NEW ROUTES FOR THE DEACETYLATION OF CHITIN

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Abstract

The efficiency of the deacetylation reaction of α- or β-chitin is greatly improved by the use of freeze – pump out – thaw cycles or ultrasound treatment. Indeed, it attains 80% (65%) when the parent β-chitin (α-chitin) is previously treated by using one of the above mentioned methods while the deacetylation of untreated chitin attains only 60% of efficiency. Also, the occurrence of simultaneous depolymerization is much less important during the deacetylation of treated chitins.

Keywords: chitin, chitosan; deacetylation; ultrasound; freeze-pump out-thaw cycles

Introduction

Chitin is a cellulose-like linear polymer predominantly formed by β(1→4)-linked 2-acetamide-2-deoxy-D-glucopyranose units. It is widely spread in nature and it is the most abundant polysaccharide after cellulose. Thus, chitin occurs as a structural material of invertebrates, such as arthropods, annelids, mollusks, algae and in the cell wall of some fungi[1]. Three polymorphs of chitin are described, namely α-, β- e γ-chitin, the α-chitin being largely dominant while the latter is supposed to be a intermediate form of the former two. The polymorph α-chitin is found where rigidity and mechanical resistance are important such as in the cuticle of arthropods while the polymorph β-chitin occur as flexible but resistant structures such as the squids pens. These polymorphs correspond to different arrangements of the polymeric chains in the solid state, that one corresponding to α-chitin being more dense packed than the arrangement of β-chitin. In the ordered regions the chains of α-chitin disposed in lamella adopt an anti-parallel arrangement which greatly favors the establishment of hydrogen bonds involving macromolecules of the same as well as of the neighbor lamella. On the other hand, the parallel arrangement adopted by the polymeric chains in β-chitin prevents the occurrence of hydrogen bonds of chains pertaining to neighbor lamella, resulting in a much less dense packing. As a consequence of these different arrangements α- and β-chitin display very distinct physico-chemical properties and reactivity, the former being less accessible to solvents and reagents.

The deacetylation of chitin results in chitosan, a copolymer of 2-acetamide-2-deoxy-D-glucopyranose and 2-amine-2-deoxy-D-glucopyranose units where the latter units predominate. Chitin can be deacetylated in homogeneous or heterogeneous conditions but its deacetylation is generally carried out through heterogeneous processes in which the polysaccharide is suspended in concentrated aqueous sodium hydroxide solution at high temperature during variable periods[1-4]. The reaction temperature ranges from 60^0C to 115^0C and the reaction time can vary from some minutes to several hours, depending on the sodium hydroxide concentration and reaction temperature. The process is more efficient the more concentrated the sodium hydroxide solution and the higher the temperature but the long reaction time does not improve the reaction efficiency while favors the simultaneous depolymerization of chitin caused by alkaline hydrolysis. Thus, several different

conditions are used to increase the reaction's efficiency and to avoid the degradation of the polysaccharide, including the use of successive treatments[4-7], of inert reaction atmospheres[8,9], the addition of diluents[5], oxygen scavengers and reducting agents[8-11], and special reaction conditions such as reactive extrusion[12], flash treatment[13] and microwave accelerated process[14]. The ultrasound treatment of chitin suspended in water has also been reported to improve the subsequent deacetylation of the treated polysaccharide[15,16]. Recently the application of freeze – pump out – thaw cycle (FPT cycle) has been claimed to result in improved deacetylation regardless of being applied to α- or β-chitin, the execution of consecutive cycles followed by deacetylation resulting in completely deacetylated chitosan (DA<0.3%) of high molecular weight (M_w>400000 g/mol)[17].

In this work the ultrasound treatment and the freeze – pump out – thaw cycles were applied to α- and β-chitin, the treated samples were submitted to deacetylation and the characteristics of the resulting chitosans were determined to allow the comparison of these routes to produce extensively deacetylated chitin with minimum depolymerization.

Material and Methods

Extraction of α- and β-chitin

The shells of *Parapenaeopsis stylifera* and the pens of *Loligo* were the raw materials for the extraction of α- and β-chitin, respectively. Initially the shells of *P. stylifera* and the squid pens were extensively washed with tap water, freeze-dried and cryo-grounded. After sieving, the fractions corresponding to 80-120μm for the squid pens and below 80μm for the shrimp shells were submitted to the treatments leading to the extraction of β- and α-chitin, respectively. The squid pens, owing to its very low content of inorganic compounds, were directly submitted to deproteinization by suspending it in 0.1M aqueous NaOH at room temperature during 24h under vigorous magnetic stirring[17]. The β-chitin was isolated after extensive washing and freeze-drying. The shells of *P. stylifera* were demineralized by treating it with 1M HCl aqueous solution during 30min at room temperature under vigorous magnetic stirring[18]. Following the extensive washing with water and freeze-drying the demineralized shells were submitted to deproteinization as described above, the α-chitin being isolated after extensive washing with water followed by freeze-drying.

Chitin deacetylation

Before carrying out the deacetylation reactions chitin was submitted to freeze-pump out-thaw cycles or to ultrasound treatment. For the execution of the freeze – pump out – thaw cycles 1g of chitin was suspended in 20g of 40% NaOH aqueous solution and the suspension was initially frozen by immersing it in liquid nitrogen (fast freeze) or in an ice/water bath (slow freeze) and then submitted to the pump out and thaw. This cycle was repeated for at least two times before submitting the chitin to the deacetylation.

For the ultrasound treatment 1g of chitin was suspended in 20g of 40% NaOH aqueous solution, the resulting suspension was poured in a glass cell kept at 40^0C and the ultrasound microsonde was immersed in it. The ultrasound irradiation proceeded for the desired at a fixed amplitude power for the desired time and the suspension was then submitted to the deacetylation.

The typical deacetylation was carried out by immersing the glass reactor containing the chitin suspension in an oil bath at 90^0C. The reaction was stopped by immersion in liquid nitrogen and the chitosan was recovered after neutralization, washing and freeze-drying.

Characterizations

The samples, α- and β-chitin and chitosans, were characterized in terms of average degree of acetylation ($\overline{DA}$) by ^{1}H NMR spectroscopy. Chitosans were suspended in acidified D_2O (pD≅3-4), vigorously stirred overnigth at room temperature and the resulting solutions were then analysed. Due to its lower solubility chitin was suspended in DCl/D_2O (20% w/w), heated at 65-70°C during 8h and the resulting solution was used to acquire the NMR spectrum. All spectra were acquired at 25°C on a Bruker AC 200 spectrometer and the $\overline{DA}$ values were calculated from the ratio of the

methyl hydrogens of acetamide groups signals to those of the hydrogen bonded to the glucopyranose ring as proposed by Hirai[20].

The weight average molecular weight ($\overline{Mw}$) of chitosans were determined by using size exclusion chromatography. Thus, the chitosans were dissolved in 0.2M AcOH/0.15M AcONa buffer (pH=4.5), the resulting solutions were filtered on 0.45μm pore size membrane (Millipore) and then injected by means of an IsoChrom LC pump (Spectra-Physics) into the chromatographic system composed by a Protein Pack glass 200 SW and a TSK gel 6000 PW columns. The detectors of polymer concentration – a Waters 410 differential refractometer - and of molecular weight – a Wyatt Dawn DSP multi angle laser-light scattering equipment – were coupled on line to the chromatographic system.

The viscosity average molecular weight ($\overline{M_V}$) of the parent chitins were determined from viscosity measurements using an automatic capillary viscometer, Viscologic TI 1 SEMATech (ϕ=0.8mm) at 25°C. The chitins were dissolved in N,N-dimethylacetamide containing 5% lithium chloride at C_p=2g/l and the values of $\overline{M_V}$ were calculated from the intrinsic viscosity by using the Mark-Houwink-Sakurada parameters (α=0.69 and K=2.4x10^{-4})[21].

Result and Discussion

α- and β-chitin

The extraction of chitin from the biomass calls for the elimination of proteins and carbonates, the main substances to which the polysaccharide is combined in nature. The shells of *P. stylifera* contain α-chitin (≅17%), a small amount of proteins (7%) and carbonates of calcium and magnesium (≅36%) while the pens of *Loligo* are composed by β-chitin (≅32%), proteins (42%) and minor amounts of carbonates (<3%). Thus, the procedures of demineralization and deproteinization must be appropriately designed to these different sources of chitin aiming to completely eliminate minerals and proteins but preserving as most as possible the native characteristics of the polysaccharide. In this manner, the squid pens were not submitted to the demineralization and mild conditions were used in the deproteinization step of both chitin sources to avoid the occurrence of deacetylation, resulting in α- and β-chitin with the characteristics resumed in Table 1. These data show that a small amount of acetamide groups (≅10%) were hydrolized during the extraction of both polymers, α- and β-chitin, as a consequence of the alkaline attack carried out in the deproteinization step and that the polymers also have similar viscosity average molecular weight.

Table 1 Characteristics of α- and β-chitin extracted from shells of *P. stylifera* and the pens of *Loligo*, respectively.

SAMPLE	$\overline{DD}$ (%)[(a)]	$\overline{M_V}$ x 10^5 (g/mol)
α-chitin	10.02±0.06	11.52±0.96
β-chitin	9.10±0.25	12.06±1.00

a) $\overline{DD}$ =100-$\overline{DA}$

Ultrasound treatments

The different susceptibilities of α- and β-chitin to the deacetylation as well as the effects of the sonication time on the reaction efficiency and on the depolymerization rate can be evaluated by examining the curves of Fig. 1 and 2. Thus, when none of the polymers were previously sonicated the reaction efficiency attained 60% and 74% for α- and β-chitin, respectively. This result shows that β-chitin is more susceptible to deacetylation than α-chitin. Also, in both cases the curves of $\overline{DD}$ versus time of sonication present the same trend, i.e. the longer the sonication time the higher the average degree of deacetylation, showing the positive effect of the sonication treatment on the

reaction efficiency as compared to the deacetylation of untreated chitin. These curves also show the same dependence of the depolymerization rate on the time of sonication, i.e. the longer the sonication time the higher the depolymerization rate but in this case the data show that α-chitin is more severely depolymerized than β-chitin.

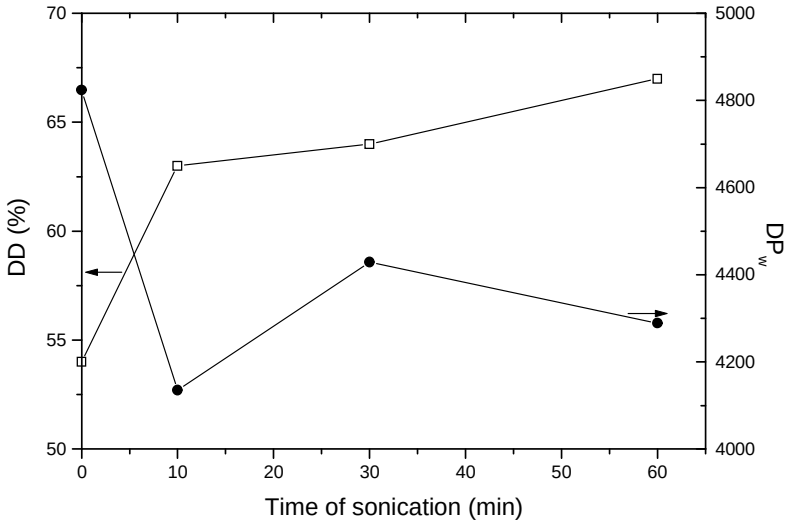

Fig. 1 Average degree of deacetylation (DD) and average degree of polymerization (DP_W) versus sonication time of α-chitin in 40% aqueous NaOH followed by deacetylation for 45min at 90°C.

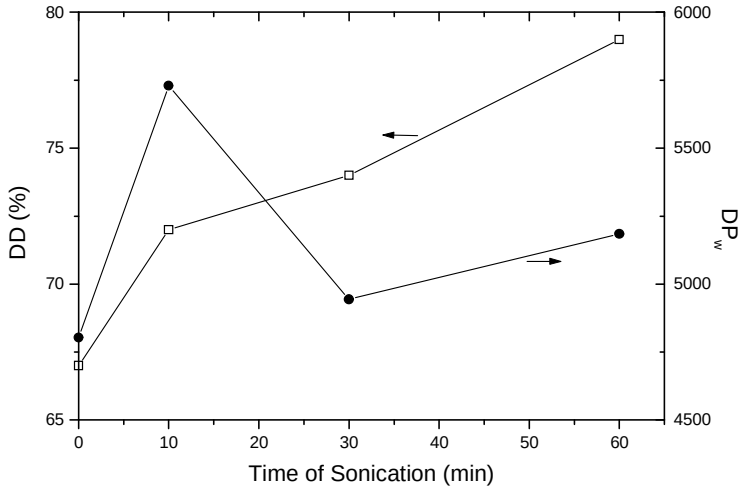

Fig. 2 Average degree of deacetylation (DD) and average degree of polymerization (DP_W) versus sonication time of β-chitin in 40% aqueous NaOH followed by deacetylation for 45min at 90°C.

Freeze – pump out – thaw cycles

The freeze – pump out – thaw cycles (FPT cycles) were applied as a treatment of α- and β-chitin before submitting these polymers to the deacetylation reaction conditions and the characteristics of parent polymers and resulting chitosans are resumed in Table 1.

Table 1: Characteristics of chitin and chitosans issued from the experiments where the FTP cycles were followed by deacetylation at 90°C during 45min.

Sample	Number of Cycles[a]	DD (%)[b]	M_w x 10^5 [c] (g/mol)	DP_w[d]	I[e]
β-chitin	-	10.02±0.06	12.06±1.00	6166±588	-
B1	2*	70.07±090	8.51±0.71	4896±467	1.81±0.15
α-chitin	-	9.10±0.25	11.54±1.00	5724±546	-
A1	8	64.12±0.08	8.42±0.70	4769±455	1.62±0.13
Chitosan C	-	77.20±0.95	4.02±0.50	2355±225	3.40±0.28
C1	8	96.50±1.20	2.26±0.18	1391±133	2.83±0.23
C2	2	96.31±1.20	2.07±0.17	1273±121	2.52±0.21
C3	2	99.75±1.20	1.67±0.14	1036±99	2.25±0.19

a)number of FPT cycles. * stands for a slow freezing step while all others correspond to fast freezing at liquid nitrogen.
b)DD = 100 – DA, where DA is the average degree of acetylation. Values determined by ^{1}H NMR.
c)M_w is the weight average molecular weight determined by size exclusion chromatography.
d)DP_w is the average degree of polymerisation; $DP_w = M_w/M_0$, where M_0 is the average molecular weight of the repeating unit.
e)I is the polydispersity index; $I = M_w/M_n$.

The data in Table 1 show that the use of a slow freeze step during the application of the FPT cycles to β-chitin is an as efficient way to produce chitosan as the use of ultrasound treatment before submitting the treated β-chitin to the deacetylation reaction. Indeed, comparing the $\overline{DD}$ values of the chitosan B1 (Table 1) and that of the chitosan produced from β-chitin which was previously submitted to 60min of sonication (Fig. 2) reveals that the deacetylation efficiencies are 79% and 74%, respectively. On the other hand, the use of a fast freeze step is slightly more efficient than the ultrasound treatment applied to α-chitin before submitting it to the deacetylation reaction. Thus, the comparison of the $\overline{DD}$ values of chitosan A1 and that of the chitosan produced from α-chitin which was previously submitted to 60min of sonication (Fig. 1) reveals that the deacetylation efficiencies are 71% and 60%, respectively. However, it should be noted in this case that eight (8) fast freeze – pump out - thaw cycles were carried out before the deacetylation of the treated α-chitin as compared to 60min of sonication.

As already mentioned, the production of extensively deacetylated chitosan is always accompanied by severe depolymerization, thus the treatment of sonication and FPT cycles were used to evaluate these methods to prepare such products.

The data in Table 2 show that when chitosan (sample C; $\overline{DD}$ =77.2%; $\overline{M_W}$ =4.02x10^5g/mol) was successively submitted to FPT cycle and deacetylation at 90^0C during 45min, it resulted in extensively deacetylated chitosan (sample C3; $\overline{DD}$ =99.75%; $\overline{M_W}$ =1.67x10^5g/mol).

The submission of β-chitin to successive sonication for 60min followed by deacetylation at 90^0C during 45min also allowed the production of extensively deacetylated chitosan ($\overline{DD}$ =99.60%; $\overline{M_W}$ =2.25x10^5g/mol). On the other hand, if untreated chitin (α- and β-chitin) is submitted to successive deacetylations, at least 5 successive reactions shall be carried out to attain similar results ($\overline{DD}$ <0.5%) but the depolymerization is much more severe in this case ($\overline{M_W}$ <0.7x10^5g/mol).

Thus, both methods, the sonication and the FPT cycles, are efficient to produce an extensive deacetylation of chitin and the depolymerization is much less severe when these treatments are applied as compared to the acetylation of untreated chitin.

Conclusions

The sonication and freeze – pump out – thaw cycles are efficient treatments aiming to improve the deacetylation of α- and β-chitin. Thus, the deacetylation efficiency attains 80% and 65% in the cases of α- and β-chitin, respectively, while it reaches only 65% when untreated chitin is submitted to deacetylation. Also, the depolymerization is less severe when treated chitins are deacetylated as compared to that occurring when untreated chitin is submitted to deacetylation.
Preliminary results indicate that the morphological changes provoked by the sonication treatment may be responsible for the improved reactivity of treated chitin toward the deacetylation reaction but further work is being currently developed to confirm it.

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