SOLID-STATE ACID HYDROLYSIS OF CHITOSAN: EVOLUTION OF THE CRYSTALLINITY AND THE MACROMOLECULAR STRUCTURE.

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Abstract

The chemical heterogeneous hydrolysis of chitosan in the solid state was performed by impregnation with concentrated hydrochloric acid. The hydrolysis kinetics was studied at different temperatures and HCl concentrations. This hydrolysis procedure increased the crystallinity index from 31 to about 55%, thanks to the elimination of hydrolysed amorphous regions. After hydrolysis, the WAXS patterns of the materials showed the presence of the anhydrous polymorph, absent in the raw original material and in the deacetylated chitosans showing in their diffractograms the only peaks of the hydrated polymorph. In spite of the washings of hydrolysed chitosan, the anhydrous polymorph was even present in the residual solid product together with the hydrated polymorph.

Introduction

Recent studies have been devoted to the use of microcrystals obtained from natural polymers as an attractive way to increase the mechanical performances of composite materials. Thus, a number of investigations concerning the preparation of whiskers (slender polymer parallelepiped rods) of cellulose[1, 2] and chitin[3, 4] has been reported. In this case, suspensions of polymer crystallites have been prepared by acid hydrolysis[5, 6] of polymer with the objective of dissolving away regions of low lateral order, so that the insoluble highly crystalline residue was converted into a stable suspension by mechanical shearing action. The obtained whiskers have been extensively used as model fillers in several kinds of polymeric matrixes, including synthetic and natural polymers[1-4].

Chitosan whiskers could be an advantageous natural filler as reinforcing phase, taking advantage of its low density, renewable character, biodegradable and bioresorbable nature. In tissue engineering, it is possible to combine the reinforcement with nanoporosity : chitosan resorbable whiskers could be incorporated into polymeric matrixes to provide the needed short-term strength and fracture resistance while tissue regeneration is occurring, then creating nanopores suitable for vascular ingrowth as the whiskers are destroyed.

In a similar way as cellulose and chitin, amorphous domains of chitosan could be removed by acid hydrolysis. The crystalline residue could be converted into a stable suspension which could be mixed with a polymer in order to obtain nanocomposite films.

In the present work, we focused on the chemical hydrolysis of chitosan in the solid state by impregnating with concentrated HCl. The hydrolysis was performed with fully deacetylated chitosan as starting sample having a relatively high crystallinity index. The hydrolysis kinetics was studied at different HCl concentrations and temperatures in emphasizing the evolution of the crystallinity and the hydrolysed macromolecular structure.

Materials and methods

The chitosan Ch1 was supplied by Mahtani Chitosan (India). The degree of acetylation was 23% and the number-average molecular weight was 1.48 x 10^5 g/mol. The chitosan Ch1 was fully deacetylated by a multi-step heterogeneous deacetylation method using a solid/liquid ratio of 1/40 (w/v) with NaOH 40% (w/w) by means the freeze-pump out-thaw cycles[7].

Fully deacetylated chitosan obtained as above were distributed into several flasks to perform the kinetics studies. They were prehydrated to a desired level before hydrolysis. Then, the hydrolysis was achieved with concentrated HCl guaranteeing a molar ratio of water to glucosamine residue r_{H2O} of 60 and non dissolution of chitosan flakes. The hydrolysed products were washed first with concentrated 1.5M HCl, then with 1.0M ammonia and finally with distilled water until neutrality to eliminate the lowest DP_n oligomers. After filtration, the remaining solid was dried by lyophilisation. The recovered hydrolysed chitosan was characterized by MALLS chromatography for study the macromolecular structure based in the molecular weight and by X-ray diffraction for study the crystalline microstructure.

Molecular weight

The molecular weight analyses of hydrolysed chitosan dissolved at 0.1% (w/v) in AcOH (0.2 M)/$AcONH_4$(0.15 M) (pH=4.5) buffer were performed by size exclusion chromatography. The chromatographic apparatus was composed of an IsoChrom LC pump (Spectra-Physics) connected with a Protein Pack 200 SW (WATERS) column and a TSK gel G6000 PW_{XL}. A multi-angle laser light scattering detector DAWN DSP (Wyatt) operating at 632.8 nm and a WATERS 410 differential refractometer were connected on line. The refractive index increment d*n*/dc was determined with an interferometer ScanRef monocolor (NFT).

Crystallinity index (CrI)

Pellets of chitosan powder were analyzed by X-ray diffraction for estimating the crystallinity index. Wide-angle X-ray scattering (WAXS) patterns were measured in reflection mode with a SIEMENS D 500 Diffractometer. The chitosan tablets were deposited on the surface of a glass plate and exposed to the Cu Kα radiation with a wavelength of 1.542 Å operating at 35 kV and 30 mA. The angle of incidence was varied between 3 and 70° by 0.06° steps. The crystallinity index was determined from the ratio of the separated peak area to the total area in the WAXS diffraction pattern.

Also, experiments were carried out at ESRF (Grenoble) on the D2AM beamline in transmission mode at 16 keV (λ = 0.7749Å). The chitosan tablets were placed on a PMMA support allowing beamline to cross the cross-section of about 500 µm thickness chitosan pellet.

Determination of degradation rates as a function of temperature

The apparent rate constants of the hydrolysis of chitosan at the solid-state by impregnating with concentrated HCl were determined at different temperatures: 5°C, room temperature (~ 22°C), 50 and 80°C. The degradation rates are reported as apparent rate constants (k), as defined in the following equation[8]:

$$\frac{1}{DP} = \frac{1}{DP_0} + kt \qquad (1)$$

where DP and DP_0 are the number-average degrees of polymerisation and t the hydrolysis time. The apparent rate constant at each temperature was then obtained directly from the slope of the time course of degradation plotted as the inverse of DP versus time.

Results and discussion

Macromolecular structure and kinetics of hydrolysis

Table 1 and Figure 1 shows the slight decrease of molecular weight of fully deacetylated chitosan in the solid state hydrolysis with 3M HCl at room temperature. After 49 hours of hydrolysis the molecular weight decreased from 1.096x10^5 to 9.709x10^4 g/mol. The polydispersity index of the

hydrolysed samples practically does not vary with the time of hydrolysis and for all results, a mono-modal molecular weight distribution was obtained.

Table 1: Characteristics of fully deacetylated chitosan Ch1-D2 and hydrolysed chitosans obtained by solid-state impregnation of chitosan Ch1-D2 with 3M HCl at room temperature.

Hydrolyzed and starting chitosan	Time (h)	$Mn/10^5$ (g/mol)	DP_n	Ip	L_{110} (nm) peak Anhyd. $2\theta \sim 15^0$	Anhyd. Polymorph (%, n/n)	CrI (%, n/n)
Ch1-D2	0	1.096	680	1.809±0.007	4.5*	~0	35.85
E1	2	1.092	677	2.07±0.005	7.4	26.7	47.16
E2	3,58	1.079	669	2.31±0.005	5.9	28.4	48.33
E3	6,5	1.064	660	2.22±0.007	5.9	28.1	48.88
E4	14	0.8085	502	2.83±0.012	5.9	29.4	50.99
E5	25	1.056	655	2.22±0.006	6.3	29.8	51.70
E6	49	0.9709	602	2.18±0.006	7.4	28.4	52.24

*L_{020} in nm of peak corresponding to hydrated allomorph which appears at $2\theta \sim 10^0$.

A higher decrease in the molecular weight was observed when the reaction was operated at 50 and at 80°C compared to the kinetics study made at room temperature (see Figure 1). The polydispersity index showed slight variations around 2.00. After only 49 hours we reduced the molecular weight from 1.096 x 10^5 to 3.91 x 10^4 g/mol at 50°C and to 9.000 x 10^3 g/mol at 80°C using 3M HCl. Nevertheless, the decrease of molecular weight with the time of hydrolysis is even relatively slow. At 50°C it does not still exist a plateau in the curve, and at 80°C it was reached a plateau after about 14 hours of hydrolysis. In order to study the evolution of the crystallinity and the macromolecular structure with the acidic hydrolysis at a lower temperature, the solid state hydrolysis of fully deacetylated chitosan was performed at 5ºC. Figure 1 reveals the smaller decrease of the molecular weight with the time of hydrolysis obtained at 5°C with 3M HCl with respect to the hydrolysis at room temperature (~22°C), 50 and 80°C. A mono-modal molecular weight distribution was obtained at 5°C, room temperature (~22°C) and at 50°C, nevertheless at 80°C a tetra-modal molecular weight distribution was obtained after 14 hours.

In order to study the effect of HCl concentration on the kinetics of the solid state hydrolysis, fully deacetylated chitosan was hydrolysed with 12M HCl at room temperature (~22°C). As in the previous kinetics with 3M HCl, with 12M HCl we observed a slight decrease of the molecular weight with hydrolysis (see Figure 1). However, in the latter case, the decrease was somewhat higher and more rapid. After 51 hours, the molecular weight decreased from 1.096x10^5 to 7.126x10^4 g/mol, and the polydispersity index close to 2.2 remained almost constant for all hydrolysed chitosan samples.

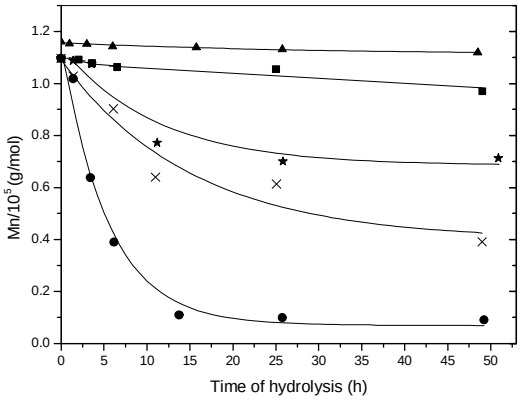

Figure 1 : Decrease in number-average molecular weight during the *solid-state* hydrolysis by impregnation of fully deacetylated chitosan with 3M HCl at: -▲- 5°C, -■- room temperature (~ 22°C), -X- 50°C, and, - ● - 80°C, - * - with 12M HCl at room temperature.

Crystalline microstructure.

From a crystallographic point of view, X-ray diffraction measurements of our hydrolysed samples exhibited an increased crystallinity in relation with the elimination of hydrolysed amorphous regions. The main peaks were not the same as those of the starting chitosans (initial and deacetylated chitosans) where only it was present the hydrated allomorph of chitosan. The anhydrous polymorph appearing after acidic hydrolysis did not disappear after washing and we observed a WAXS diffraction pattern that contained both hydrated and anhydrous chitosan polymorphs. Figure 2 shows the WAXS diffraction pattern obtained for the hydrolyzed samples at the different studied temperatures and for the fully deacetylated chitosan before hydrolysis.

For our non-hydrolysed chitosans (initial and deacetylated) we could assume a structure where less ordered regions (amorphous) were combined considering both amorphous parts and bundles of chains of crystalline character preferably constituted of the hydrated chitosan polymorph. The acid hydrolysis carried out preferably in the amorphous regions increases the crystallinity of chitosan due to their own elimination with the hydrolysis and posterior washings and also because the end of chains which were retained to the original crystalline bundle of chains could crystallize as anhydrous polymorph form. Thus, the hydrolysed chitosans will contain in their structure both the hydrated and anhydrous polymorphs.

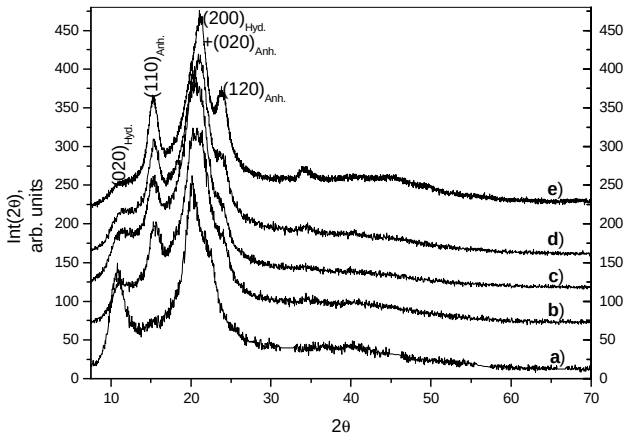

Figure 2 : WAXS diffraction patterns obtained for: a) fully deacetylated chitosan Ch1-D2 prepared by heterogeneous deacetylation procedure (chitosan without contribution of anhydrous polymorph); for hydrolysed chitosan prepared by solid-state impregnation of chitosan Ch1-D2 with 3M HCl during 6h: b) at 5°C, c) at room temperature ~ 22°C, d) at 50°C, e) at 80°C. It allows us to calculate an anhydrous polymorph proportion of: 33% at 5°C and at 50°C, 28% at room temperature and 41% at 80°C.

The WAXS diffraction patterns of the hydrolysed chitosans (Figure 2) exhibit a peak at near 2θ =15° corresponding to the (110) reflection of the anhydrous crystalline allomorph. This peak is absent in the diffractogram of the starting chitosan Ch1-D2 (Figure 2) in which only the peaks corresponding to the hydrated crystalline allomorph are present. This anhydrous polymorph is present in all samples obtained from our particular solid-state hydrolysis. In previous works the anhydrous polymorph has been obtained by annealing the hydrated chitosan at about 240ºC in water[9, 10]. Recently, Okuyama *et al.*[11] obtained the anhydrous polymorph using milder conditions, at room temperature. They transformed a chitosan/acetic acid salt to the anhydrous form when kept in 100% relative humidity (RH) at room temperature for several days to remove acetic acid by progressive hydrolysis of the salt.

For the hydrolysis at 50 and 80°C, the anhydrous allomorph was more developed than at room temperature (see Figure 2). This increase in the percent of the anhydrous allomorph could be attributed to the role of temperature allowing a higher mobility of the segments of chains produced by the hydrolysis and then, their higher crystallization as anhydrous allomorph.

From a crystallographic point of view, X-ray diffraction measurements of our hydrolysed samples exhibited an increased crystallinity in relation with the elimination of hydrolysed amorphous regions. For kinetics at room temperature and at 50ºC, the crystallinity index remained constant for the first three hours. After this time, the values increased from 48 to 52% at room temperature at 49 hours and decreased from 48 to 44% at 50ºC at 49 hours. At the beginning, we could estimate the hydrolysis at both temperatures as similar because this hydrolysis corresponds essentially to the depolymerisation of amorphous regions that normally allows an easy access of the hydrolysis reactive. For longer times, the role of temperature was important, because at 50°C one can suppose a better access of the reactive to the crystalline domains of the material. This was revealed through a more pronounced decrease of crystallinity index at 50°C for longer hydrolysis times and also a more rapid decrease of molecular weight of chitosan with hydrolysis. This was responsible of a destructive effect on the original crystallinity of chitosan that was less pronounced at room temperature. The highest values of the crystallinity index for all times were observed in the kinetics study at low temperature as 5°C. At low temperatures close to 0°C, the reactivity of the reagents of hydrolysis toward crystalline domains is more difficult. Essentially, we notice an increase of the crystallinity due to the crystallization of ends of chains, products of the hydrolysis of amorphous domains, under the anhydrous form, and, practically, there should be no destruction of the native crystalline domains of chitosan. The percent of anhydrous allomorph obtained at 5 °C is quite similar to that obtained at 50°C and is higher to those in the kinetics at room temperature.

Depolymerization rates as a function of temperature.

The time course of degradation at 5°C, room temperature (~22°C), 50 and 80°C is presented in Figure 3. Independently of the temperature, the plots of the degree of scission ($\alpha=1/DP_n$) versus time show two different domains with a frontier located at near 6 hours. This behaviour particularly emphasized for the three highest temperatures is negligible at 5°C. For reaction times below five hours the hydrolysis proceeded at relatively faster rates than at longer times. Thus, especially at room temperature, 50 and 80°C, two different modes of reaction should contribute to the de-polymerisation. We could propose that in the first range, the access to the amorphous domains of chitosan for the reagents of hydrolysis is preeminent and relatively easy and then, is responsible for a relatively fast depolymerisation. At 5°C the observed behaviour is practically due to the hydrolysis of amorphous regions of chitosan. At longer times at room temperature, 50 and 80°C, the reaction principally concerns the crystalline domains, where glycosidic bonds are less accessible for hydrolysis and a slower de-polymerisation takes place.

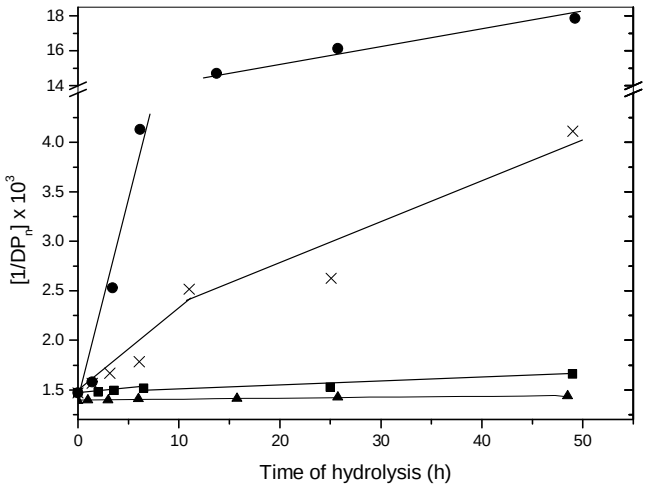

Figure 3 : Time course of degradation of fully deacetylated chitosan in 3M HCl at different temperatures (see Tables 1 for kinetics study at room temperature), where the inverse of DP_n is plotted versus time. -▲- 5ºC (chitosan Ch1-D2' with Mn=1.160 x 10^5 g/mol), -■-room temperature ~ 22ºC (chitosan Ch1-D2 with Mn=1.096 x 10^5 g/mol), -X- 50ºC (chitosan Ch1-D2), -●- 80ºC (chitosan Ch1-D2).

The results of the time course of degradation at the different temperatures studied (see Figure 3) allowed us to calculate the activation energy parameters through the plot of the logarithm of the rate constants (k) versus the inverse of the absolute temperature (Arrhenius plot, not shown). The activation energies (Ea) calculated from the slopes of the obtained line for all hydrolysis time at 5ºC and obtained at the shorter hydrolysis times for the kinetics at room temperature, 50 and 80ºC was 63.5 ± 8.3 kJ/mol, and for the longer times at room temperature, 50 and 80ºC was 66.3 kJ/mol. This Ea should represent the Ea for solid state acidic hydrolysis of the glycosidic linkage in the amorphous and crystalline domains of chitosan, respectively. Ea values obtained in this work were much lower than those reported by Vårum *et al.*[12] for hydrolysis of chitosan (F_A=0.002) in 0.4N HCl (Ea = 152.2 kJ/mol) in homogeneous conditions, and also lower than Ea for the solid-state hydrolysis of chitin in 11.0N HCl (94.1 kJ/mol) reported by Rypley[13]. Holme *et al.*[14] also studied the degradation of chitosan hydrochloride (F_A=0.02) in the solid state, and they determined Ea around 110 kJ/mol.

It was also possible to calculate the pre-exponential factor A from the Arrhenius plot, which represents the frequency of collisions between two molecules in the proper orientation necessary to the reaction. A-values were equal to 1.0 x 10^6 ± 27.4 h^{-1} and 2.3 x 10^6 h^{-1} for the amorphous and crystalline domains of chitosan, respectively.

Conclusions

The solid-state acid hydrolysis of fully deacetylated chitosan constitutes an efficient method for increasing its crystallinity. The kinetics of hydrolysis and the evolution of the crystallinity index are dependent on temperature and HCl concentration.

There exist two mechanism of hydrolysis. One for the amorphous domains (favoured at low temperature). Another for the crystalline domains (favoured at high temperature and not ocurred at low temperature as 5°C).At low temperature as 5°C, the highest crystallinity indexes were obtained. It was possible to increase this index from 33 to about 55% using 3M HCl.The anhydrous polymorph chitosan appeared after hydrolysis was preserved with the time of hydrolysis and after the posterior washings. Its characteristic peak at 2θ=15° appeared in all WAXS diffraction patterns of hydrolyzed chitosan. At 50 and 80 °C the highest percents of anhydrous polymorph of chitosan were obtained.

The apparent crystallite width calculated from the Scherrer expression for the peak corresponding to the anhydrous polymorph observed at 2θ=15° was found between 5.1 and 7.6 nm for all obtained hydrolyzed.

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