EFFECT OF DRY HEAT ON SOME PHYSICAL AND CHEMICAL PROPERTIES OF CHITOSAN

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Abstract

It is believed that heat-induced reactions on chitosan entail the formation of crosslinkages and involve the amino groups but the nature of the chemical changes is still poorly understood. The occurrence of the Maillard reaction has been described for chitosan and reducing sugars, where the amino groups of the first would react with the reducing ends of the last. The chitosan molecule has both carbonyl and amino residues, so this reaction could take place with any other carbohydrate if the conditions were favoured. We have studied the solid-state behaviour of chitosans of different average molecular weight prepared by deacetylation and chemical or enzymatic depolymerization. Samples prepared by nitrous acid depolymerization showed an absorption band and chemical shifts in the ^{1}H-NMR spectrum which can be attributed to the occurrence of a Maillard-type reaction which would be facilitated by the lyophilisation process. When stored under mild conditions (40ºC and 79% relative humidity) this chitosan developed a yellowish color, decreased its water solubility and changed the behaviour in water in accordance with a possible crosslinking process produced by the progress of the Maillard reaction. The colorimetric determination of chitosan in the samples supported the participation of the amino groups in a crosslinking reaction.

Introduction

It has been reported that exposure of chitosan to high temperatures can change its properties. Samples of this biopolymer submitted to heat have shown decreased aqueous solubility and development of color. Although the nature of the chemical changes is still poorly understood, it is believed that heat-induced reactions in chitosan involve the amino groups and the formation of crosslinkages [1].

The proposed mechanism corresponds to the Maillard reaction, also called non-enzymatic browning or glycation, which occurs between the free amino groups of proteins and the reducing ends of sugars [2]. The first stable reaction products formed during the early stage are the so-called Amadori compounds. They are the result of the condensation between the molecule containing the aminoacid and the carbohydrate followed by a subsequent rearrangement [3]. Amadori compounds are degraded in the advanced stage to the highly reactive α-dicarbonyl compounds [4]. During the final stage high molecular weight coloured compounds, melanoidins, are formed by the cross-linking reaction between a low molecular weight chromophore and a non-coloured high molecular weight biopolymer [5].

The occurrence of the Maillard reaction has been described for chitosan and reducing sugars, where the amino groups of the first react with the reducing ends of the last. Chitosan has been submitted to reaction with glucose, glucosamine, maltose and fructose in acetic acid solution at 65º C in order to

obtain water soluble derivatives [6]. The chitosan molecule itself has both carbonyl and amino groups, so the Maillard reaction could take place without any other carbohydrate in favourable conditions. The development of this reaction is considered to impart undesirable properties to the product, such as brown colour and scarce solubility. However, if this process could be controlled in order to keep the biocompatibility and other useful properties of the polymer, dry heat might be used to produce crossliked chitosan for controlled release applications without the need of chemical reagents. We have undertaken a preliminary study of the occurrence of the Maillard reaction on chitosan samples obtained by deacetylation and depolymerization by nitrous acid.

Materials and Methods

Chitins from snow krab (*Paralomis granulosa*) or from lobster (*Palinurus vulgaris*) were deacetylated with NaOH in our laboratory to obtain chitosans with different average molecular weight (Mw) as determined by viscosimetry: 643, 324 and 191 kDa (CHT 1, 2 and 3, respectively). CHT 1 and CHT 2 were depolymerised with nitrous acid (Final average Mw 67 and 50 kDa; CHT 4 and 5, respectively). Furthermore, fractions of chitooligosaccharides prepared by depolymerization of CHT 3 with chitosanase followed by membrane ultrafiltration with cellulose acetate membranes of 3 and 10 kDa cut-off were used.
All products were dissolved in 0.1M acetic acid, the pH adjusted to 6, freeze dried and stored at 40ºC and 79% relative humidity in a desiccator containing a saturated potassium bromide solution. Samples were taken at times in the range 0 to 14 days and kept at -20º in glass bottles with silica gel prior to further analysis.
Absorption spectra were recorded on a GBC 920 UV/VIS spectrometer. NMR spectra were performed on an AMX 500 Bruker spectrometer. Samples were dissolved (1% w/v) in D_2O/DCl 9:1 (v/v) and transferred to 5 mm NMR tubes. All chemical shifts were determined relative to internal TSP (sodium 3-(trimethylsilyl)-propionate-d4). Typical conditions used for ^1H NMR acquisition were 500,14 MHz; acquisition time 3s; actual pulse repetition time 4 s; number of scans 64; 316 K.

Results and Discussion

The naked eye observation of the chitosan samples allowed seeing the fibrous appearance after freeze drying. A colour development was noticeable with time, the rate of the low molecular weight sample being the highest. Samples were pale yellow but turned to yellowish brown at 14 days. When manipulated for weighing fibres were difficult to separate from 2 days of storage. When samples were suspended in water they formed swollen gels from 7 days. The formation of swollen gels had been observed in samples of chitosan heated with saturated steam at 120ºC for 1 hour or with dry heat at 160º for 2 hours [1].

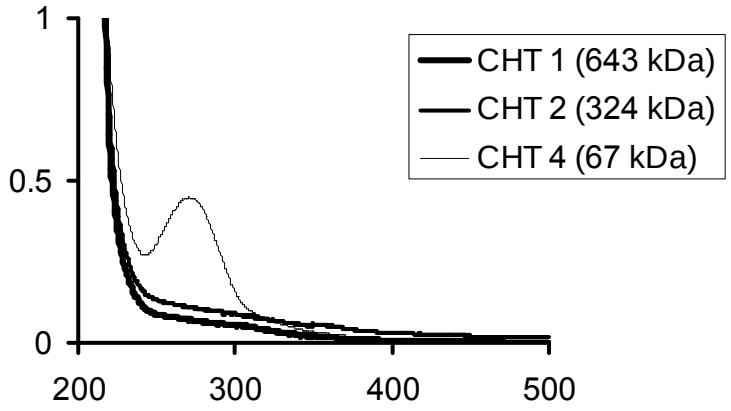

Figure 1. UV/VIS absorption spectra of chitosan samples of different average Mw.

The appearance of an absorption band between 280 and 300 nm exhibiting featureless end absorption in UV/VIS spectrum has been reported in the study of some melanoidin-type colorants [5]. In spite of extensive studies, the chemical species responsible of the brown coloration of the Maillard reaction products remains undefined but the appearance of the described band seems to be caused by the chromophore groups of the coloured precursors of the melanoidins. This band has been observed in the analysis of glucosamine, mixtures of chitosan and glucose or chitosan solutions heated at 105º C under 15 psi [10]. Figure 1 shows the spectra of the aqueous solution of three chitosans of different average Mw before the storage. CHT 4 presented a band that could correspond to the one mentioned before. The relatively short average chain length was unlikely to produce this different profile: the samples of chitooligosaccharides obtained by enzymatic depolymerization did not show this band whereas the same profile was found in CHT 5 (results not shown). This showed that the preparation of the sample was decisive: CHT 4 and CHT 5 had been obtained by nitrous acid depolymerization.

The mechanism of the nitrous acid depolymerization involves a deamination of a deacetylated unit forming 2,5-anhydro-d-mannose at the new reducing end [7]. The new aldehyde group is more available for reactions since it does not participate in intramolecular hemiacetals. Tommeraas et al. [8] have found that during the lyophilisation of trimers of 2-amino-2-deoxy-D-glucopyranose with this reducing end dissolved in a buffer containing ammonium acetate, a Schiff base reaction analogous to that of the Maillard browning occurs. It seems that the amino groups are deprotonated due to the increase of pH to give a strong nucleophile, which can react with the aldehyde of the reducing end. This produces a reversible imino bond with the release of a water molecule, and, as water evaporates during lyophilisation, the reaction is facilitated. These authors could detect the Schiff base in the ^{1}H-NMR spectrum of the samples at pH 5.7. When pH was 4, below the pK_a value of the amino group, they observed the chemical shifts corresponding to 5-hydroxymethylfurfural (5-HMF), a common by-product of Maillard browning produced by two eliminations of water followed by chain cleavage. In the ^{1}H-NMR spectrum of CHT 4 at acidic pH we could detect a chemical shift corresponding to the imino proton of a Schiff base and four signals corresponding to 5-HMF (Figure 2 and Table 1).

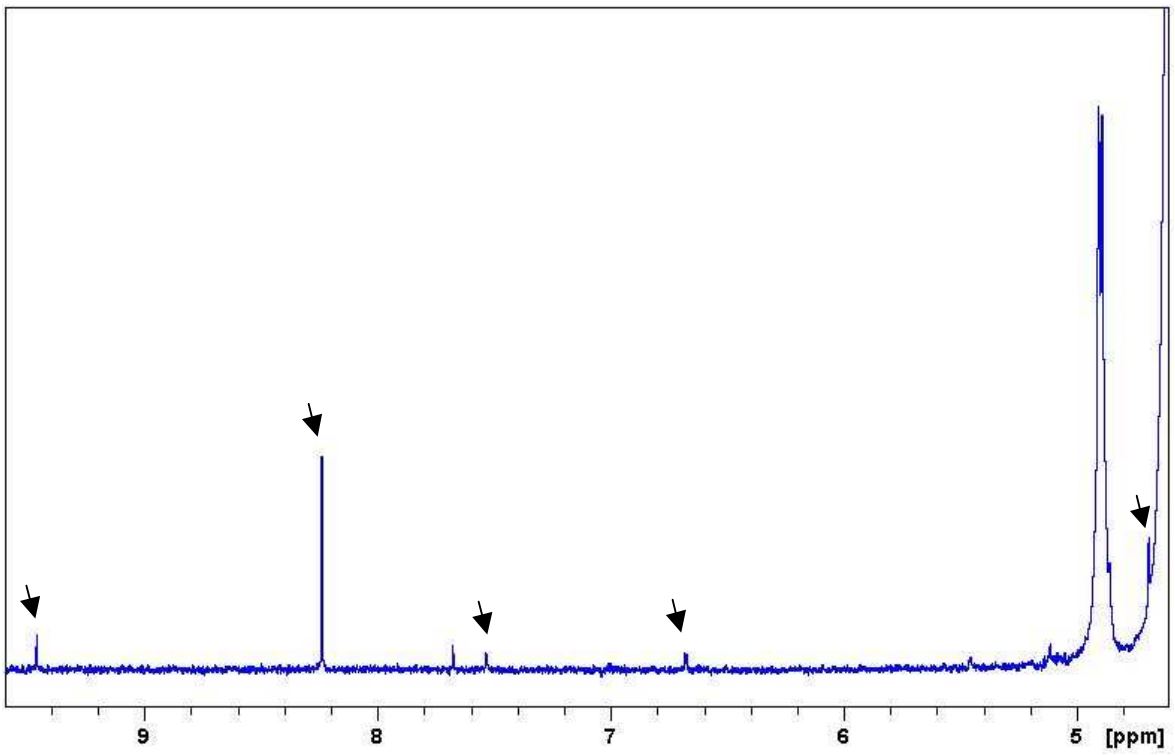

Figure 2. Selected area of the ^{1}H-NMR spectrum (500.14 MHz) of CHT 4. The resonances of the imino proton of a Schiff base and HMF are indicated

The occurrence of these different species can be due to the heterogeneity of the molecules present in our chitosan sample, which most probably have different rates of reaction. These chemical shifts could not be observed in the ^{1}H-NMR spectra of CHT 1 and CHT 2. Based in these results the presence of the active reducing ends followed by the conditions of the lyophilisation are responsible of the occurrence of the Maillard reaction in CHT 4 and 5 before the storage.

Table 1. ^{1}H NMR (500.14 MHz) chemical shifts (ppm) of the marked signals of CHT 4 in Figure 2.

Schiff base	H-1: 8.23			
HMF	H-1: 9.48	H-3: 7.53	H-4: 6.68	H-6: 4.69

The browning of chitooligosaccharides obtained by enzymatic reaction has been described in similar conditions and the occurrence of a reaction between free amino groups and reducing groups was supported by the fact that they showed less progress of browning when treated with a reducing agent [9]. In this case the presence of the adsorption band at time 0 was attributed to the high number of reducing groups in the samples. We could find any evidence of the occurrence of the Maillard reaction in our oligomers fractions.

In order to gain a greater understanding on the effects of dry heating on chitosan produced by nitrous acid depolymerization we analyzed the samples of CHT 4 submitted to storage. The UV/VIS spectra of the aqueous solutions of samples showed an increase of the band corresponding to melanoidin-type colorants (results not shown). However from 2 days of storage it was not possible to obtain the spectra because there was undissolved material. In order to remove the insoluble aggregates it was decided to filter the samples with 0.45 µm pore-sized cellulose acetate membranes. With the aim to quantify this step the soluble fraction was weighed once freeze-dried. Figure 3 shows the decrease in water soluble material found in the samples of chitosan stored from 1 to 14 days in terms of percentage. A sharp decrease occurs during the first 24 hours while after this time the decrease flattens. The absorption spectra of the filtered samples presented the band attributed to the Maillard reaction products and it decreased with time of storage. The absorption at 278 nm was used to plot the decrease of this peak versus time of storage (Figure 3). It was concomitant with the decrease of soluble material which showed removal of coloured macromolecules by the filtration.

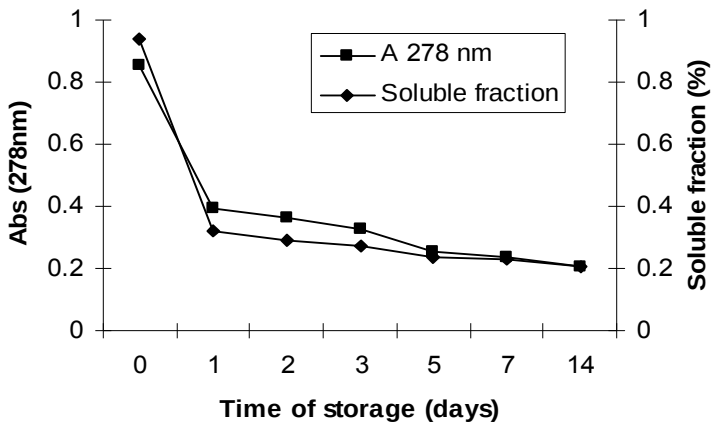

Figure 3. Plot of the absorption at 278 nm and soluble fraction of CHT 4 after filtration in terms of percentage versus time of storage.

The ^{1}H NMR spectra of the stored samples showed the signals corresponding to both the Schiff base and the 5-HMF, although they could not be used to obtain a reliable quantification (results not shown).

In an effort to investigate the chemical changes occurring during the storage the colorimetric assay of chitosan with Cibacron Brilliant Red was performed. Figure 4 shows the plot of the determination of chitosan in terms of percentage of the weighed sample versus time of storage. This method is based on the reaction of this dye with the chitosan protonated groups. A decrease in the number of protonated groups can be observed, more pronounced in the first 2 days of reaction. This could be attributed to the crosslinking reactions involving the amino groups, which are available any more for reaction with the dye.

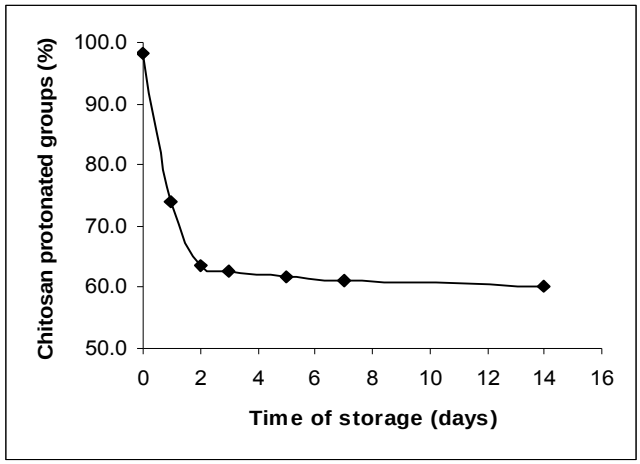

Figure 4. Colorimetric assay of CHT 4 in terms of percentage of protonated groups determined.

Conclusions

Samples of chitosan prepared by nitrous acid depolymerization have shown an absorption band and chemical shifts in the NMR spectrum which can be attributed to the linkage of the reactive reducing ends with the amino groups in a Maillard-type reaction which is facilitated by the lyophilisation process. During dry heating storage these samples showed a browning and decrease of aqueous solubility that can be the consequence of the progress of the Maillard reaction. Based of the colorimetric determination the amino groups are likely to be involved in a crosslinking of chitosan molecules. A deeper characterization of the obtained products is needed to investigate the chemical changes occurring in these systems.

Acknowledgements

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