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Chemical modifications of carboxylated chitosan

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C-6-carboxylated chitosan obtained by oxidation of chitosan was selectively modified in order to obtain derivatives similar to bacterial antigens. Selective *O*-acetylation of 6-carboxyl chitosan afforded a modified polysaccharide with the 2-amino group available for further modifications to create carbonyl groups. A deaminative degradation reaction allowed the formation of oligosaccharides with terminal aldehyde groups. Reductive alkylation with lactose introduced lactityl branches which were oxidized with galactose oxidase to give aldehyde groups in its D-galactose residues. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Chitosan, a linear polysaccharide of 2-amino-2-deoxy-D-glucopyranose is an abundant resource obtained by *N*-deacetylation of chitin. Chitosan and derivatives are important in various biomedical and pharmaceutical applications (Sandford & Hutchings, 1987; Hirano *et al.*, 1987). Many selectively modified chitosan products have shown unique biological activities and physicochemical properties. Thus, the high blood anticoagulant activities of sulfated derivatives of chitosans and 6-carboxylmethylchitosans have been reported by Hirano *et al.* (1985). Yalpani and Hall (1984) by reductive alkylation of chitosan with a series of reducing sugars obtained branched derivatives soluble in water. Recently, Zhang and Hirano (1995) reported the preparation and physico-chemical properties of *N*-unsaturated higher fatty acyl derivatives of chitosan.

Many bacteria such as *Neisseria meningitidis* group B, *Streptococcus* group B, *Haemophilus influenzae* types d and e and *Salmonella typhi*, produce extracellular polysaccharides which contain amino and carboxyl groups in the repeating moiety (Jennings, 1983) *Salmonella typhi* synthesizes the Vi antigen, a polymer of *N*-acetyl-2-amino-2-deoxy-D-galacturonic acid, 90% acetylated on carbon 3 which is used as human vaccine (Tacket *et al.*, 1986).

Horton and Just (1973) treated chitosan with chromium trioxide in the presence of perchloric acid and obtained, by protection of the secondary hydroxyl group, the selective oxidation of the primary alcoholic function on carbon 6. The 2-amino group in the carboxylated chitosan provides a convenient function for selective modifications.

In this work, selective modifications of the oxidized chitosan in order to obtain carbonyl groups capable of further modifications such as reductive amination with proteins are presented.

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EXPERIMENTAL

Materials

Chitosan was purchased from Sigma Chemical Co. and used without further purification. It was characterised by FT-IR and FT-Raman spectroscopies. Vi antigen of *Salmonella typhi* was a gift from Biomanguinhos, Instituto Osvaldo Cruz, Brazil.

Methods

FT-IR spectra of KBr pellets were recorded in the 4000–400 cm^{-1} region using a Bruker IFS 66v instrument. Thirty-two scans were taken with a resolution of 4 cm^{-1}. Derivation, including Savitzky–Golay algorithm (Maddam & Mead, 1982) with 25 smoothing points was performed using the OPUS/I.R. version 1.4 software incorporated into the hardware of the instrument. Raman spectra of the powdered samples packed into stainless-steel cups were recorded in the 3500–10 cm^{-1} region with a Bruker FRA 106 equipment interfaced to the IFS spectrometer. A

Nd:YAG laser of wavelength 1064 nm was used. Fifty scans were taken with a 200 mW power.

^{13}C NMR spectra were recorded at 22°C in D_2O/H_2O (1:1 v/v) at 52 MHz on a Bruker 200 spectrometer using DSS as internal reference. Microanalysis were performed in Laboratorio de Recursos Renovables of Universidad de Concepción, Chile.

Oxidation of chitosan

Chitosan was oxidized according to Horton and Just (1973). Briefly, chitosan (4.6 g) in acetic acid glacial 100% was freed of most of the water by repeated evaporations. Acetic acid (580 ml) and 60% perchloric acid (5.5 ml) were added with stirring, followed by 1.38 g of chromium trioxide dissolved in 2.0 ml of water and 14 ml of acetic acid. After stirring for 30 min at room temperature, the same amount of oxidant was added, and after 1 h 0.92 g of chromiun trioxide in 35 ml of water. The precipitate was dissolved in water and neutralized with 0.05 N NaOH. A white solid (30.0% yield) insoluble in water and methyl alcohol was obtained. Anal. Calc. for $[(C_8H_{13}NO_5)_{0.3}\cdot (C_6H_9NO_5)_{0.7}\cdot 1.25\,H_2O]_n$: C, 38.46; H, 6.07; N, 6.79. Found: C, 38.39; H, 6.22; N, 6.77.

Partial hydrolysis of the oxidized chitosan

The oxidized chitosan (0.1 g) was heated for 1 h at 90°C with 37 ml of 0.08 M HCl, cooled and poured into 185 ml of acetone. The precipitate was separated by centrifugation, washed three times with fresh acetone, dissolved in water and freeze-dried.

Schiff base of the oxidized chitosan

The oxidized chitosan (0.64 g) was suspended in 30 ml of methyl alcohol and 1.7 g of *p*-chlorobenzaldehyde (Fluka) in 3.0 ml of methyl alcohol was added. The mixture was stirred for 16 h at room temperature and the solid was filtered off, washed with methyl alcohol and dried. The dried material was extracted in a Soxhlet for 10 h with methyl alcohol, washed with diethyl ether and dried *in vacuo* to give a yellow solid (79.8% yield). Anal. Calc. for $[(C_8H_{13}NO_5)_{0.3}\cdot (C_{13}H_{12}NO_5Cl)_{0.7}\cdot 2H_2O]_n$: C, 47.39; H, 5.59; N, 4.80. Found: C, 47.04; H, 5.40; N, 5.34.

Selective *O*-acetylation of the Schiff base

The Schiff base of the oxidized chitosan (0.86 g) was stirred with anhydrous pyridine (17.3 ml), acetic anhydride (2.6 ml) and triethylamine (0.17 ml) for 96 h. Diethyl ether (50 ml) was added and the solid was filtered off and washed with diethyl ether.

Hydrolysis of the *O*-acetylated Schiff base

The product of the preceding experiment was stirred at room temperature with 0.5 M HCl (50 ml) for 24 h. To the resulting solution 130 ml of ethyl alcohol were added and the mixture was stirred for 24 h. The solid was filtered off, washed thoroughly with diethyl ether and dried *in vacuo* to give (with 55.4% yield) a white solid, soluble in water. Anal. Calc. for $[(C_{12}H_{17}NO_7)_{0.3}\cdot (C_8H_{11}NO_6)_{0.7}\cdot 3H_2O]_n$: C, 37.80; H, 6.43; N, 4.79. Found: C, 37.70; H, 6.09; N, 4.71.

***N*-acetylation of the *O*-acetylated oxidized chitosan**

The *O*-acetyl derivative of the oxidized chitosan (0.050 g) was stirred for 96 h with anhydrous pyridine (1.0 ml), acetic anhydride (0.16 ml) and triethylamine (0.02 ml). The solid was centrifuged, washed with diethyl ether and dried.

Deamination

The *O*-acetyl derivative of the oxidized chitosan (0.050 g) was dissolved in 30 ml of distilled water and 12 ml of a 50% aqueous solution of sodium nitrite and 3.7 ml of acetic acid were added. After 2 h methyl alcohol (25 ml) was added and the solution was evaporated *in vacuo*. The residue was dissolved in a minimum volume of water and applied on a column (80×2.0 cm i.d.) of Sephadex G-75. The column was eluted at 5 ml/h with distilled water. Fractions (2 ml) were collected, assayed with phenol–sulfuric acid reagent (Dubois *et al.*, 1956) and pooled. Pools were freeze-dried. The void volume (58 ml) was determined using blue dextran 2000.

Reductive alkylation

The oxidized chitosan (0.4 g) was suspended in 20 ml of methyl alcohol–acetic acid (3:1 v/v) and 1.33 g of lactose in 15 ml of water and 1.0 g of sodium cyanoborohydride (Sigma) were added. The mixture was stirred for 6 days at room temperature, filtered and the solid was washed exhaustively with methyl alcohol and dried to give (43.9% yield) a white powder, insoluble in water. Anal. Calc. for $[(C_8H_{13}NO_5)_{0.3}\cdot (C_{18}H_{31}NO_{15})_{0.28}\cdot (C_6H_9NO_5)_{0.42}\cdot 2.25H_2O]_n$: C, 37,92; H, 6.95; N, 4.44. Found: C, 37.89; H, 6.95; N, 4.50.

Oxidation with galactose oxidase

The reductive alkylation product was stirred for 24 h with 10.0 ml of phosphate buffer (pH 7.0) at 25°C. Then, catalase (Sigma) (14000 U) and galactose oxidase (Sigma) (90 U) were added and the mixture was incubated at 25°C for 72 h. The reaction mixture was

extracted four times with 1 ml of a 1% aqueous phenol solution and centrifuged. The residue was washed with ethyl alcohol (4×10 ml), diethyl ether and dried *in vacuo* to give a white powder with 82.8% yield.

RESULTS AND DISCUSSION

The degree of *N*-acetylation of commercial chitosan determined by FT-IR spectroscopy according to Domszy and Roberts (1985) was 30.0%. The FT-Raman spectrum of chitosan showed characteristic absorption bands at 3298.7 cm^{-1} assigned to N–H stretching, at 2886.2 cm^{-1} assigned to C–H stretching, at 1458.4 cm^{-1} assigned to C–N stretching of a primary amine and at 1378.2 cm^{-1} due to the C–O deformation of a secondary alcoholic group and at 896.7 cm^{-1} characteristic of a C–H vibration of β-anomeric residues (Mathlouthi & Koenig, 1986).

Treatment of chitosan with CrO_3 allowed the introduction of a carboxyl function. The FT-IR spectrum of the oxidation product showed a shoulder at 1700 cm^{-1}, while the second-derivative spectrum had a band at 1707 cm^{-1} (C=O stretching of carboxyl group). Partial hydrolysis with 0.08 M HCl gave with 90% yield, a water-soluble product which was analysed by ^{13}C NMR spectroscopy. Data are shown in Table 1. Two signals are present in the carbonyl region, assigned to carboxyl and *N*-acetyl carbonyl carbons. The presence of a signal at 60.82 ppm due to primary alcoholic carbon (Zhang and Hirano, 1995) suggests that the *N*-acetylglucosaminyl residues were not oxidized. The microanalysts' results agree with this assumption and indicate that all of the free glucosaminyl residues were selectively oxidized giving the 6-carboxylated chitosan.

The selective *O*-acetylation on C-3 of the carboxylated chitosan was carried out protecting the amino group by formation of a Schiff base (Moore & Roberts, 1982). Reaction of the carboxylated chitosan with *p*-chlorobenzaldehyde gave with good yield, a light yellow solid insoluble in water and methyl alcohol. Its FT-IR spectrum showed a weak absorption band at 1643.5 cm^{-1} (C=N stretching of a Schiff base) and the characteristic bands of the aromatic ring. On the other hand, the FT-Raman spectrum shows a strong signal at 1643.7 cm^{-1} (C=N stretching) in addition to the signals at 1593.9 cm^{-1} (C=C stretching aromatic ring) and 1220.8 cm^{-1} (C–H deformation of aromatic ring (Fig. 1). Acetylation of the Schiff base afforded (with 87.7% yield) a yellow solid that showed new bands at 3089.4 cm^{-1} (C–H), 1747.1 cm^{-1} (C=O) and 1229 cm^{-1} (C–O) in the FT-IR spectrum, assigned to the acetyl group. Mild hydrolysis with HCl gave a water-soluble compound with 55.4% yield. Its FT-IR spectrum showed no absorption bands due to the aromatic imino group but a band at 1657.2 cm^{-1} assigned to the C=O stretching of the acetyl group. The ^{13}C NMR spectrum (Table 1) showed three bands in the carbonyl carbon region indicating the introduction of the acetyl function. *N*-acetylation with acetic anhydride gave a modified polysaccharide with the same substitution pattern as the Vi antigen of *Salmonella typhi* (Fig. 2). Their FT-IR spectra are very similar (Fig. 3). The FT-IR spectrum of acetylated 6-carboxyl chitosan showed a band at 1744.3 cm^{-1} that in the second derivative spectrum is resolved in two signals at 1754.3 cm^{-1} (C=O stretching of acetyl group) and 1724.7 cm^{-1} (C=O stretching of carboxyl group). It is noteworthy that the spectrum of Vi polysaccharide also shows only an absorption band

Table 1. ^{13}C NMR data for carboxylated chitosan (A) and its 3-*O*-acetyl derivative (B)

Assignment	^{13}C (ppm)	
	A	B
C_1	98.37	98.09
C_2	56.67	54.74
C_3	70.94	71.26
C_4	77.09	—
C_5	75.57	—
C_6	60.82	60.66
COOH	176.71	174.11
CH_3CON	175.65	171.03
CH_3CON	22.88	21.18
CH_3COO	—	165.68
CH_3COO	—	13.29

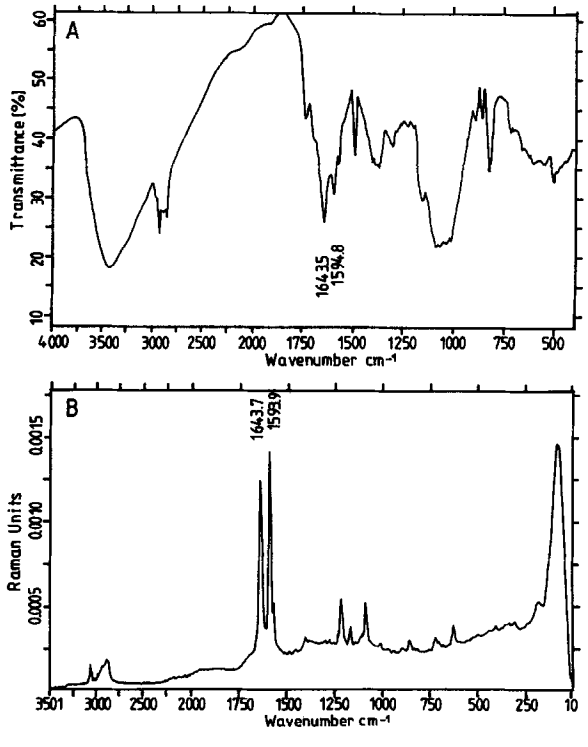

Fig. 1. FT-IR and FT-Raman spectra of the Schiff base of carboxylated chitosan.

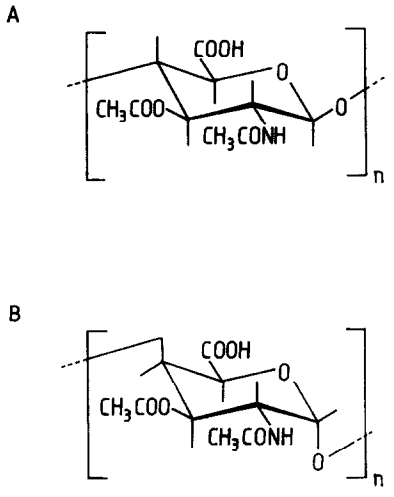

Fig. 2. Primary structures of (A) *N,O*-diacetyl derivative of carboxylated chitosan. (B) Vi antigen of *Salmonella typhi.*

around 1740 cm^{-1}, while the second derivative spectrum shows two absorption bands at 1743.1 and 1722.1 cm^{-1}.

As found earlier (Michell, 1990; Matsuhiro & Rivas, 1993) FT-IR spectra in the second derivative mode gave more resolved signals and constitute an excellent tool for monitoring chemical modifications of polysaccharides in the solid state. FT-Raman spectroscopy gave complementary information and when combined with FT-IR can be used to extensively characterize polysaccharides.

Deamination of carboxylated chitosan

The deamination reaction with nitrous acid of glycosaminoglycans has been used for the quantification of amino sugars and for structural studies (Cifonelli, 1976). Deaminative cleavage of basic polysaccharide at pH 4.5 provides oligosaccharides with terminal aldehyde groups (Riesenfeld & Rodén, 1990).

Size-exclusion chromatography of the deamination reaction product gave five fractions. The main fraction (36.8% yield) was analysed by FT-IR spectroscopy. The spectrum shows a weak signal at 1789.2 cm^{-1} assigned to the C=O stretching of an aldehyde function, while the second derivative spectrum presents in the carbonyl region three signals at 1789.5, 1772.1 (C=O stretching of acetyl and 1734.1 cm^{-1} (C=O stretching of carboxylate). At 835.6 cm^{-1} both spectra present a strong signal assigned to the C–O–C stretching of the 2,5-anhydromannosyl residue (Tipson & Parker, 1980).

The formation of the aldehyde function was corroborated by formation of a derivative with *p*-chloroaniline. The FT-IR spectrum of the reaction product showed the characteristic bands of the Schiff base at 1641.3 cm^{-1} (C=N stretching of the imino group), 1591 cm^{-1} (C=C stretching of the aromatic

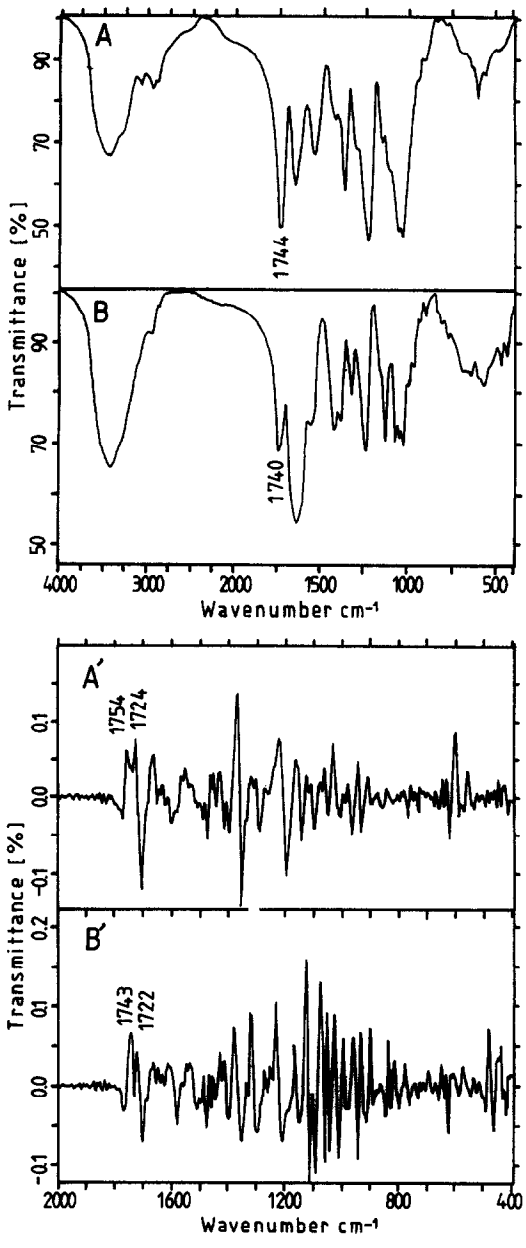

Fig. 3. FT-IR spectra of (A) *N,O*-diacetyl derivative of carboxylated chitosan; (B) Vi antigen of *Salmonella typhi.* A', second derivative of A; B', second derivative of B.

residue), 1506.5 cm^{-1} (C=C deformation), 874 cm^{-1} (1–4 substitution in the aromatic ring) and 755.4 cm^{-1} (C–Cl stretching).

Reductive alkylation and enzymic oxidation

Reaction of carboxylated chitosan with lactose under reductive conditions gave a modified polysaccharide with a secondary amine function. Unlike the lactitylchitosan reported by Yalpani and Hall (1984), the reaction product was insoluble in water. The second derivative FT-IR spectrum showed a new band at 1631.2 cm^{-1} assigned to the N–H deformation vibration of a secondary amine. Microanalysis results

indicated that approximately 40% of the free amino groups were alkylated. The presence of lactityl branches was corroborated by enzymic oxidation with galactose oxidase and creation of an aldehyde function in position 6 of the D-galactose unit. The second derivative of the FT-IR spectrum showed a new band at 1725.8 cm^{-1} assigned to the C=O stretching of an aldehyde group.

Although the lactityl 6-carboxylchitosan is not soluble in water, the oxidation reaction in heterogeneous phase allowed the introduction of aldehyde groups.

The changes introduced in the 6-carboxylated chitosan may be applied to bacterial polysaccharide modifications for the introduction of a carbonyl function which is capable of forming glycoconjugates with proteins.

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REFERENCES

Cifonelli, J. A. (1976) Chemical methods of analysis. In *Methods in Carbohydrate Chemistry*, ed. R. L. Whistler and J. N. BeMiller, Vol. 7, pp. 139–141. Academic Press, New York.

Domszy, J. G. and Roberts, G. A. F. (1985) Evaluation of infrared spectroscopic techniques for analysing chitosan. In *Makromolecule Chemie* **186,** 1671–1677.

Dubois, M., Gilles, K., Hamilton, J., Rebers, P. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. In *Analytical Chemistry* **28,** 350–356.

Hirano, S., Tanaka, Y., Hasegawa, M., Tobetto, K. and Nishioka, A. (1985) Effect of sulfated derivatives of chitosan on some blood coagulant factors. In *Carbohydrate Research* **137,** 205–215.

Hirano, S., Sato, N., Yoshida, S. & Kitagawa, S. (1987) Chemical modification of chitin and chitosan, and their novel applications. In *Industrial Polysaccharides: Genetic Engineering, Structure/Properties Relations and Applications*, ed. M. Yalpani, pp. 163–176. Elsevier, Amsterdam.

Horton, D. and Just, E. K. (1973) Preparation from chitan of (1→4)-2-amino-2-deoxy-β-D-glucopyranuronan and its 2-sulfoamino analog having blood-anticoagulant properties. *Carbohydrate Research* **29,** 173–179.

Jennings, H. J. (1983) Capsular polysaccharides as human vaccines. In *Advances in Carbohydrate Chemistry and Biochemistry* **41,** 155–206.

Maddam, W. F. and Mead, M. L. (1982) The measurement of derivatives i.r. spectra-I. Background studies. In *Spectrochimica Acta* **38A,** 437–444.

Mathlouthi, M. and Koenig, J. L. (1986) Vibrational spectra of carbohydrates. In *Advances in Carbohydrate Chemistry and Biochemistry* **44,** 7–89.

Matsuhiro, B. and Rivas, P. (1993) Second-derivative Fourier transform infrared spectra of seaweed galactans. In *Journal of Applied Phycology* **5,** 45–51.

Michell, A. J. (1990) Second-derivative F.T.-I.R. spectra of cellulose I and II and related mono- and oligosaccharides. In *Carbohydrate Research* **197,** 53–60.

Moore, G. K. and Roberts, G. A. F. (1982) Reaction of chitosan: 4. Preparation of organosoluble derivatives of chitosan. In *International Journal of Biological Macromolecules* **4,** 246–249.

Riesenfeld, J. and Rodén, L. (1990) Quantitative analysis of N-sulfated, N-acetylated, and unsubstituted glucosamine amino groups in herapin and related polysaccharides. In *Analytical Biochemistry* **188,** 383–389.

Sandford, P. A. and Hutchings, G. P. (1987) Chitosan. A natural, cationic biopolymer: commercial applications In *Industrial Polysaccharides: Genetic Engineering, Structure/Properties Relations and Applications*, ed. M. Yalpani, pp. 163–176. Elsevier, Amsterdam.

Tacket, C. O., Ferrecio, C., Robbins, J. B., Tsai, C. and Schulz, D. (1986) Safety and immunogenicity of two *Salmonella typhi* Vi capsular polysaccharide vaccines. In *Journal of Infectious Diseases* **44,** 342–345.

Tipson, R. S. and Parker, F. S. (1980) Infrared spectroscopy. In *The Carbohydrates IB*, ed. W. Pigman and D. Horton, pp. 1394–1433. Academic Press, New York.

Yalpani, M. and Hall, L. D. (1984) Some chemical and analytical aspects of polysaccharide modifications. 3. Formation of branched-chain, soluble chitosan derivatives. In *Macromolecules* **17,** 272–281.

Zhang, M. and Hirano, S. (1995) Novel N-unsaturated fatty acyl and N-trimethylacetyl derivatives of chitosan. In *Carbohydrate Polymers* **26,** 205–209.