Biomacromolecules **2007,** *8, 1942−1950*

Thermodynamic Aspects of the Heterogeneous Deacetylation of *β*-Chitin: Reaction Mechanisms

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Received January 8, 2007; Revised Manuscript Received March 20, 2007

This paper aims at giving a better understanding of the reaction mechanisms involved in the heterogeneous deacetylation of *β*-chitin in relation with the influence of soda concentration (30−55% (w/v)) and the type of sodium hydroxide hydrates formed in solution. The role of temperature (35−110 °C) and of the amount of sodium acetate generated in the reaction medium was also investigated. We demonstrated that the type of soda hydrate formed before deacetylation starts and its relative abundance drive the reaction efficiency. Thus, in the first part of this work, we evidenced that activation energies and the global reaction order associated to sodium hydroxide varied as a function of soda concentration. Therefore, we revealed that deacetylation efficiency was emphasized when the less hydrated soda was used, whereas anhydrous soda showed no or very low activity. We also pointed out that various parameters could be responsible for the progressive dehydration of the reaction medium, responsible for the transformation of the most reactive hydrates into less effective species. We underlined that this progressive dehydration could be caused by either one or all of the three following phenomena: alkaline hydrolysis of the polymer, the delivery of sodium acetate in the medium, and the evaporation of water when we process deacetylation at high temperatures and in open reactors. Beside kinetics reasons, we revealed that the transformation of soda hydrates as the deacetylation proceeded was also ascribable for the low reaction efficiency at long reaction times. Thanks to our investigations, we concluded that the amount of water present in the system chitin/soda/water/sodium acetate was the angle stone of complex equilibriums governing the reaction, and we propose soda mono- and dihydrates to be the most active reactants for the chitin deacetylation.

Introduction

Chitin and chitosan are linear copolymers of (1→4) linked 2-acetamido-2-deoxy-*β*-D-glucan (GlcNAc) and 2-amino-2-deoxy-*β*-D-glucan (GlcN). Although chitin is insoluble in both aqueous and usual organic solvents, once the copolymer becomes soluble in dilute acidic media, it is termed chitosan. Chitin exists according to two polymorphic forms named α and *β*. In the α-crystallographic cell, the chain segments are antiparallel inside a polymer sheet and the packing structure is strongly stabilized by hydrogen bonds in the three unit cell directions.[1] Since chain segments are parallel inside a polymer sheet in the *β*-chitin cell, there is no hydrogen bond between two successive chain segments.[2,3] Consequently, *β*-chitin exhibits a better reactivity,[4−7] swelling,[8,9] and solvation, both in organic solvents[10] and aqueous solutions, than α-chitin.

If chitin is the most abundant polysaccharide in biomass with cellulose, in contrast, chitosan only occurs in some cell walls of fungi[11] and, thus, is essentially produced from the chemical deacetylation of chitin. In contrast, chitosan exhibits more versatile biological and eliciting properties than chitin and finds numerous applications in agriculture,[12] biomedicine,[13−15] or food industry.[16,17] Since the bioactivity[18] of chitosan and its behavior in aqueous solution[19] strongly depend on its physicochemical properties, the control of the degree of deacetylation (DA), polymerization (DP), and the distribution of its repeating units during deacetylation become of a major interest in the development of this polysaccharide.

Unfortunately, in almost all cases, the industrial deacetylation of chitin is still mainly based on old procedures leading to polymers with uncontrolled characteristics. For example, the alkaline degradation of chitin occurring during deacetylation gives rise to highly polydisperse chitosans with unspecific properties. Among the methods proposed to deacetylate chitin, the chemical deacetylation in heterogeneous media involving strong alkaline solutions remains the most studied, especially at the industrial scale. However, the heterogeneous process has not much evolved for the past three decades. Particularly, the weak efficiency of the reaction at long reaction times is a major obstacle for the production of chitosan with low DAs and high molecular weights. Thanks to the calculation of the pre-exponential factor of the Arrhenius equation and assuming that deacetylation locally operates as a homogeneous reaction, we recently demonstrated that the crystalline mesh of *β*-chitin was more accessible than α-chitin and was responsible for its enhanced reactivity. Conversely, activation energies were found equivalent for both chitins, contradicting previous studies,[20,21] but it proved that the accessibility of the reactive sites (GlcNAc units) in the α- or *β*-crystalline domains drove both the reaction efficiency and the frequency factor of collision. However, in such severe conditions, chitin underwent an important hydrolysis, for which the reaction rate was strongly linked to the crystalline packing of the copolymer[7] and was temperature-dependent and oxygen-sensitive.[22] We succeeded to overcome these problems by performing freeze−pump out−thaw (FPT) cycles of the heterogeneous reaction medium before the deacetylation started. First, this technique highly decreased the oxygen concentration in the porous chitinous matrix and,

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10.1021/bm070021m CCC: $37.00

consequently, reduced the hydrolysis rate. Second, by acting as a thermomechanical piston, this process attacked the crystalline structure of the copolymer and allowed it to be permeated with the alkaline solution before starting the reaction. Subsequently, the polysaccharide was activated by the alkaline solution at the very beginning of the reaction, and its acetylated sites were fully accessible sooner. Compared to those reported in the literature, our process allowed us to reach lower DAs in shorter reaction times, to better prevent the copolymer from hydrolysis, and to produce statistical copolymers with lower polydispersity indexes.

However, if we demonstrated that using FPT cycles increased the deacetylation rates during the first minutes of reaction, the soda activity leveled off at a plateau after a few minutes and prevented chitin from a full deacetylation with reasonable molecular weights in a single reaction. This phenomenon was already observed,[23,24] but it remains the main stake to the development of chitin and chitosan. This obstacle is usually overcome by deacetylating chitin several times with intensive washings with water between two successive reactions. Because the reasons of the low deactylation efficiency at long reaction times were not clearly established until now, Roberts[25] claimed that the washing steps could either (a) decrease the soda concentration remaining in chitin particles, responsible for the inaccessibility to GlcNAc residues, or (b) eliminate poor reactive chitin−sodium hydrate complexes formed during the deacetylation. Unfortunately, no experiment validated these assumptions. Consequently, we already demonstrated the first hypothesis by evidencing that the accessibility of GlcNAc sites was one of the key parameters in the deacetylation effectiveness, and the present work first aims at studying the major role played by soda hydrates formed in solution with respect to soda concentration and temperature and their influence on activation energies and soda activity. In the second part, we evidence the role played by the progressive dehydration of the alkaline solution on the reaction efficiency and conclude with the general reaction schemes driving the overall process of the heterogeneous deacetylation.

Materials and Methods

Raw Materials: Preparation. As in a previous work,[6] β-chitin was extracted from squid *Loligo* pens provided by Mahtani Chitosan. Its water content was estimated by thermogravimetric analysis using a DuPont Instrument TGA 2000.

Thermodynamic Studies of the Heterogeneous Deacetylation. *(A) Influence of Soda Concentration and Temperature.* The roles of temperature and soda concentration were followed during a first deacetylation under argon atmosphere. β-Chitin was finely divided at ambient temperature and was introduced in a reinforced Schlenck tubelike reactor and was purged with argon and was preheated at the desired temperature. Kinetics of deacetylation was triggered when adding NaOH solutions (22.2 mL per g of chitin) of different concentrations. The role of soda concentration was studied at 90 °C for concentrations ranging between 20 and 55% (w/v) and that of temperature, within 35 and 110 °C, for two soda concentrations, 30 and 50% (w/v). All reactions were stopped by immersing the reactor in liquid nitrogen. Reaction media were then centrifuged, and supernatants were separated from the insoluble parts. The latter were extensively washed with deionized water at pH 8.5 (fixed with ammonia) until the conductivity reached that of water and then were analyzed.

(B) Influence of Sodium Acetate. To (a) improve the diffusion of the reactants and products through the chitin matrix, (b) allow the reaction to run in hermetic conditions, and (c) prevent the alkaline solution from loosing water by evaporation, the influence of sodium acetate was performed by means of freeze−pump−thaw (FPT) cycles with 50% (w/v) NaOH at 90 °C. Six FPT cycles were operated to completely degas the reaction medium and to guarantee the absence of dioxygen. After the last cycle, the reactors were filled with nitrogen. Reactions were then performed according to the same procedure as above. After the deacetylation was stopped and the reaction medium was centrifuged, 5 mL of the supernatants was filtered through 0.45 μm PTFE membranes and was injected in high-performance liquid chromatography (HPLC) to determine the amount of sodium acetate delivered during the reaction. HPLC was performed by means of a Lichrosorb C$_{18}$ pump (5 μm, 4.6 × 250 mm, L5C$_{18}$-25F 32705). The solutions were eluted with acetonitrile/water (4/1, v/v) at pH 2.2 and 30 °C at a flow rate of 1 mL/min and were detected with a UV detector at $\lambda = 210$ nm (reference at $\lambda = 500$ nm). Solutions of sodium acetate trihydrate (24.4−220 mM) in 50% (w/v) sodium hydroxide were prepared, were filtered twice on PTFE 0.45 μm membranes, and were used as standards. The amount of sodium acetate in the supernatant was calculated from peak areas. The remaining supernatants were dialyzed through membrane tubing (Spectra/Por Biotech CE, molecular-weight cut-off (MWCO): 500 g/mol) against water at pH 8.5 and insoluble fractions were washed with deionized water at pH 8.5. Soluble and insoluble fractions were finally lyophilized and characterized.

Table 1. DAs, Weight-Average Molecular Weights, Water Contents, and Conditions of Production of the Starting Materials[f]

product	DA (%)[26]	M_w (g/mol)	water content[e] (% w/w)
β-chitin	90.3 ± 1.9[a]	1 300 000[c]	8.5
1D/β	52.0 ± 0.1[b]	1 060 000[d]	9.56

[a] Determined by ^{1}H NMR spectroscopy in 20% (w/w) DCl/D$_2$O. [b] Determined by ^{1}H NMR spectroscopy in 20% (w/w) DCl/D$_2$O and D$_2$O (pD 3−4). [c] Determined by viscometry in DMAc/LiCl 5% (w/w) with $\alpha = 0.69$ and $K = 2.4 \times 10^{-4}$ L/g.[29] [d] Determined by SEC in the AcOH/AcONH$_4$ (pH 4.5) buffer. Errors on molecular weights did not exceed 5%. [e] Determined by thermogravimetric analysis. Errors did not exceed 1% of the value. [f] β-Chitin: chitin extracted from squid *Loligo* pens; 1D/β: chitosan deacetylated from β-chitin during 45 min at 90 °C with 50% (w/v) NaOH.

Determination of DAs and Molecular Weights. Depending on their apparent solubility in deuterated solvents, DAs of the samples were determined either by liquid ^{1}H NMR or solid-state ^{13}C CP/MAS NMR spectroscopies, following the procedures developed in previous studies.[6] For both liquid and solid NMR spectroscopies, DA was calculated as proposed by Hirai et al.[26] According to previous papers,[6,7,22] size-exclusion chromatography was performed on chitosan samples whenever they showed good solubility in the AcOH (0.2 M)/AcONH$_4$ (0.15 M) buffer (pH 4.5). The refractive index increment d*n*/d*c* ranged from 0.183 to 0.190 cm^3·g^{-1} according to the DA.[27] When the samples were not soluble in the buffer, they were reacetylated by means of acetic anhydride in a hydroalcoholic medium and were neutralized and washed with water (pH 8.5).[28] Their molecular weights were subsequently determined by viscometry in *N*,*N*-dimethylacetamide/lithium chloride (95/5%, w/w), using a Viscologic TI 1 SEMATech viscometer operating at 25 ± 0.1 °C, with $\alpha = 0.69$ and $K = 2.4 \times 10^{-4}$ L/g as Mark−Houwink−Kuhn−Sakurada (MHKS) constants.[29]

Results and Discussion

Influence of Soda Concentration and Temperature. The influence of temperature and sodium hydroxide concentration on the deacetylation was performed on a β-chitin extracted from squid pens exhibiting the characteristics reported in Table 1, first line.

To follow the influence of sodium acetate on the deacetylation, β-chitin was initially deacetylated by means of FPT cycles with 50% (w/v) NaOH at 90 °C for 45 min to work on a chitosan exhibiting a great solubility in mild acidic aqueous solutions

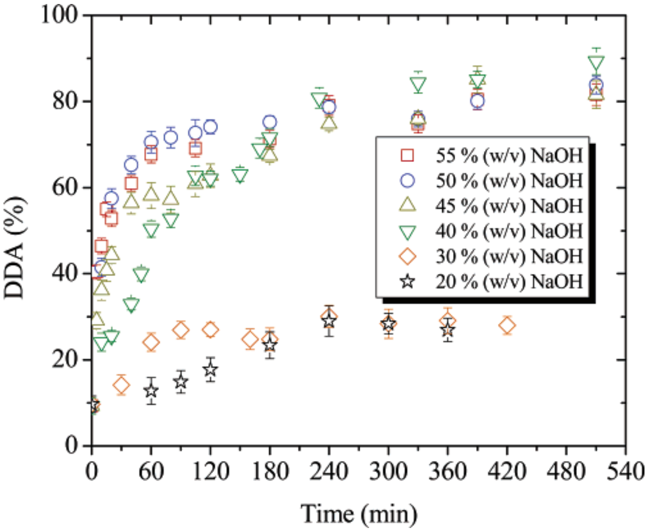


Figure 1. Heterogeneous deacetylation of β-chitin at 90 °C with 20−55% (w/v) NaOH. DDA (%) = 100 − DA (%). Error bars represent the standard deviations over three different experiments.

without extensive hydrolysis (Table 1, second line). Only the insoluble fraction recovered after the first deacetylation was subjected to further reactions. β-Chitin was preferred to the more common form α-chitin for its weaker network of hydrogen bonds. Indeed, we previously demonstrated that β-chitin particles were more easily swollen by the alkaline solution thanks to its more open crystalline mesh.[6] Subsequently, it became more rapidly amorphous, and deacetylation was more efficient and led to more homogeneous samples in terms of DA and DP. The rapid swelling of β-chitin under the action of soda associated with a fast transformation in an amorphous form allowed us to consider deacetylation to behave locally like a homogeneous reaction and to use the related kinetics equations without introducing noticeable errors in the calculations. This assumption only requires β-chitin to be rapidly swollen by soda when the reaction starts. In the case of concentrated soda, the deacetylating solutions remained fluidic at high temperatures and ensured β-chitin to swell very quickly. Just the opposite, at temperature where soda became more viscous, β-chitin was milled before starting the deacetylation. Thus, the time constant associated with the swelling of β-chitin under the action of soda is negligible compared to the time scale of our experiments and cannot influence our results by delaying the kinetics. This issue is totally solved in the case of FPT cycles since we previously evidenced this technique to swell indifferently α,β-chitins and chitosan with soda before the reaction started.

As shown in Figure 1, the first studies on the influence of soda concentration were operated at 90 °C, in a range of concentrations located within 20−55% (w/v). Only the insoluble fractions recovered after deacetylations were considered and analyzed since soluble and insoluble fractions were deacetylated at the same rate in the case of β-chitin.[6] The calculated activation energies subsequently do not depend on how much material is soluble in the reaction mixture, and the degrees of deacetylation (DDAs) of the insoluble fractions were each time taken as representative for the whole sample.

At 90 °C, 40% (w/v) NaOH was required to rapidly transform β-chitin into chitosan, which confirms the previous claims of Chang et al.,[21] even if lower concentrations could be used for slower deacetylations. According to the observed behaviors, 30 and 50% (w/v) NaOH were chosen as representative soda concentrations to follow the influence of temperature upon deacetylation (Figure 2).

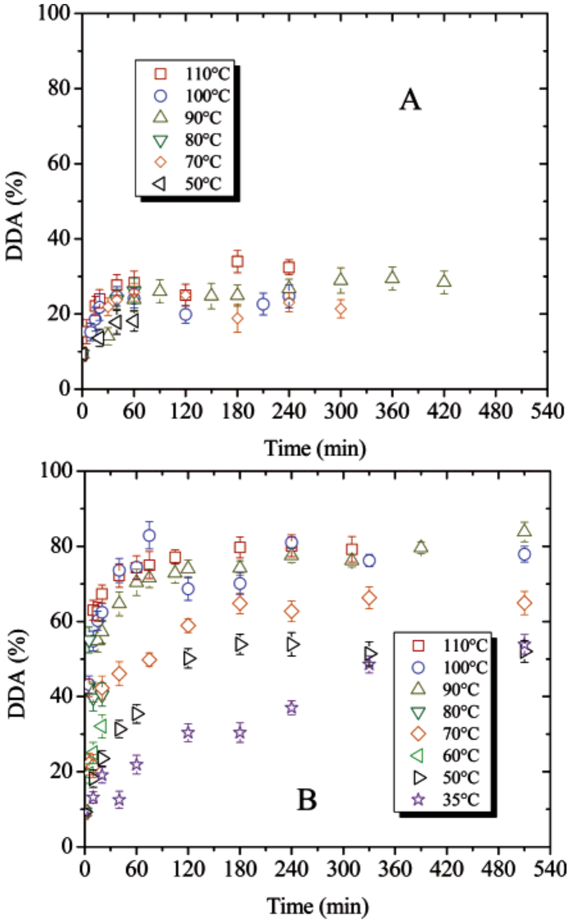
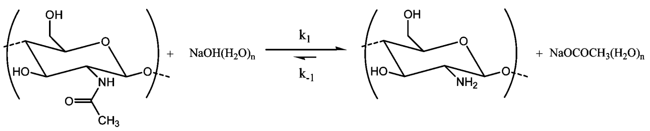


Figure 2. Heterogeneous deacetylation of β-chitin at temperatures within the 35−110 °C range, with (A) 30 and (B) 50% (w/v) NaOH. Error bars represent the standard deviations over three different experiments.

Scheme 1. Deacetylation of GlcNAc Units by Soda Hydrates; $n \geq 0$



Whatever the temperature of the reaction within 35−110 °C, we observed the same trend as in Figure 1. Heterogeneous reactions with 30% (w/v) NaOH led to a weak deacetylation and water-insoluble materials, even after several hours. According to Figures 1 and 2, the critical factor in favor of an efficient deacetylation was the soda concentration, whereas temperature seemed to play a secondary role on the reaction rate. Moreover, the plateau reached at the end of the reaction using 30% (w/v) NaOH seemed to be independent of the temperature at which the deacetylation was performed. On the other hand, deacetylation with 50% (w/v) NaOH exhibited two different behaviors whether the temperature was below or above 60 °C, if we consider the slope of the curves within the time range 0−60 min. According to Scheme 1, one can consider that kinetics are driven by eq 1:

$$-\frac{\mathrm{d[GlcNAc]}}{\mathrm{d}t} = k[\mathrm{NaOH}]^{a}\,[\mathrm{GlcNAc}]^{b} \qquad (1)$$

where [GlcNAc] is the concentration of the acetylated residues remaining in the polymer chain during deacetylation and is calculated from the DA.

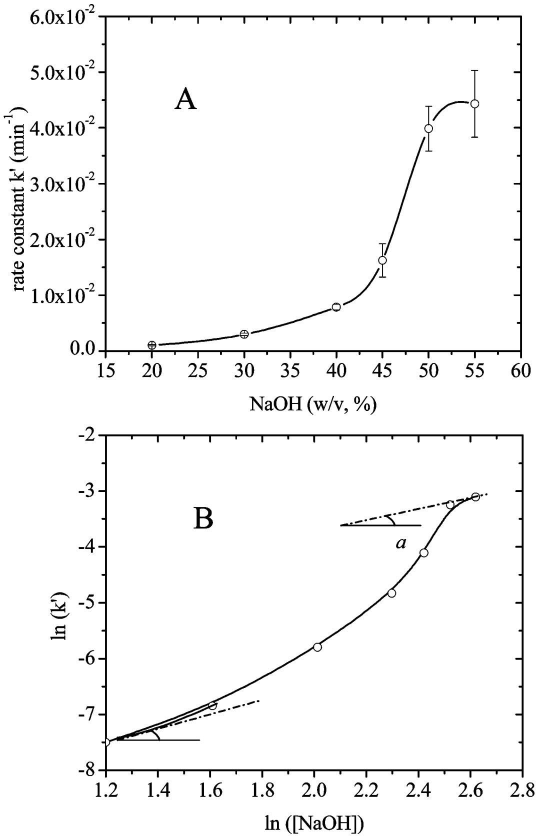


Figure 3. Heterogeneous deacetylation of β-chitin at 90 °C: (A) evolution of the rate constant k' as a function of the soda concentration and (B) determination of the reaction order a of soda by means of the slop of ln $k' = f(\ln([\mathrm{NaOH}]))$.

Considering that soda is in large excess all along the reaction ($k_1 >> k_{-1} \approx 0$), and the reaction order regarding [GlcNAc] is 1, eq 1 becomes:

$$-\frac{\mathrm{d[GlcNAc]}}{\mathrm{d}t} = k'[\mathrm{GlcNAc}] \tag{2}$$

In the first stages of the deacetylation, ln([GlcNAc]) plotted versus time exhibited a linear behavior for each soda concentration and temperature, thus confirming the hypothesis that $b = 1$ (data not shown). The reaction order regarding [NaOH], namely a, was also calculated at 90 °C from eq 3 with the variation of k' as a function of the soda concentration (Figure 3A) and plotting ln k' versus ln([NaOH]) (Figure 3B).

$$\ln k' = \ln k + a \ln([\mathrm{NaOH}]) \tag{3}$$

Figure 3A shows that the deacetylation at 90 °C became more and more efficient above a soda concentration of about 40% (w/v), but this trend leveled off when reaching 50% (w/v). The first derivative of the curve shown in Figure 3B gives a values ranging from 3 to 7 for intermediate soda concentrations, with low and high limit values near 1.5 and 1.1 for 20 and 50−55% (w/v) NaOH, respectively. To our knowledge, this last result is the first experimental confirmation that deacetylation is a first-order reaction toward both [GlcNAc] and [NaOH] whenever the soda concentration exceeds 50% (w/v). Moreover, the variation of a also suggested that different species, that is, soda hydrates, in addition to the anhydrous form had to be considered with their own activity:

$$[\mathrm{NaOH}]^a = \sum_{i=0}^{n} x_i[\mathrm{NaOH(H_2O)}_i]^{a_i} \tag{4}$$

where x_i and a_i are the molar ratio and reaction order of the i soda hydrate, respectively, and n is the number of hydrates in solution. Although there are infinite solutions to this equation, if the hypothesis above is correct, the activation energy of deacetylation should also be dependent on soda concentration and on the type of soda hydrate formed. Values of k' were easily calculated from the slopes obtained in Figure 2 and then were plotted against temperature (Figure 4).

For each soda concentration, k' plots show two ranges of variation below and above a critical temperature (90 and 70 °C for 30 and 50% (w/v) NaOH, respectively) suggesting different activation energies. However, the determination of activation energies by means of the Arrhenius relation necessarily required the pre-exponential factor A to be constant in the temperature range considered.

$$k' = A\mathrm{e}^{-(E_a/RT)} \tag{5}$$

$$A = Z \times p \tag{6}$$

A is usually shared into a collision factor Z and a steric parameter p, which represent the proportion of reactant having the required kinetic energy to overcome the activation energy and the proportion of reactant having the right orientation to become activated species, respectively. If Z corresponds to the probability for a reactant to access a reactive site, A becomes very sensitive to the number of acetylated groups accessible by soda.[6,7] As claimed above, the crystalline mesh of β-chitin was sufficiently open to allow soda to swell the porous material and to make the majority of the acetylated residues accessible to undergo deacetylation. The decrease of the crystallinity ratio of β-chitin was subsequently not a factor notably influencing A, which can be then considered as constant all along the reaction. ln $k' = f(1/T)$ was then plotted in Figure 5.

We obtained three reliable activation energies for the heterogeneous deacetylation of β-chitin: 80.3 ± 9.6 kJ·mol^{-1} when using 30% (w/v) NaOH and 71 ± 4 and 40 ± 3 kJ·mol^{-1} with 50% (w/v) NaOH, for temperatures below and above 70 °C, respectively. The activation energy below 90 °C corresponding to the deacetylation with 30% (w/v) NaOH could not be determined because of important uncertainties in the calculations. The value calculated for 50% (w/v) NaOH at temperatures over 90 °C is very close to that determined in our previous studies ($E_a = 42.8 \pm 1.8$ kJ·mol^{-1}),[6] when we took the DA of the insoluble and soluble materials into account in the calculation of the activation energies. As above-mentioned, deacetylation and alkaline hydrolysis of β-chitin can be considered as being locally homogeneous since the porous material rapidly swells under the action of soda and becomes amorphous after a few minutes of reaction.[6] The insoluble and soluble fractions we recovered after deacetylation usually exhibit the same DDA and consequently show the same kinetics plots. Then, we decided to work on the insoluble fraction only, considered to be representative of the whole sample. If this assumption had no noticeable effect on the determination of activation energies, it had a dramatic influence on the determination of A, which quantitatively depends on how much material underwent the reaction. The collision factor Z in eq 6 was then surely underestimated, and this explained why ln $A = \lim_{1/T\to 0}(\ln k')$ shifted to lower values. This observation strongly justifies our

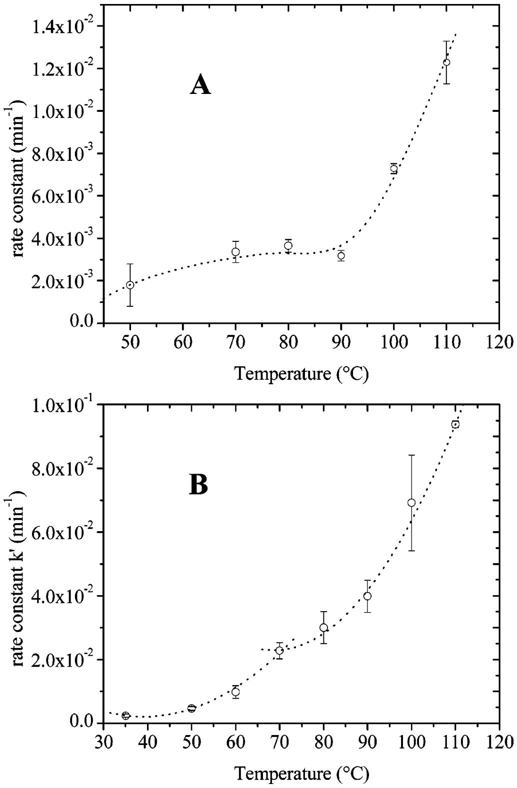


Figure 4. Evolution of the rate constant *k*′ as a function of temperature during heterogeneous deacetylation of β-chitin with (A) 30 and (B) 50% (w/v) NaOH.

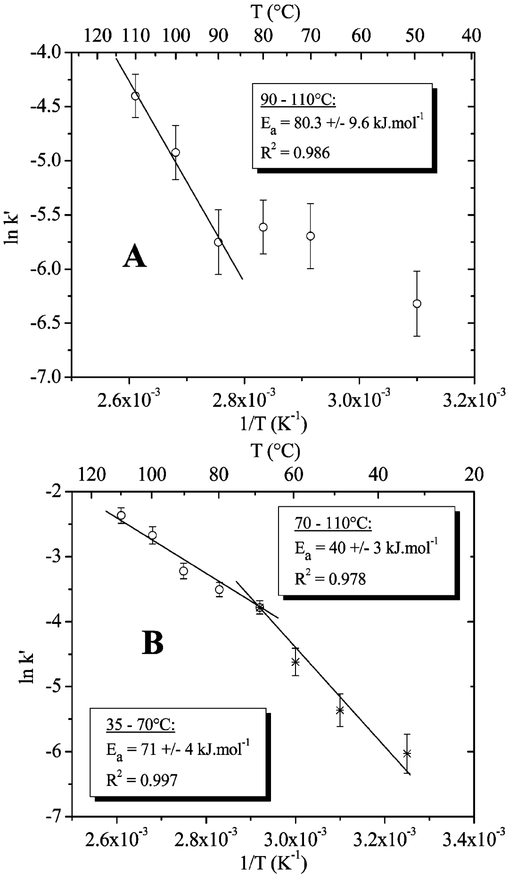


Figure 5. Determination of the activation energies for the heterogeneous deacetylation of β-chitin using a soda concentration of (A) 30 and (B) 50% (w/v).

choice to consider both soluble and insoluble materials in our previous studies when evaluating chitin reactivity toward deacetylation. The activation energies of the other soda concentrations were also calculated according to the same procedures as for 30 and 50% (w/v) NaOH in the 90−110 °C range. The plot $E_a = f([\mathrm{NaOH}])$ clearly revealed a decrease of E_a with an increasing soda concentration in the 20−50% (w/v) range before reaching a constant plateau for 50−55% (w/v) (data not shown). This particularly reveals that deaceylation gets more and more effective as soda concentration increases but also that sodium hydroxide reaches its highest global activity near 50% (w/v). Higher concentrations reduce the global activity of soda. According to this decisive result, 50% (w/v) NaOH seems to be the concentration of choice to deacetylate chitin in the most efficient way: the use of a higher soda concentration would lead to increasing the alkaline hydrolysis rate without being more efficient in terms of DA.

The existence of several activation energies together with the important variation of *a* suggested the involvement of different reactants according to the soda concentration used. Indeed, it has been well-known for a long time that sodium hydroxide may exist in the form of different hydrates according to its concentration and temperature.[30,31] With respect to soda concentrations used in this study, the composition diagram of sodium hydroxide hydrates reveals that before the reaction starts, only sodium mono- and dihydrates should form for NaOH ≥ 50% (w/v), whereas the more hydrated structures observed at lower concentrations all melt above 10 °C. Thus, differences in activation energies and reaction order observed for 30 and 50% (w/v) NaOH shall be the consequence of the different sodium hydroxide hydrates formed before raising temperature. Moreover, as for cellulose, chitin has been shown to strongly interact with mildly concentrated soda by coordinating one or two of its hydroxyl groups to soda hydrates, assuming hydrogen bonds interactions.[32] According to these two phenomena, and taking into account that the reaction order *a* dropped when we used more and more concentrated soda, we strongly suggest that $\mathrm{NaOH(H_2O)}_n$, with $n = 1$ or 2, are the hydrates showing the greatest affinity toward chitin and form the most reactive complexes regarding the deacetylation (Scheme 1). Regarding eq 4, if the activity of the hydrates with $n > 2$ exhibits low activity (i.e., $a_{i>2}$ tends to zero), any parameter in favor of the formation of soda mono- and dihydrates (increase of $x_{i\leq 2}$) should enhance the deacetylation efficiency (increase of $[\mathrm{NaOH}]^a$).

However, at high soda concentrations, the structure of the formed sodium hydroxides, their complexes with the polymer chains, and their respective proportions become extremely sensitive to the amount of water present in the reaction medium. If this assumption is correct, one shall prevent the system from losing water to keep constant the proportion of soda mono- and dihydrates present in the reaction medium. Unfortunately, during deacetylation, the amount of water may slightly decrease because of the combined action of three phenomena. First, water evaporation when deacetylating at high temperatures is mainly responsible for losing water. Second, the sodium acetate delivered during the process can also be responsible for a drift of soda concentration by complexing three equivalents of water per acetate molecule.[33] Third, the alkaline hydrolysis, which consumes one water molecule per chain scission, can also influence the structure of soda hydrates and their complexes. If we may easily prevent the reaction medium from the evaporation of water by processing deacetylation in a sealed reactor, we aimed at pointing out if the relative influences of sodium acetate and alkaline hydrolysis during deacetylation could affect the

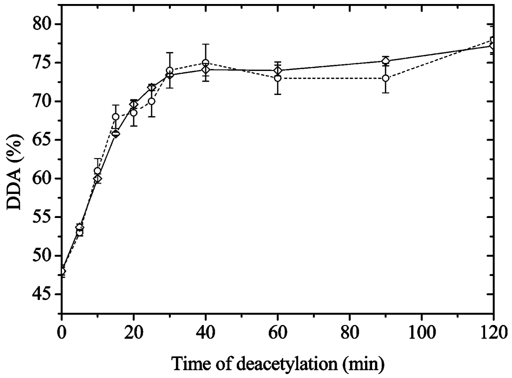


Figure 6. Second heterogeneous deacetylation kinetics of β-chitin (starting DA = 52%) by means of FPT cycles at 90 °C with NaOH 50% (w/v). DDA determined by HPLC (○, ---) and ^{1}H NMR spectroscopy (◇, —) on both soluble and insoluble fractions.

type of soda hydrate formed in our system where water was rare and played a role in the plateau occurring at long reaction times.

Influence of Sodium Acetate. To follow the role of sodium acetate on the deacetylation, we developed a new tool of analysis consisting of evaluating the sodium acetate delivered in the reactor by HPLC. As a proof of the method reliability, β-chitin was first deacetylated by means of FPT cycles with 50% (w/v) NaOH at 90 °C for 45 min (noted 1D/β) to work on a chitosan exhibiting a great solubility in mild acidic conditions. The DA and weight-average molecular weight (M_w) were equal to 52% and 1.06×10^6 g·mol^{-1}, respectively (Table 1, second line). Second deacetylation kinetics was then followed using the FPT cycles technique at 90 °C using 50% (w/v) NaOH. After reaction, the media were centrifuged, and 5 mL of the supernatants was collected, was filtered on PTFE membranes, and was directly injected in HPLC. Solutions were neutralized in situ thanks to the mobile phase (acetonitrile/water (4/1, v/v) at pH 2.2), and the amount of $NaOC(O)CH_3$ recovered after different times was estimated by comparing the peak area at t_r = 3.44 min to a calibration curve drawn for different $NaOC(O)CH_3$ concentrations (sodium acetate trihydrate was used as starting material) in 50% (w/v) NaOH (data not shown). The efficiency of the method was estimated from the comparison of the DDA calculated by HPLC to that determined by ^{1}H NMR (on both soluble and insoluble fractions recovered after deacetylation). Both methods revealed quite similar values all along the reaction and proved that HPLC could also be used to estimate the global DDA of a deacetylated sample with a very good accuracy by the determination of $NaOC(O)CH_3$ concentration (Figure 6).

This important feature confirmed again that once the copolymer was amorphous, deacetylation behaved like a homogeneous reaction by delivering an equimolar amount of $NaOC(O)CH_3$ with respect to the number of the deacetylated GlcNAc units. Thanks to this powerful tool, two sets of experiments were conducted to check the influence of sodium acetate on the deacetylation in the same conditions as those used in the first part of this paper. However, we preferred to deacetylate β-chitin by means of FPT cycles for the following two reasons. First, this technique allowed us to work in a sealed reactor and in hermetic conditions, and water evaporation was thus avoided. Second, we demonstrated in previous works that FPT cycles were a method of choice for completely expelling dioxygen from the reactor and for preventing β-chitin from oxidative hydrolysis.[22] Consequently, only thermal degradation could occur in these conditions.

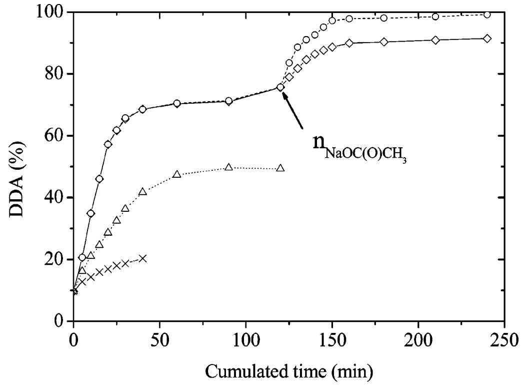


Figure 7. First and second heterogeneous deacetylation kinetics of β-chitin in the presence of sodium acetate. All reactions were carried out at 90 °C with 50% (w/v) NaOH by means of FPT cycles. $n_{NaOC(O)CH_3}$ represents the amount of sodium acetate delivered in the reaction medium after 2 h of reaction. Deacetylation conditions: first deacetylation with the initial addition of (a) $n_{NaOC(O)CH_3}$ (△, •••) or (b) 10 $n_{NaOC(O)CH_3}$ mol of anhydrous sodium acetate (×, -•-•); (c) first deacetylation of 120 min, neutralization, dialysis against distilled water, and then second deacetylation up to 120′ with (◇, —) or without (○, ---) $n_{NaOC(O)CH_3}$ mol of sodium acetate trihydrate.

The starting β-chitin was deacetylated with 50% (w/v) NaOH at 90 °C for 2 h. The amount of $NaOC(O)CH_3$ delivered at the end of the reaction was evaluated by HPLC and was noted $n_{NaOC(O)CH_3}$. The first set of experiments consisted of adding either $n_{NaOC(O)CH_3}$ or 10 $n_{NaOC(O)CH_3}$ mol of anhydrous sodium acetate in the reactor containing β-chitin and the alkaline solution and then starting the deacetylation. Interestingly, when adding $n_{NaOC(O)CH_3}$ mol of sodium acetate at the reaction start, the deacetylation seemed less efficient, and it even stopped after only 40 min with 10 $n_{NaOC(O)CH_3}$ because the solution completely turned into a solid (Figure 7).

Consequently, the following question arose: How could $NaOC(O)CH_3$ decrease the deacetylation efficiency? As the pH of the alkaline solution remained the same during the reaction run with or without sodium acetate, this could not be assigned to a change in the deacetylation efficiency. Alternately, sodium acetate could not take part in a reaction equilibrium that would be illustrated by the following constant:

$$K = \frac{[\mathrm{GlcN}][\mathrm{NaOC(O)CH_3(H_2O)_3}]}{[\mathrm{GlcNAc}][\mathrm{NaOH}]}$$

This would have implied that a reaction of reacetylation had occurred, which is not realistic at such a high pH. To definitely invalidate this assumption, a second set of experiments involving sodium acetate additions was carried out. After β-chitin was deacetylated with 50% (w/v) NaOH at 90 °C for 2 h, the product was neutralized and dialyzed against water (pH 8.5, MWCO: 500 g/mol) to remove salts and was submitted to a new deacetylation for 2 h in the presence of $n_{NaOC(O)CH_3}$ mol of sodium acetate trihydrate to keep the same proportions of reactants and water as when the reaction was stopped (Figure 7, (◇, —) curve). If $NaOC(O)CH_3$ was really involved in a reaction equilibrium, deacetylation should not restart. Kinetics was then compared to a benchmark experiment consisting of a first deacetylation of 120 min followed by neutralization, washings by means of dialysis against distilled water, and a second deacetylation of 120 min (Figure 7, (○, ---) curve). By comparing the DDA reached after 240 min, the second deacetylation with $n_{NaOC(O)CH_3}$ of sodium acetate trihydrate restarted, although it was less effective compared to the benchmark

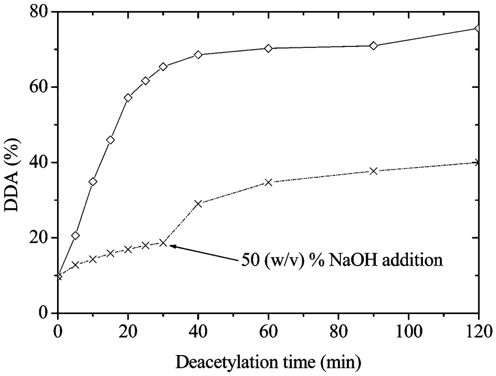


Figure 8. Restart of the reaction containing 10 $n_{NaOC(O)CH_3}$ mol of anhydrous sodium acetate (×, -·-·) with the addition (after 30′) of a small amount of fresh and preheated 50% (w/v) NaOH to reach the same sodium acetate concentration as in a normal deacetylation (◇, —).

experiment. Thus, sodium acetate was necessarily not taking part in a reaction equilibrium as simple as mentioned above.

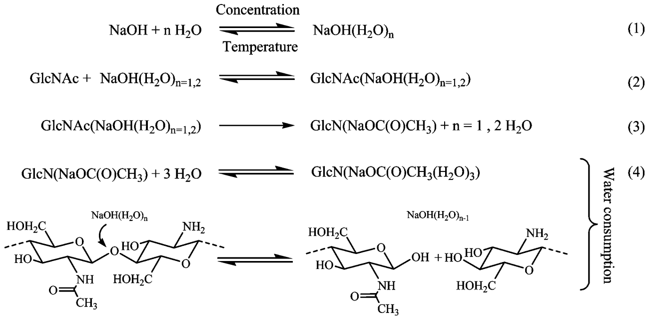
Interestingly, when introducing 10 $n_{NaOC(O)CH_3}$ mol of anhydrous $NaOC(O)CH_3$ before deacetylation started, we observed small particles of soda precipitating on the flask bottom after only 10 min, which may evidence the progressive dehydration of the alkaline solution or at least a change in the proportion of soda hydrates in favor of its anhydrous state. The complete solidification of the reaction medium even occurred after only 40 min. As suggested above, in our conditions of deacetylation, dehydration of the alkaline solution can be provoked either by thermal degradation or by sodium acetate. The main challenge was to evaluate the relative importance of these two phenomena. The evaluation of the water consumption by nonoxidative hydrolysis was deduced from previous studies on the alkaline degradation of chitin and chitosan.[22] Assuming a statistical alkaline hydrolysis of chitin during deacetylation, the rate constant of hydrolysis k_h could be calculated from eq 7:

$$\frac{1}{DP_w} - \frac{1}{DP_{w,0}} = \frac{k_h t}{2} \qquad (7)$$

with $DP_{w,o}$ and DP_w, the starting and final weight-average degrees of polymerization, and t, the reaction time.

In our conditions, the consumption of water caused by alkaline hydrolysis can be neglected, since it would only consume less than 0.1% of the free water in the case of a complete hydrolysis. To evaluate the influence of sodium acetate on the dehydration, let us consider that either the soda mono- or dihydrate is formed exclusively in the reactor before starting the reaction. In our conditions of deacetylation and taking into account the soda concentration and the proportion between chitin and the deacetylating solution, the 10 $n_{NaOC(O)CH_3}$ mol of anhydrous sodium acetate introduced in the reactor before the reaction started would have complexed 12 and 18% of the free water present in the solution, in the case of the soda mono- and dihydrate, respectively. Even though these are rough approximations, however, they give evidence for the real influence of sodium acetate on the soda concentration and can partly explain the behaviors observed in Figure 7. Indeed, the addition of 10 $n_{NaOC(O)CH_3}$ mol of anhydrous sodium acetate is sufficient to artificially increase the soda concentration of around 6% (w/v) and to begin to provoke its precipitation, even at 90 °C. This assumption is realistic, since at such a high concentration the soda behaves like a weak base and most of the salt is still nondissociated. According to eq 4, it means that x_0 is high with

Scheme 2. Equilibriums Occurring during Deacetylation between Chitin, Soda Mono- and Dihydrates, Water, and Sodium Acetate



regard to $x_{i\neq0}$ and that anhydrous soda shows very low activity compared to the mono- and dihydrates. Soda under its anhydrous form shall be then strongly responsible for the decrease of its global activity for concentrations higher than 50% (w/v). At a lower scale, $n_{NaOC(O)CH_3}$ mol of anhydrous sodium acetate is still sufficient to decrease the deacetylation efficiency by complexing only 2 or 3% of the free water, to favor the formation of anhydrous soda, and to decrease the global activity of soda once we overtake 50% (w/v) (Figure 3B).

Even though these approximated values could have been roughly estimated, they confirmed for the very first time that deacetylation efficiency, besides kinetics reasons, is partially linked to the dehydration provoked by the change in the proportions of soda hydrates. To measure the role played by dehydration on the deacetylation plateau at long reaction times, we tried to restart the reaction containing 10 $n_{NaOC(O)CH_3}$ mol of anhydrous $NaOC(O)CH_3$ thanks to the addition of a small amount of fresh 50% (w/v) NaOH solution in the reactor, in such a proportion that the sodium acetate concentration decreased to the value expected for a deacetylation in normal conditions (Figure 8).

Nevertheless, the DDA reached after 2 h of reaction was lower than that obtained in a normal deacetylation, which gives evidence that the drift in the proportions between the different soda hydrates is not the only cause of the low reaction efficiency at long reaction time. We can also exclude statistical/kinetics explanations, since the number of remaining acetylated sites during the reactions carried out with an extra amount of sodium acetate is much higher than that in the control experiment. One can then expect soda to find an acetylated site easier than in a chitin with a low DA and for the reaction with high DAs to be more effective. It seems then that GlcN units and sodium acetate exhibit greater affinity than GlcNAc/soda hydrates complexes. Consequently, it would mean that the system behaves like it was locally frozen once an acetylated site reacted with soda because the complex formed by the sodium acetate and the GlcN units seems so strong that it prevents the surrounding GlcNAc units from being deacetylated. This new complex is certainly stabilized thanks to a local network of hydrogen bonds via water molecules involving the amino groups of the numerous GlcN units, the hydroxyl groups of the glycosidic ring, and the sodium acetate freshly delivered. Washings with water between two successive reactions would be then preponderant to eliminate those nonreactive complexes.

From these results, it strongly seemed that both the nature of the sodium hydrates formed in the deacetylating mixture and their relative proportion drive the reaction efficiency via a cascade of equilibriums (Scheme 2). We particularly assume that sodium hydroxide mono- and dihydrates exhibit the greatest activity toward chitin deacetylation, whereas that of anhydrous soda tends to zero.

According to the soda concentration used before the reaction started, sodium hydroxide hydrates were formed in different proportions in the alkaline solution (equilibrium 1). At room temperature and for soda concentrations over 40% (w/v) anhydrous sodium hydroxide, the mono- and dihydrates shall form in majority and complex with the β-chitin: all other hydrates usually generated at lower soda concentrations all melt above 10 °C. This explains the different activation energies calculated for 30 and 50% (w/v) NaOH and the variation of the global activity of soda according to its concentration. Before the reaction starts, the global cohesive energy resulting from the complexation between soda hydrates and chitin is linked both to the number of water molecules it may contain and to the relative proportion of each type of complex (equilibrium 2). This assumption is supported by the lower activation energy determined for [NaOH] = 50% (w/v) and $T > 70$ °C, where the less hydrated forms of soda are favored. One shall somehow prevent soda from being too concentrated, otherwise, the very low activity of anhydrous soda shall prevail upon those of the mono- or dihydrate and consequently shall decrease the global activity (Figure 3B). When deacetylation occurs, the cohesive energy between GlcN units and the reacted soda hydrate must be so high that it requires washings with water to renew the GlcNAc/sodium hydrate active complexes (equilibrium 3). This is supported by the kinetics plots of Figure 8, exhibiting the influence of sodium acetate concentration and the low dissociation ability of soda to break the complex between the polymer and reacted hydrates independently from statistical reasons.

If the decreasing number of GlcNAc units and the difficulty to dissociate the reacted complexes are mainly responsible for the deacetylation plateau at long reaction times, then we also highlighted that dehydration of the reaction mixture can influence its appearance. Evaporation of water or delivery of sodium acetate during reaction can slightly change the soda concentration or at least the proportions between each soda hydrate in such a way that it reduces its activity by favoring its anhydrous form (equilibrium 4). The anticipated stop of the reaction after only 40 min when introducing 10 $n_{\mathrm{NaOC(O)CH_3}}$ mol of anhydrous $\mathrm{NaOC(O)CH_3}$ particularly illustrated the deleterious effect of dehydration. Moreover, the fact that soda particles easily recrystallized from the 50% (w/v) NaOH solution by pushing dehydration and that the soda particles were responsible for the reaction stop at long deacetylation times reinforced our proposition to consider $\mathrm{NaOH(H_2O)}$ and $\mathrm{NaOH(H_2O)_2}$ as the most active soda hydrates. Thus, the key parameter to explain the low efficiency of deacetylation at long reaction times is the cumulative actions of three phenomena: a kinetics/statistical reason, the high stability of the GlcN/sodium acetate complexes, and the drift in the proportions of the soda hydrates formed in solution in favor of anhydrous NaOH (back to equilibrium 1).

Conclusion

The first purpose of this work was to obtain deeper understanding of the reaction mechanisms involved during the deacetylation of chitin. In a first set of experiments, we suggested that different sodium hydrates were formed in the deacetylating solution according to the soda concentration at the origin of the different activation energies and the global reaction order of soda calculated for 30−50% (w/v) NaOH. This assumption was then confirmed experimentally thanks to the study of the influence of the sodium acetate delivered in the alkaline solution during deacetylation and the role played by alkaline hydrolysis. We propose that the deacetylation success depends on a narrow window of soda concentration, close to saturation, where mono- and dihydrates are formed in high proportions. On the other hand, one shall prevent the alkaline solution from dehydration to avoid the concentration drift of soda in favor of its anhydrous state responsible for the anticipated reaction stop. This definitely demonstrates the utility of our method of deacetylation involving both FPT cycles and washing steps with water between two successive reactions. Indeed, if the main goal of FPT cycles was to enhance reactivity of chitin toward deacetylation, then the FPT cycles also prevented the copolymer from oxidative hydrolysis by sucking out dioxygen in the reaction medium. On the other hand, the washing steps with water between two successive deacetylations enabled the (a) elimination of the sodium acetate formed during the reaction, which also takes part in the dehydration of the reaction medium, and (b) renewing of the reacted sodium acetate hydrates/GlcN units complexes. We now propose to combine these two procedures to deacetylate chitins in a more efficient way and to produce chitosans with higher molecular weights.

Acknowledgment. These studies are part of the CARAPAX project from the fifth European framework Program “Quality of Life and Management of Living Resources”. Authors thank “Mahtani Chitosan” for providing them with fresh squid pens of high quality and Viton, C. for his help in evaluating the amount of sodium acetate delivered in the reaction medium by HPLC.

References and Notes

(1) Minke, R.; Blackwell, J. *J. Mol. Biol.* **1978**, *120*, 167.
(2) Gardner, K. H.; Blackwell, J. *Biopolymers* **1975**, *14*, 1581.
(3) Blackwell, J. *Biopolymers* **1969**, *7*, 281.
(4) Kurita, K.; Tomita, K.; Tada, T.; Ishii, S. *J. Polym. Sci., Part A* **1993**, *31*, 485.
(5) Kurita, K.; Ishii, S.; Tomita, K.; Nishimura, S.-I.; Shimoda, K. *J. Polym. Sci., Part A* **1994**, *32*, 1027.
(6) Lamarque, G.; Viton, C.; Domard, A. *Biomacromolecules* **2004**, *5*, 992.
(7) Lamarque, G.; Viton, C.; Domard, A. *Biomacromolecules* **2004**, *5*, 1899.
(8) Saito, Y.; Putaux, J.-L.; Okano, T.; Gaill, F.; Chanzy, H. *Macromolecules* **1997**, *30*, 3867.
(9) Saito, Y.; Okano, T.; Gaill, F.; Chanzy, H.; Putaux, J.-L. *Int. J. Biol. Macromol.* **2000**, *28*, 81.
(10) Austin, P. R.; Castle, J. E.; Albisetti, C. J. In *Chitin and Chitosan*; Skjåk-Braek, G., Anthonsen, T., Sandford, P., Eds.; Elsevier Applied Science: London, 1989; p 749.
(11) Peberdy, J. F. In *Biochemistry of Cell Walls and Membranes in Fungi*; Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Gossey, M. W., Copping, L. G., Eds.; Springer-Verlag: Berlin, 1990; p 5.
(12) Vander, P.; Vårum, K. M.; Domard, A.; El Gueddari, N. E.; Moerschbacher, B. *Plant Physiol.* **1998**, *118*, 1353.
(13) Peluso, G.; Petillo, O.; Ranieri, M.; Santin, M.; Ambrosio, L.; Calabro, D.; Avallone, B.; Balsamo, G. *Biomaterials* **1994**, *15*, 1215.
(14) Gérentes, P.; Vachoud, L.; Doury, J.; Domard, A. *Biomaterials* **2002**, *23*, 1295.
(15) Hörner, V.; Pittermann, W.; Wachter, R. In *Advances in Chitin Science*; Domard, A., Roberts, G. A. F., Vårum, K. M., Eds.; Jacques André Publisher: Lyon, France, 1997; Vol. II, p 671.
(16) Muzzarelli, R. A. A. *Carbohydr. Polym.* **1996**, *29*, 309.
(17) Sugano, M.; Watanabe, S.; Kishi, A.; Izume, M.; Ohtakara, A. *Lipids* **1988**, *23*, 187.
(18) Rabea, E.; Badawi, M. E.-T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W. *Biomacromolecules* **2003**, *4*, 1457.
(19) Lamarque, G.; Lucas, J.-M.; Viton, C.; Domard, A. *Biomacromolecules* **2004**, *6*, 131.
(20) Methacanon, P.; Malinee, P.; Pothsree, T.; Pattaraarchchai, J. *Carbohydr. Polym.* **2003**, *52*, 119.
(21) Chang, K. L. B.; Tsai, G.; Lee, J.; Fu, W.-R. *Carbohydr. Res.* **1997**, *303*, 327.
(22) Lamarque, G.; Cretenet, M.; Viton, C.; Domard, A. *Biomacromolecules* **2005**, *6*, 1380.

(23) Domard, A.; Chaussard, G. In *Advances in Chitin Science*; Suchiva, K., Chandrkrachang, S., Methacanon, P., Peter, M. G., Eds.; MTEC Publishers: Bangkok, Thailand, 2002; Vol. V, p 1.
(24) Roberts, G. A. F.; Wood, F. A. In *Advances in Chitin Science*; Peter, M. G., Domard, A., Muzzarelli, R. A. A., Eds.; Universität Potsdam Publisher: Potsdam, Germany, 2000; Vol. IV, p 34.
(25) Roberts, G. A. F. In *Advances in Chitin Science*; Domard, A., Roberts, G. A. F., Vårum, K. M., Eds.; Jacques André Publisher: Lyon, France, 1997; Vol. II, p 22.
(26) Hirai, A.; Odani, H.; Nakajima, A. *Polym. Bull.* **1991**, *26*, 87.
(27) Schatz, C.; Viton, C.; Delair, T.; Pichot, C.; Domard, A. *Biomacromolecules* **2003**, *4*, 641.
(28) Vachoud, L.; Zydowicz, N.; Domard, A. *Carbohydr. Res.* **1997**, *302*, 169.
(29) Terbojevich, M.; Carraro, C.; Cosani, A. *Carbohydr. Res.* **1988**, *180*, 73.
(30) Pickering, S. U. *J. Chem. Soc.* **1893**, *63*, 890.
(31) Cohen-Adad, R.; Tranquard, A.; Péronne, R.; Negri, P.; Rollet, A.-P. *C. R. Acad. Sci. Paris* **1960**, *251*, 2035.
(32) Roy, C.; Budtova, T.; Navard, P.; Bedue. O. *Biomacromolecules* **2001**, *2*, 687.
(33) Jing-Hua, L.; Jian-Kuo, Z.; Shan-King, H. *Thermochim. Acta* **1991**, *188*, 17.

BM070021M