5	248,000
	1 h × 3	+	7.5	206,000

+ : presence of $NaBH_4$; − : absence of $NaBH_4$.

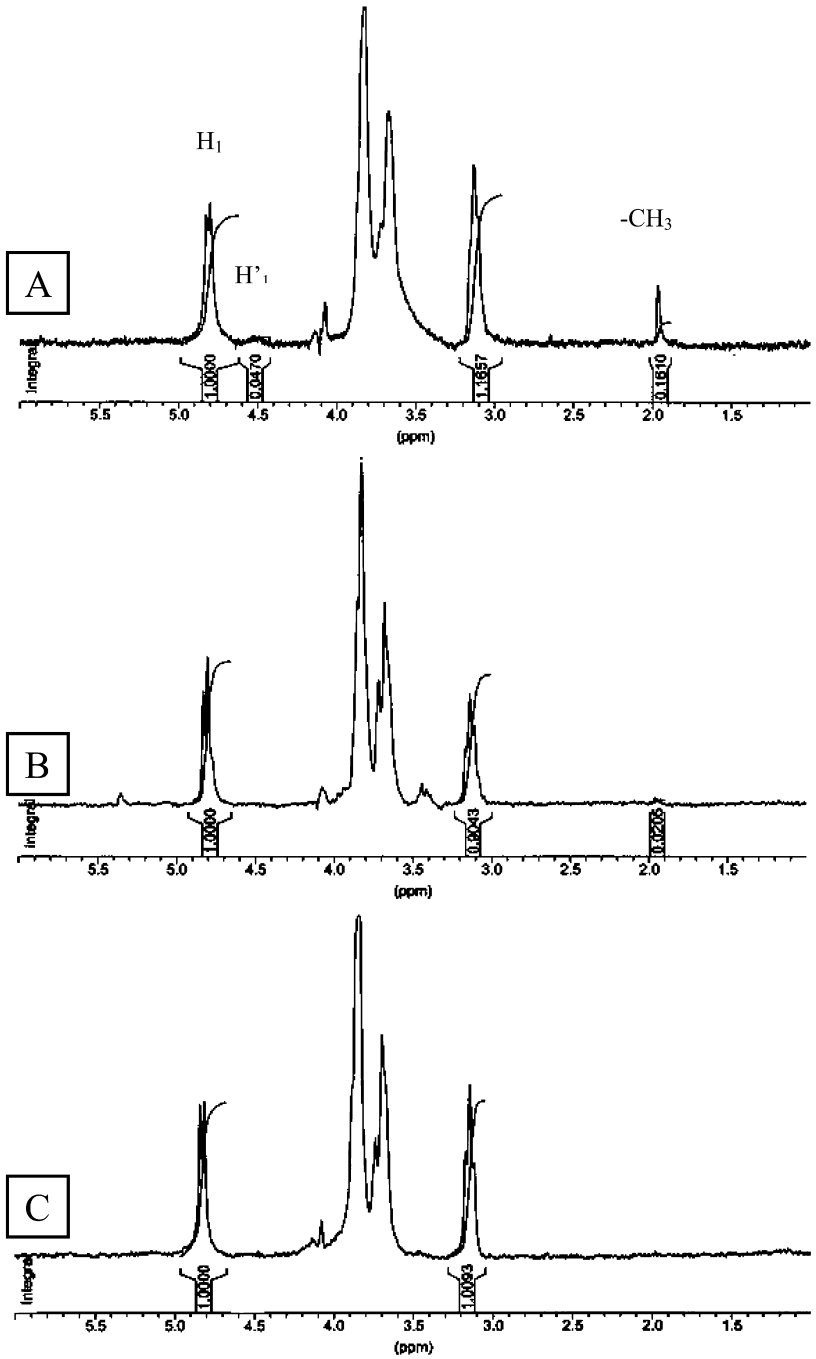

Fig. 7. A and B. ^{1}H NMR spectra of chitosans prepared from β-chitin (40% NaOH solution in presence of $NaBH_4$ under nitrogen atmosphere at 80 °C, 3 h × 2 (A, DA = 5%) and 3 h × 3 (B, DA = 0%), C: ^{1}H NMR spectra of chitosan prepared from α-chitin (50% NaOH solution in presence of $NaBH_4$ under nitrogen atmosphere at 120 °C, 3 h × 4 (DA = 0%)).

DA). We have shown that besides the process, the role of the source of chitin is essential and moreover its quality. As an example, when the deacetylation Kurita process was performed on β-chitin from squid pens isolated according to Kurita conditions the observed molecular weights were distinctly smaller than those obtained when chitin was isolated according our process (Table 12). The isolation conditions used by Kurita, too severe, affect the resulting chitin and it is normal that a decrease of the molar mass of the corresponding chitosan was observed.

Within the isolation process we have finalised, the influence of the order of the different steps related to the elimination of proteins and minerals on the quality of the isolated chitin and corresponding chitosan was studied. The obtained results when the demineralization was preceded by deproteinization allow to observe that concerning the chitin,

Table 11
Effect of the addition of thiophenol in the reaction medium when the chitin deacetylation was carried out using KOH in anhydrous solution according Broussignac process

Chitin source	KOH 50%; $T = 120$ °C		Acetylation degree (DA, %)	Viscosity-average molar mass (M_v, g/mol)
	Duration (h)	Thiophenol		
Squid (β chitin)	1 h × 3	−	5	248,000
	1 h × 3	+	8	180,000
	1 h × 2	−	5	29,000
	1 h × 2	+	9	266,000

+ : presence of thiophenol; − : absence of thiophenol.

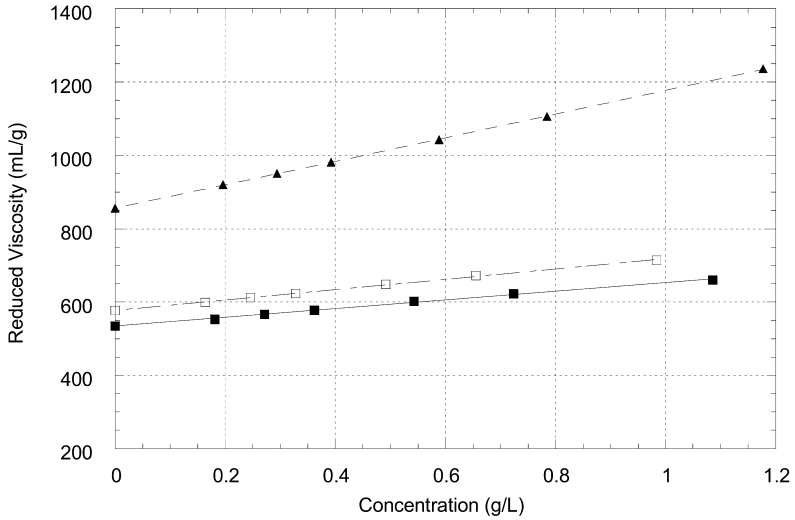

Fig. 8. Influence of the order of isolation and bleaching steps on the molar mass of chitosan reflected by intrinsic viscosity (example of grey shrimp). ■: demineralization, deproteinization then bleaching, $M_v = 111{,}000$ g/mol, DA = 9.9%; □: deproteinization, demineralization then bleaching, $M_v = 123{,}000$ g/mol, DA = 13%; ▲: demineralization, deproteinization, no bleaching, $M_v = 207{,}000$ g/mol, DA = 9.9%.

the residual content in mineral elements stays high when for the chitosan a slight increase of the molecular weight of the chitosan and a larger DA were observed. On the other hand, the pigment elimination step applied to β-chitins leads to chitosan with a molecular weight clearly higher than which obtained when chitin was first submitted to a bleaching step (Fig. 8). The average molecular weight is related with intrinsic viscosity by the Mark Houwink law ($[\eta] = KM^a$). The larger the intrinsic viscosity, the larger the molecular weight. In fact, we estimate that it is not necessary to proceed to bleaching when the extracted chitin will be used to prepare chitosans. This observation allows to think that it may be still possible to improve the physico-chemical characteristics of chitosans prepared from α-chitins.

4. Conclusion

As a synthesis of this work we have shown that the isolation process of chitin we have proposed presents the advantages to be adaptable to any source when native chitin is associated with minerals and/or proteins and to supply pure, white chitins which keep their original properties (molecular weight and DA). We may consider that the

Table 12
Role of the chitin isolation process on the chitosan characteristics

Duration of deacetylation with NaOH 40% w/w; 80 °C	Chitosan from chitin 1			Chitosan from chitin 2		
	DA (%)	$[\eta]$ (ml/g)	M_v (g/mol)	DA (%)	$[\eta]$ (ml/g)	M_v (g/mol)
3 h × 2	3	1906	590,000	4.5	1327	369,000
3 h × 3	3	1675	500,000	1	750	174,000

Chitin 1. Tolaimate et al. process: demineralization with 0.55 M HCl, 2 h × 2, ambient temperature; deproteinization: 0.3 M NaOH, $T = 80$ °C, 1 h × 2; chitin 2. Kurita et al. process: demineralization with 1 M HCl, one night, ambient temperature; deproteinization: 2 M NaOH, one night at ambient temperature then 4 h at 100 °C.

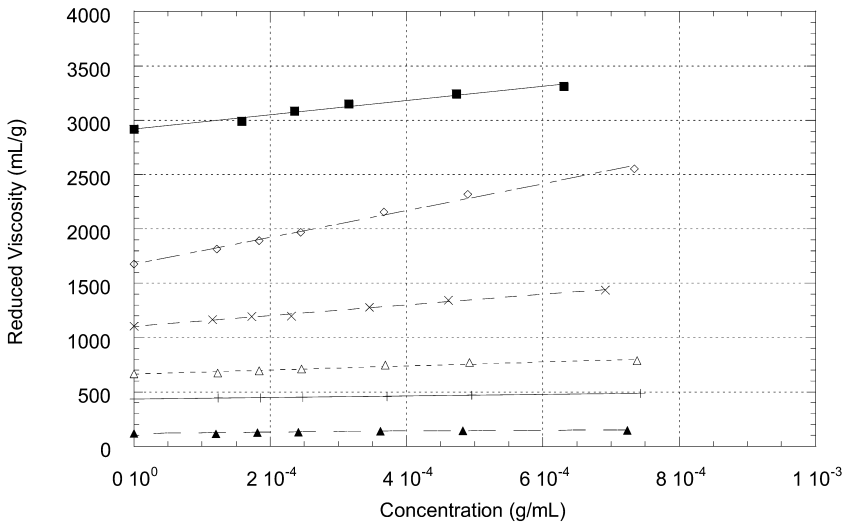

Fig. 9. Chitosans ($0.5 < DA < 6\%$) with controlled molar mass from 15,000 to 1,040,000 g/mol. ■: NaOH + $NaBH_4$; 3 h × 2; DA = 5%, M_v = 1,040,000 g/mol; ◇: NaOH; 3 h × 3; DA = 1%, M_v = 500,000 g/mol; ×: KOH; 1 h × 2; DA = 5%, M_v = 290,000 g/mol; Δ: KOH; 2 h; DA = 5%, M_v = 150,000 g/mol; +: KOH; 2 h × 3; DA = 1.6%; M_v = 85,000 g/mol; ▲: KOH; 24 h; DA = 0.5%, M_v = 15,000 g/mol.

prepared chitins with such a process are the purest ever obtained using an isolation process.

We have also studied the role of parameters which determine the quality of a chitosan sample. We have particularly shown that these characteristics, which have to be evaluated using exact and reliable methods, are influenced by many factors introduced at each stage of its preparation. Besides the different parameters related to the deacetylation process, the nature of the source, the physical structure of original chitin and its isolation process are also factors which determine the final characteristics of the chitosan sample.

The adjustment of these different factors allows to prepare chitosans with controlled physico-chemical characteristics either from α-chitins from crustaceous, or β-chitin from squid pens. We have prepared pure samples with a large range of molecular weight and DA.

But β-chitin, compared with α-chitin, allows to prepare a larger range of chitosan samples (Fig. 9). The experimental conditions to prepare completely deacetylated chitosan with large viscosity-average molar masses ($600{,}000-10^6$ g/mol) were determined which is very interesting in the objective to prepare highly deacetylated chitosan with low polymer degradation.

References

[1] Peter M. J Macromol Sci, Pure Appl Chem 1995;A32:629–40.
[2] Muzzarelli RAA. Chitin. New York: Pergamon Press; 1977.
[3] Roberts GAF. Chitin chemistry. London: Macmillan Press; 1992.
[4] Tracey MV. Rev Pure Appl Chem 1957;7:1.
[5] Conrad L. In: Chitin in Encycl. Polym. Sci. Technol., 1st ed., vol. 3, p. 695–705. 1964.
[6] Muzzarelli RAA. In: Chitin in Encycl. Polym. Sci. Technol., 2nd ed., vol. 3, p. 431–441. Wiley, New York, 1985.
[7] Jeuniaux C. In: Florkin M, Stotz E, editors. Comprehensive biochemistry. Amsterdam: Elsevier; 1971. p. 595.
[8] Richards AG. The integument of arthropods. Minneapolis: University of Minnesota Press; 1951.
[9] Minke R, Blakwell J. J Mol Biol 1978;120:167.
[10] Gardner KH, Blackwell J. Biopolymers 1975;14:1581.
[11] Kurita K, Tomita K, Tada T, Ishii S, Nishimura SI, Shimoda K. J Polym Sci, Part A: Polym Chem 1993;31:485.
[12] Kurita K, Ishii S, Tomita K, Nishimura SI, Shimoda K. J Polym Sci, Part A: Polym Chem 1994;32:1027.
[13] Kurita K, Tomita K, Ishii S, Nishimura SI, Shimoda K. J Polym Sci, Part: A Polym Chem 1993;31:2393.
[14] Sannan T, Kurita K, Iwakura Y. Makromol Chem 1976;177:3589.
[15] Lee VFP. PhD Thesis, Université de Washington, Seattle, 1974.
[16] Austin PE, Castle JE, Albisetti CJ. In: Skjak-Braek G, Anthonsen T, Sandford P, editors. Chitin and chitosan. Essex: Elsevier; 1989. p. 749.
[17] Brzeski MM. Chitin and chitosan-putting waste to good use. INFOFISH Int, Issue 5/87 1987;5:38–40.
[18] Rutherford FA, Dunson WA. In: Zikakis JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 135–43.
[19] Allan GG, Altman LC, Bensinger RE, Ghosh DK, Hirabayashi Y, Neogi AN, Neogi S. In: Zikakis JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 119–33.
[20] Pangburn SH, Trescony PV, Heller J. In: Zikakis JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 3–19.
[21] Gordon DT. In: Zikakis JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 97–117.
[22] Nagai T, Sawayanagi Y, Nambu N. In: Zikakis JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 21–39.
[23] Ember L. Chem Engng News 1997;15:26.
[24] Spreen KA, Zikakis JP, Austin PR. In: Zikakis JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 57–75.
[25] Shigemasa Y, Saito K, Sashiwa H, Saimoto H. Int J Biol Macromol 1994;16:43.
[26] Hutadilok N, Mochimasu T, Hisamori H, Hayashi K, Tachibana H, Ishii S, Hirano H. Carbohydr Res 1995;268:143.
[27] Nordtveit RJ, Varum KM, Smidsrod O. Carbohydr Polym 1996;29:163.
[28] Peluso G, Petillo O, Ranieri M, Santin M, Ambrosio L, Calabro D, Avallone B, Balsamo G. Biomaterials 1994;15:1215.

[29] Wang W, Bo S, Li S, Quin W. Int J Biol Macromol 1991;13:281.
[30] Errington N, Harding SE, Varum KM, Illum L. Int J Biol Macromol 1993;15:113.
[31] Varum KM, Anthonsen MW, Geasdalen H, Smidsrod O. Carbohydr Polym 1994;25:65.
[32] Zydowicz N, Vachoud L, Domard A. In: Domard A, Jeuniaux C, Muzzarelli R, Roberts E, editors. Advances in chitin science, vol. 1. Lyon: Andre J; 1996. p. 262.
[33] Tolaimate A, Desbrieres J, Rhazi M, Alagui A, Vincendon M, Vottero P. Polymer 2000;41:2463.
[34] Rhazi M, Desbrieres J, Tolaimate A, Alagui A, Vincendon M, Vottero P. Polym Int 2000;49:337.
[35] Broussignac P. Chim Ind Genie Chim 1968;99:1241.
[36] Rinaudo M, Milas M, Le Dung P, Int. J Biol Macromol 1992;14:121.
[37] Rinaudo M, Le Dung P, Gey C, Milas M. Int J Biol Macromol 1993; 15:281.
[38] Muzzarelli RAA, Tanfani F, Emanuelli M, Gentile S. J Appl Biochem 1980;2:380.
[39] Madhavan P, Ramachaudran Nair KG. Fishery Technol 1974;11:50.
[40] Anderson GG, De Pablo N, Romo CR. In: Allan GG, Fox JR, Kong N, Proceedings of First International Conference on Chitin and Chitosan, Muzzarelli RAA, Priser ER, editors, Mit Sea Grant Program, 78-7, 1978, p. 108.
[41] Mima S, Miya M, Iwamoto R, Yoshikawa S. In: Hirano S, Tokura S, editors. Chitin and chitosan. The Japanese Society of Chitin and Chitosan; 1982. p. 21.
[42] Shimahara K, Ohkouchi K, Ikeda M. Chitin chemistry. London: Macmillian Press; 1992. p. 56.
[43] Moorjani MN, Achuta V, Khasim DI. J Food Sci Technol 1975;12: 187.
[44] Whistler RL, BeMiller JN. J Org Chem 1962;27:1161.
[45] Hakman RH. Aust J Biol Sci 1954;7:168.
[46] Hakman RH, Goldberg M. Carbohydr Res 1974;38:35.
[47] Takeda M, Abe E. Norisho Susan Koshusho Kenkyu Hokoku 1964; 13:109.
[48] Takeda M, Abe E. Norisho Susan Koshusho Kenkyu Hokoku 1962; 11:339.
[49] Kurita K, Sannan T, Iwakura Y. Makromol Chem 1977;178:3197.
[50] Kurita K, Sannan T, Iwakura Y. Makromol Chem 1977;178:2595.
[51] Hirano S, Ohe Y, Ono H. Carbohydr Res 1976;47:315.
[52] Tolaimate A, Thesis, University Cadi Ayad, Marrakech, Morocco, 256, C2000.
[53] Muzzarelli RAA, Tanfani F, Emanuelli M, Gentile S. J Appl Biochem 1980;2:380.
[54] Wolform ML, Maher GG, Chany A. J Org Chem 1958;23:1990.
[55] Wolform ML, Shen Han TM. J Am Chem Soc 1959;81:1764.
[56] Batista I, Roberts GAF. Makromol Chem 1990;191:429.
[57] Roberts GAF. BP Appl 1987;8:704–19.
[58] Domard A, Rinaudo M. Int J Biol Macromol 1983;5:49.
[59] Gummow BD. PhD Dissertation, (CNAA), Nottingham Polytechnic, UK, 1984.
[60] Rinaudo M, Le Dung P, Milas M, Fr. Pat. 9302182, 1993.