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Chemical modifications of 1 → 4-2-amino-2-deoxy-α-D-galactan

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Abstract

Oxidation of 1 → 4-2-amino-2-deoxy-α-D-galactan (poly-α-D-galactosamine) with nitrogen oxides allowed the formation of a carboxyl group at the C-6 position. The carboxylated polysaccharide by acetylation afforded a derivative similar to the antigenic Vi polysaccharide of *Salmonella typhi*. The reaction of the modified poly-α-D-galactosamine with caprolactam assisted by carbodiimide introduced at the C-6 position a secondary amide function with a new terminal acidic group. The caproic acid residue was used as a linker for the conjugation of the modified poly-α-D-galactosamine with tetanus toxoid and with bovine serum albumin.

Keywords: Poly-D-galactosamine; Modification; Vi polysaccharide; Conjugation

1. Introduction

1 → 4-2-amino-2-deoxy-α-D-galactan, known as poly-α-D-galactosamine is obtained from the culture fluid of the microorganism *Paecilomyces* sp. I-1. Poly-α-D-galactosamine may constitute an important starting material for fine chemicals and biological active derivatives. It is known that it exhibits antitumour effect against solid tumours transplanted in mice (Ishitani, Suzuki, & Suzuki, 1988). It shows similar physicochemical properties to chitosan (1 → 4-2-amino-2-deoxy-β-D-glucan), but, the easy production of this polysaccharide and its stability against enzymes or microorganisms capable of hydrolysing glucosamine residues are advantages over chitosan (Takagi & Kadowaki, 1985).

The chemical modifications of polysaccharides allow the preparation of derivatives with new properties and a variety of applications. For example, *N*-acylation of chitosan produced materials that may be used for immobilising proteins, as media for gel chromatography and for medical and pharmaceutical purposes (Hirano, Sato, Yoshida, & Kitagawa, 1987; Hon, 1996; Tojima et al., 1999). Water-soluble derivatives of chitosan were prepared by reductive alkylation with glycoses (Yalpani, 1985) and hydrophobic derivatives were obtained by substitution of chitosan with alkyl chains with six or more carbon atoms (Desbrieres, Martínez, & Rinaudo, 1996).

Many bacteria, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Salmonella typhi* produce capsular polysaccharides with immunogenic properties. Vaccines of polysaccharide antigens are licensed as commercial products (Lee, 1996). The capsular polysaccharide of *S. typhi*, known as Vi polysaccharide is a polymer of *N*-acetyl-2-amino-2-deoxy-α-D-galacturonic acid 1 → 4 linked, and about 90% acetylated on carbon 3 (Tacket et al., 1986). It is used in humans for protection against typhoid fever, a common and serious disease in developing countries. Polysaccharide vaccines are ineffective in infants; however, the immune response is enhanced when the polysaccharide vaccines are covalently linked to protein carriers (Jennings, 1983; Lee, 1996). Conjugation of the carboxyl groups of Vi polysaccharide with the amino function of proteins resulted in low yields and poor solubility due to the conformation and the large molecular weight of the polysaccharide (Szu, Stone, Robbins, Scheerson, & Robbins, 1987). Szu et al. (1994) obtained conjugates by using linkers bound to Vi polysaccharide and to the protein. Conjugates with more immunogenic properties than the polysaccharide alone were obtained by reaction of Vi polysaccharide with adipic acid dihydrazide derivatives of the toxin of *Escherichia coli* or the recombinant exoprotein A of *Pseudomonas aeruginosa* (Kossaczka et al., 1997).

The selective oxidation of primary alcohol groups in carbohydrates is an important chemical modification, because it allows the introduction of new functions, which can be used for further modifications. The highly selective TEMPO-mediated reaction of water-soluble polysaccharides with

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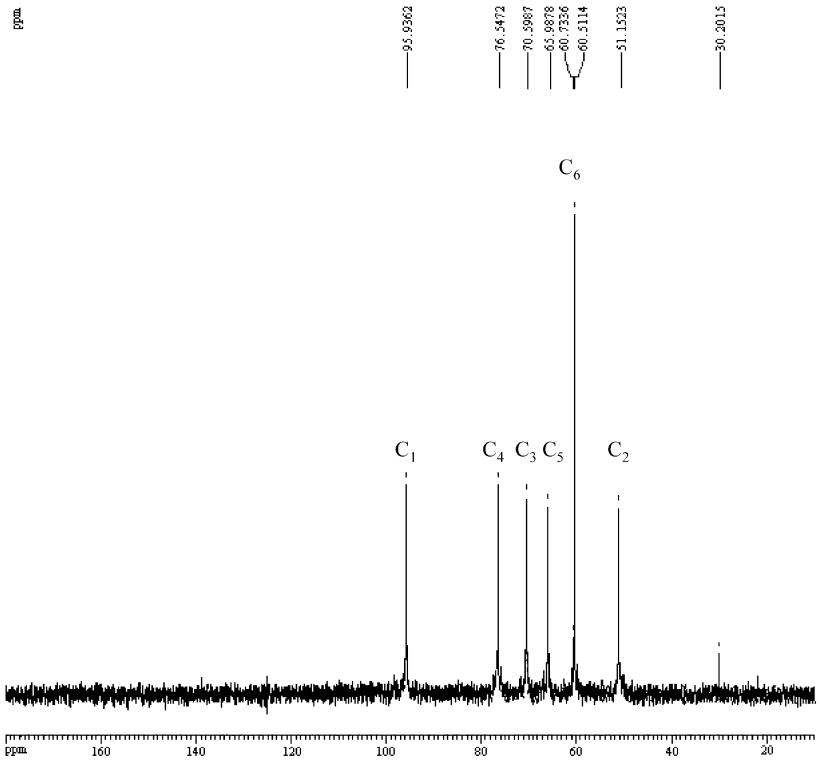

Fig. 1. ^{13}C NMR spectrum of poly-α-D-galactosamine. Acetone at 30.20 ppm as internal standard.

hypobromite affords the complete oxidation of primary alcoholic groups (de Nooy, Besemer, & Van Bekkum, 1995). Oxidation of insoluble polysaccharides, such as cellulose was accomplished with gaseous nitrogen dioxide (Nevell, 1963). It was found that the predominant reaction was the transformation of the primary alcohol function to carboxylic acid, but this reaction was accompanied by some ketone formation and partial degradation. Painter (1977) improved this reaction by dissolution of cellulose in 85% phosphoric acid and addition of solid sodium nitrite to the syrupy solution. de Nooy, Pagliaro, Van Bekkum, and Besemer (1997) reported the use of sodium nitrate as an oxidant in the presence of catalytic amount of sodium nitrite. In 85% phosphoric acid, the oxidising nitrogen oxides are formed in situ.

Horton and Just (1973) reported the selective oxidation of primary hydroxyl groups of the perchlorate salt of chitosan. Using chromium trioxide as oxidant, a quantitative conversion of the primary alcoholic group into carboxyl acid was achieved. In this laboratory, the reaction failed

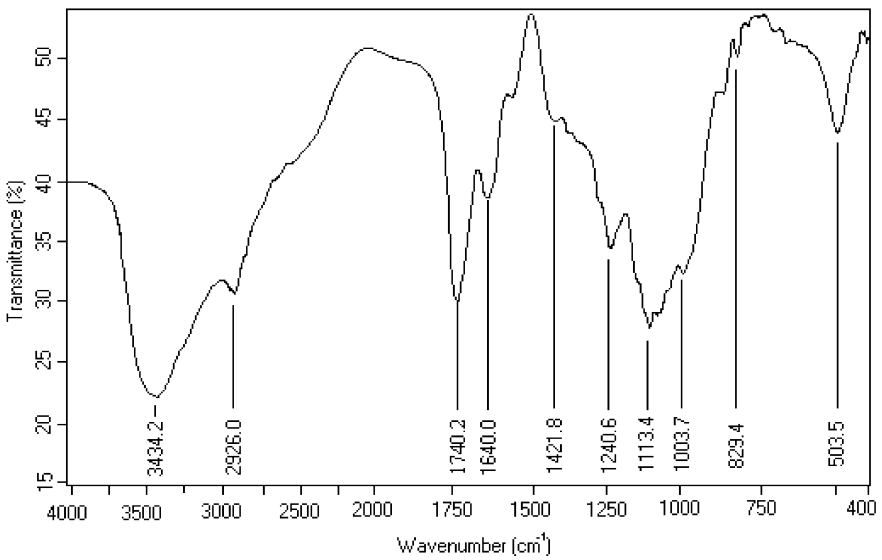

Fig. 2. FT-IR spectrum of oxidised poly-α-D-galactosamine.

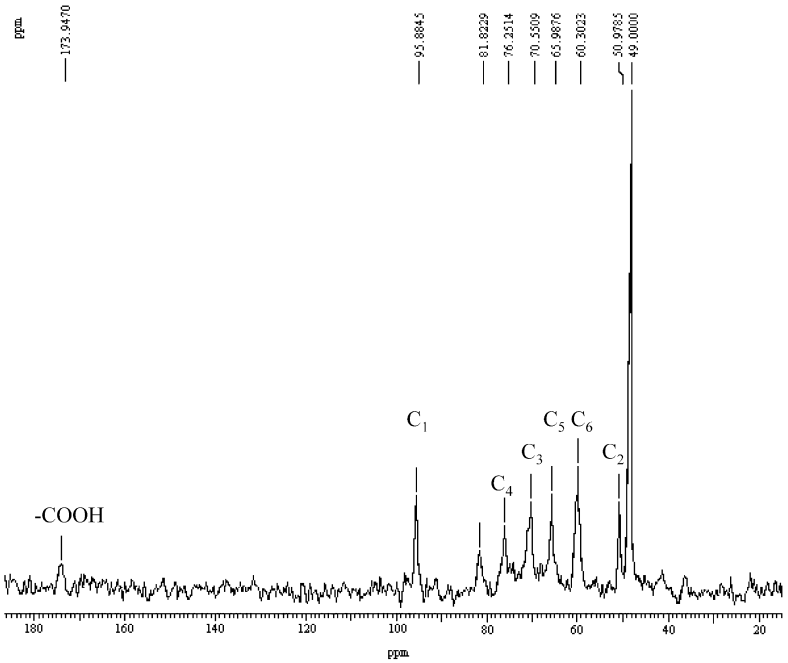

Fig. 3. ^{13}C NMR spectrum of the oxidised poly-α-D-galactosamine. Methanol at 49.00 ppm as internal standard.

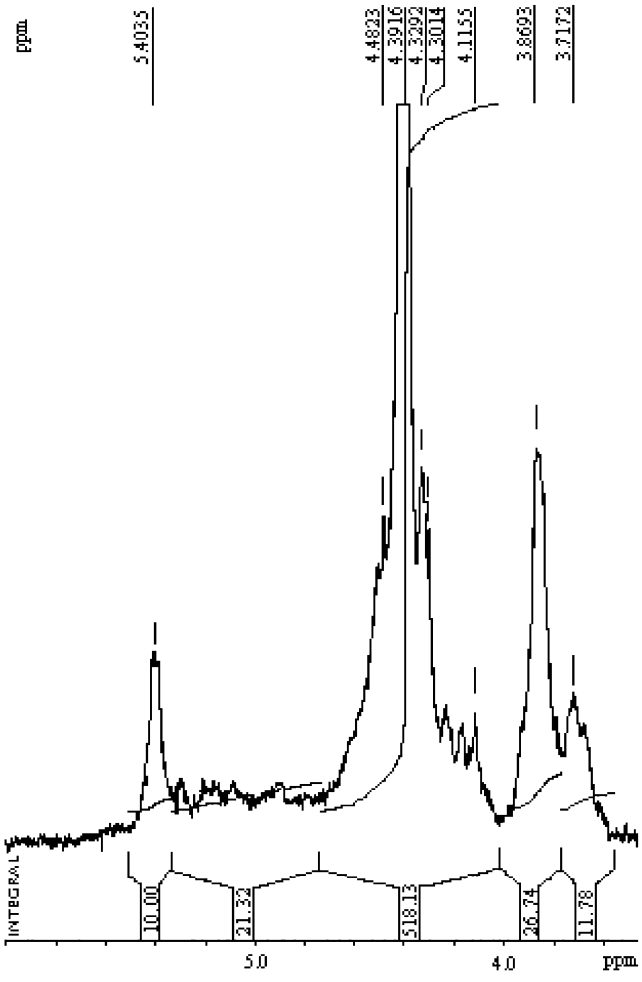

Fig. 4. ^{1}H NMR spectrum of the oxidised poly-α-D-galactosamine.

when it was applied to poly-α-D-galactosamine. The FT-IR spectrum of the product obtained by treatment of the polysaccharide with chromium trioxide in perchloric acid was identical to that of the starting material and no new bands in the carbonyl region were found. Similar results were obtained when the oxidation mediated by 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) radical (de Nooy et al., 1995) was tried. A product with an FT-IR spectrum superposible with that of the starting material was recovered in 95% of yield. TEMPO-mediated oxidation of water-insoluble polysaccharides is difficult. Chang and Robyt (1996) by TEMPO–NaBr–NaClO oxidation of cellulose obtained ~85% oxidation of primary alcoholic groups. Various cellulose samples were oxidised with this system, but only in the case of regenerated and mercerised celluloses (Isagai & Kato, 1998) and amorphous cellulose (Tahiri & Vignon, 2000) the selective oxidation of primary alcohol function was fully accomplished.

The current work presents the results of the preparation of an analog of Vi polysaccharide by chemical modification of poly-α-D-galactosamine and the introduction of a linker for the coupling to proteins.

2. Experimental

2.1. Materials

Poly-α-D-galactosamine was purchased from ICN. Caprolactam was from Aldrich Chemical Co. 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide methiodide (EDC), 2-(*N*-morpholino)ethanesulfonic acid (MES), and bovine serum albumin (BSA) (A-6918) were from Sigma. Purified

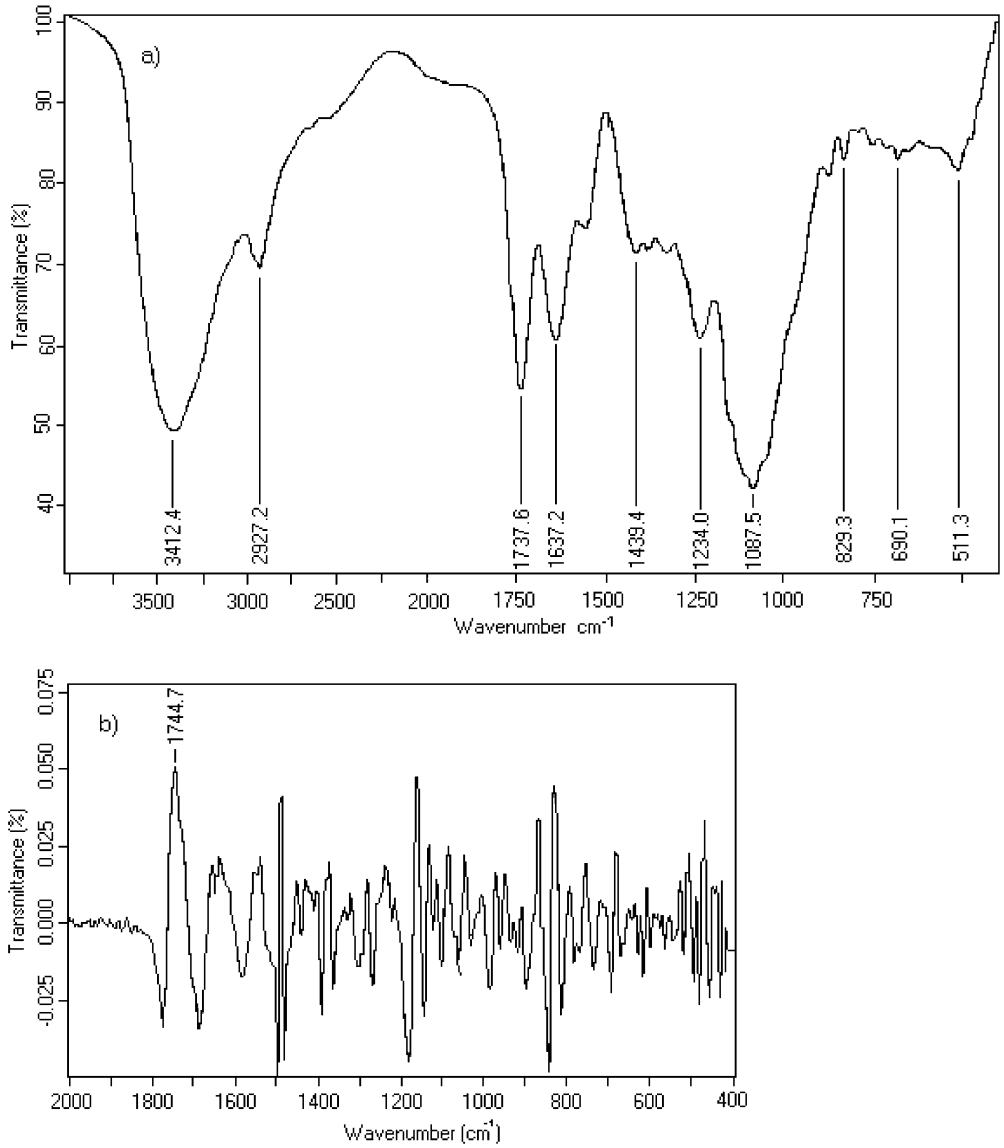

Fig. 5. (a) FT-IR spectrum of the acetylation product of oxidised poly-α-D-galactosamine and (b) second-derivative FT-IR spectrum of the acetylation product of oxidised poly-α-D-galactosamine.

tetanus toxoid of approximately 150 kDa was provided by Instituto de Salud Pública, Chile.

2.2. Methods

FT-IR spectra of KBr pellets were recorded in the 4000–400 cm^{-1} region using a Bruker IFS 66*v* instrument. Thirty-two scans were taken with a resolution of 2 cm^{-1}. Derivation, including Savitzky–Golay algorithm with 25 smoothing points was performed using the OPUS/I.R. version 1.4 software incorporated into the hardware of the instrument (Matsuhiro, 1996). ^{1}H NMR spectra were recorded at 70 °C in D_2O on a Bruker 200 spectrometer with water suppression, using external methanol as reference (δ 3.40 ppm). ^{13}C NMR spectra were registered on Bruker Avance DRX400 equipment (100 MHz) at 70 °C. Microanalysis were performed at the Facultad de Química, Universidad Católica de Chile. The amino sugar content in poly-α-D-galactosamine was determined by the Elson Morgan assay (Carney, 1986) using D-glucosamine as standard.

2.3. Partial hydrolysis of the polysaccharide

Poly-α-D-galactosamine (0.200 g) was heated for 1 h at 90 °C with 36 ml of 0.08 M HCl, cooled and poured into 100 ml of acetone. The precipitate was separated by centrifugation, washed three times with acetone, dissolved in water and freeze-dried.

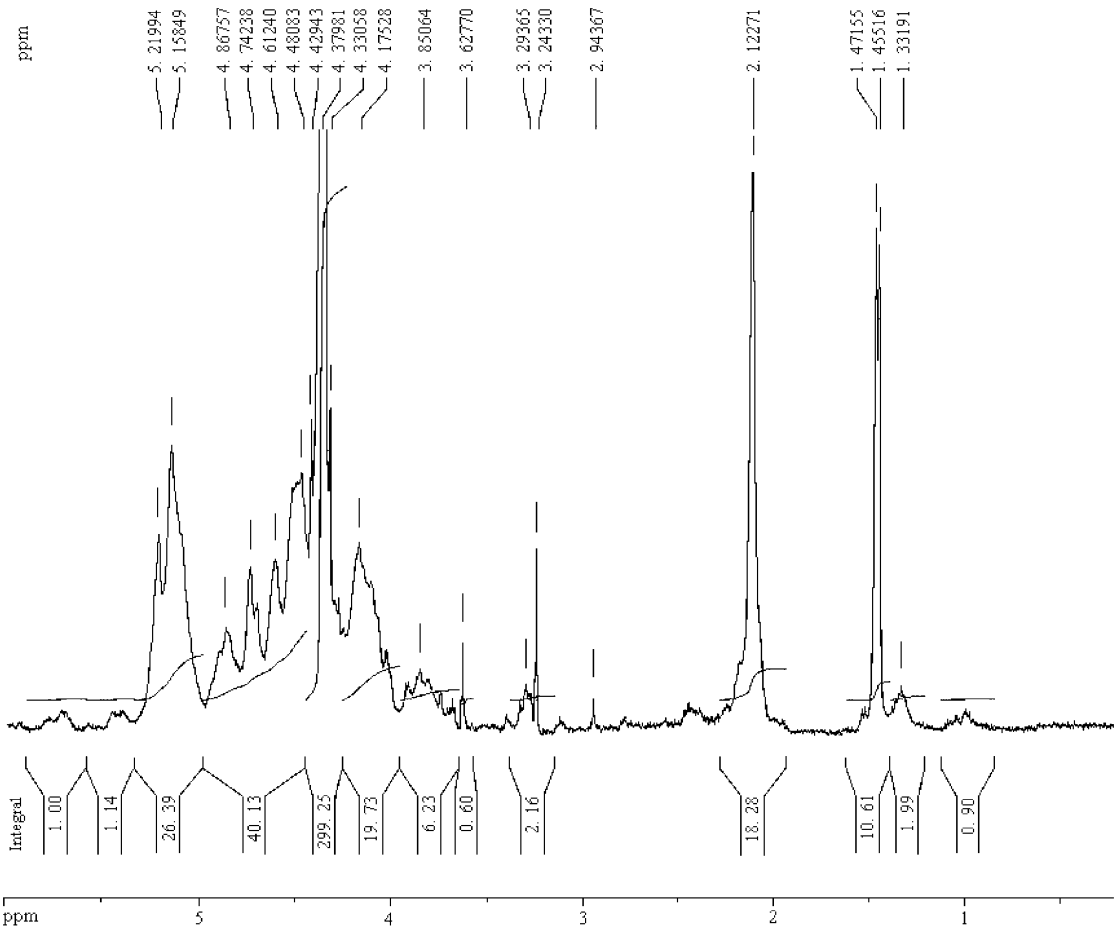

Fig. 6. ^{1}H NMR spectrum of the acetylation product.

2.4. Oxidation of poly-α-D-galactosamine with nitrogen oxides

Poly-α-D-galactosamine was oxidised according to de Nooy et al. (1997). Briefly, the polysaccharide (0.400 g) was dissolved in 30 ml of 85% phosphoric acid and, sodium nitrate (0.410 g) and sodium nitrite (0.020 g) were added. After stirring 40 h at 4 °C, the resulting solution was poured into 150 ml of ethyl alcohol. The precipitate was washed with ethyl alcohol, acetone, and dissolved in distilled water. The product was purified by dialysis, concentrated in vacuo and freeze-dried. A white solid (50% yield) soluble in water was obtained. The content of uronic acid was determined following the method described by Blumenkrantz and Asboe-Hansen (1973), using D-galacturonic acid as standard.

2.5. Acetylation of the oxidised polysaccharide

The oxidised polysaccharide obtained by the method described in Section 2.4, was stirred with 3 ml of acetic anhydride–pyridine mixture v/v and of triethylamine (0.02 ml) during 96 h. The resulting solution was poured into 50 ml of ethyl alcohol and the solid was separated by centrifugation, washed with ethyl alcohol and diethyl ether. The product was dried in vacuo to give (with 88% yield) a white solid slightly soluble in water.

2.6. Amidation with caprolactam

EDC (0.080 g) was dissolved in 2.5 ml of 65% aqueous acetone and the acetylated derivative (0.490 g) was added. The pH was adjusted to 6.5 by addition of solid sodium carbonate and 0.090 g of caprolactam in 1 ml of water was added. The resulting solution was stirred for 18 h at 25 °C and it was then dialysed extensively against distilled water, concentrated in vacuo and freeze-dried. A white solid in 62% yield was obtained.

2.7. Coupling of modified polysaccharide to protein

2.7.1. To tetanus toxoid

The modified polysaccharide–caprolactam derivative (0.020 g) and EDC (0.040 g) in 2.0 ml of buffer phosphate (pH 7.0) were mixed with a 2 ml solution of tetanus toxoid (0.020 g) in the same buffer. The resulting solution was stirred 24 h at 4 °C, dialysed against phosphate buffer (pH 7.0) for 24 h and against 0.2 M NaCl for 48 h. Afterwards, it

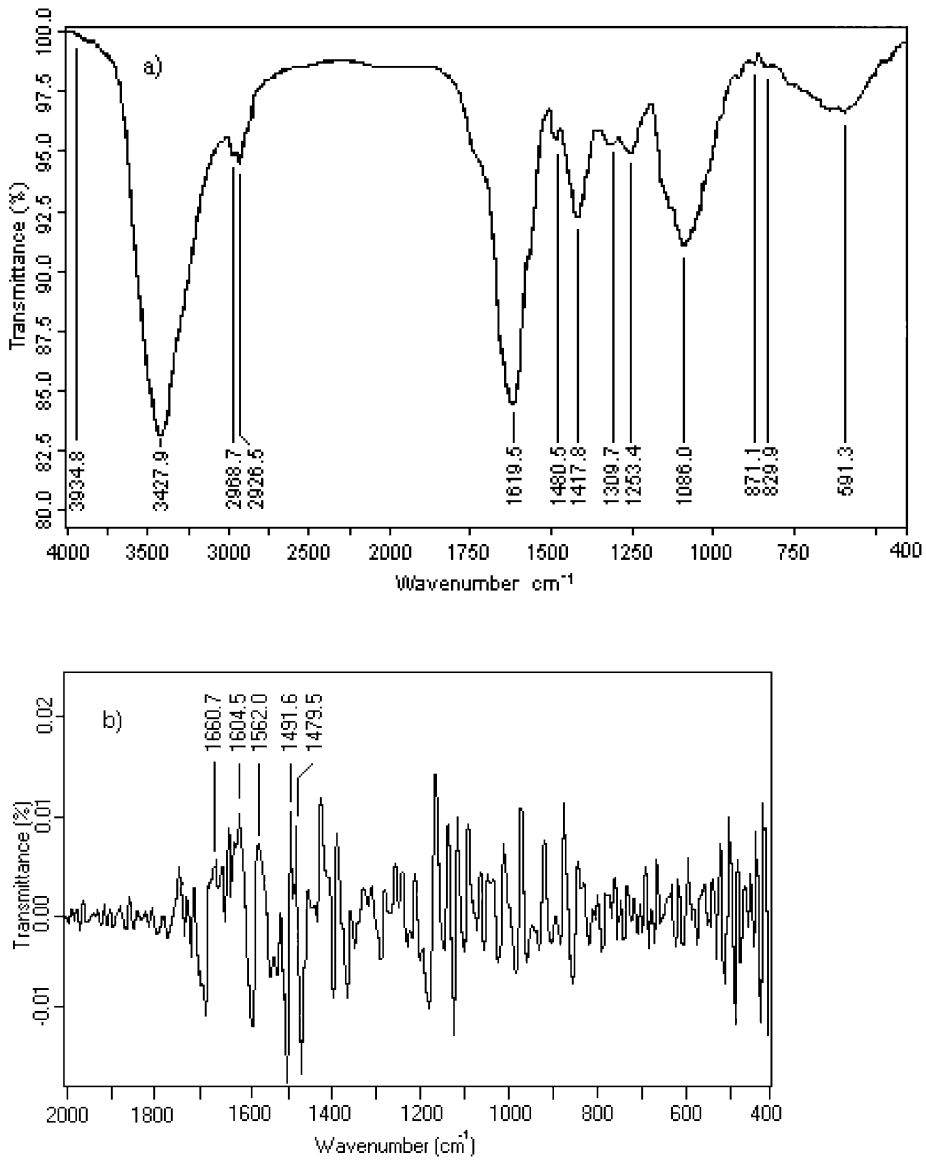

Fig. 7. FT-IR spectra of the reaction product of the modified poly-α-D-galactosamine with caprolactam: (a) normal spectrum and (b) second-derivative FT-IR spectrum.

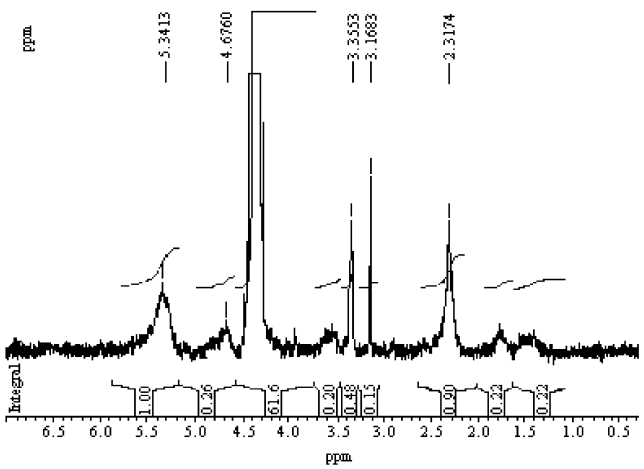

Fig. 8. ^{1}H NMR spectrum of the reaction product of the modified poly-α-D-galactosamine with caprolactam.

was chromatographed on a 1.5 × 100 cm^2 column of Bio-Gel A 0.5 using 0.2 M NaCl as eluant. Fractions of 2.5 ml were collected. The dead volume (53 ml) was determined with a 0.2% Blue dextran 2000 solution. Elution was monitored spectrophotometrically with the Bradford reagent for proteins and with the phenol–sulfuric acid reagent for sugars (Boyer, 1993; Chaplin, 1986). Aliquots of solutions of the protein and the polysaccharide–caprolactam derivative were separately chromatographed on Bio-Gel A 0.5 in the same conditions. Elutions were monitored for proteins and sugars as stated above, using BSA and glucose as standards, respectively.

2.7.2. To bovine serum albumin

The reaction was conducted as in Section 2.7.1 but at pH 5.6 employing buffer MES.

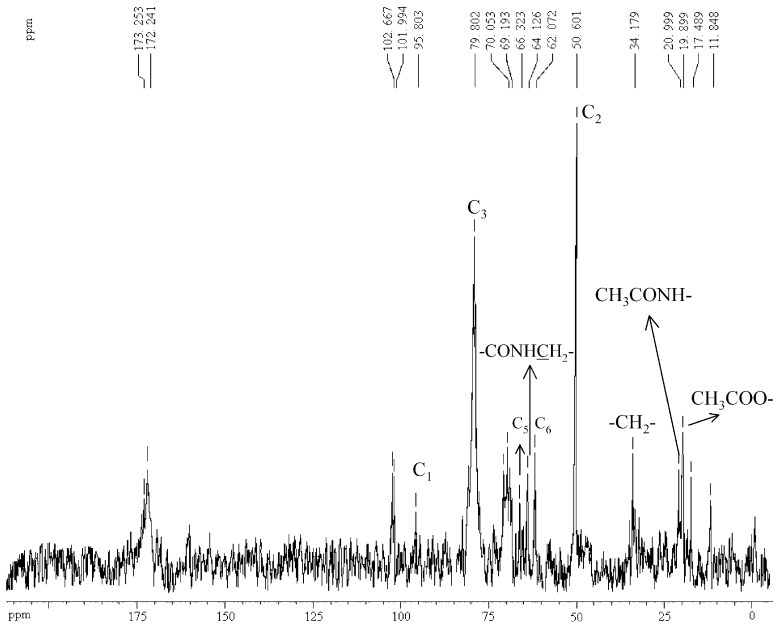

Fig. 9. ^{13}C NMR spectrum of the reaction product of the modified poly-α-D-galactosamine with caprolactam.

3. Results and discussion

Poly-α-D-galactosamine is a white solid, insoluble in water and soluble in diluted formic acid and acetic acid. Microanalysis indicated that contained 6.51% of nitrogen. The FT-IR spectrum presents characteristic absorption bands at 3404.5 cm^{-1} assigned to N–H and O–H stretchings, at 2933.8 cm^{-1} assigned to C–H stretching, at 1526.3 cm^{-1} assigned to N–H bending and, at 1384.2 cm^{-1} due to the C–O deformation of a secondary alcoholic group. The broad band centred at 1631.5 cm^{-1} is resolved into two bands, in the second-derivative mode, at 1630.1 cm^{-1} assigned to the C=O stretching vibration of the *N*-acetyl group and at 1588.9 cm^{-1} assigned to the N–H deformation vibration of a primary amine (Conley, 1966).

Partial acid hydrolysis of poly-α-D-galactosamine gave with 91% yield, a water-soluble product which was analysed by ^{13}C NMR spectroscopy (Fig. 1). The low signal at 22.0 ppm indicates the presence of an acetyl substituent. In the ^{1}H NMR spectrum, integration of the signals at δ 5.29 ppm assigned to the anomeric proton and at δ 2.11 ppm corresponding to methyl protons in the spectrum registered without water suppression indicated that the degree of acetylation was 8.8%. According to the literature 8% of the galactosamine residues in poly-α-D-galactosamine are *N*-acetylated and about 10% are N-substituted by other than acetyl groups (Takagi & Kadowaki, 1985). The amino sugar content in poly-α-D-galactosamine determined by the Elson Morgan method was 72.8%, which is in accordance with the value reported by Takagi and Kadowaki (1985).

Treatment of poly-α-D-galactosamine with nitrogen oxides in phosphoric acid allowed the introduction of a carboxyl function. The FT-IR spectrum of the oxidation product showed a strong signal at 1740.2 cm^{-1} assigned to the C=O stretching of carboxyl group (Fig. 2). The ^{13}C NMR spectrum (Fig. 3) showed two signals in the carbonyl carbon region the signal at 173.94 ppm indicates the formation of carboxylic groups (Horton & Just, 1973; Lillo & Matsuhiro, 1997), the significant decrease of the signal at 60.30 ppm is indicative that the oxidation occurred at the primary alcohol groups. The ^{13}C NMR spectrum showed no signal in the ketone carbonyl carbon region indicating that the secondary hydroxyl group on carbon 3 was not oxidised. The ^{1}H NMR spectrum of the oxidised product did not show signals in the aldehyde proton region. The relative integration values, in the spectrum registered without water suppression of the signal at 5.40 and 3.71 ppm assigned to the anomeric and the protons of the primary alcoholic group, respectively (Fig. 4), indicated that 50% of the primary alcoholic group was oxidised. The product showed to contain 50% of uronic acid by the colorimetric method of Blumenkrantz and Asboe-Hansen (1973).

Acetylation of the oxidised poly-α-D-galactosamine afforded a solid (in 88% yield) slightly soluble in water. Its FT-IR spectrum (Fig. 5(a)) shows a signal 1737.6 cm^{-1}, which in the second-derivative mode is resolved into two signals (Fig. 5(b)). Both spectra are very similar to those previously published by Lillo and Matsuhiro (1997) of Vi polysaccharide, and of the per-*O*-acetyl derivative of C-6-carboxylated chitosan. The ^{1}H NMR spectrum (Fig. 6) showed new signals at 2.12, and 1.47–1.45 ppm assigned to the methyl protons of CH_3CONH- group, and methyl protons of CH_3COO- group, respectively. The amidation of the modified poly-α-D-galactosamine with caprolactam in the presence of EDC gave a water-soluble derivative. Its FT-IR spectrum shows a new weak band at 2926 cm^{-1} (C–H

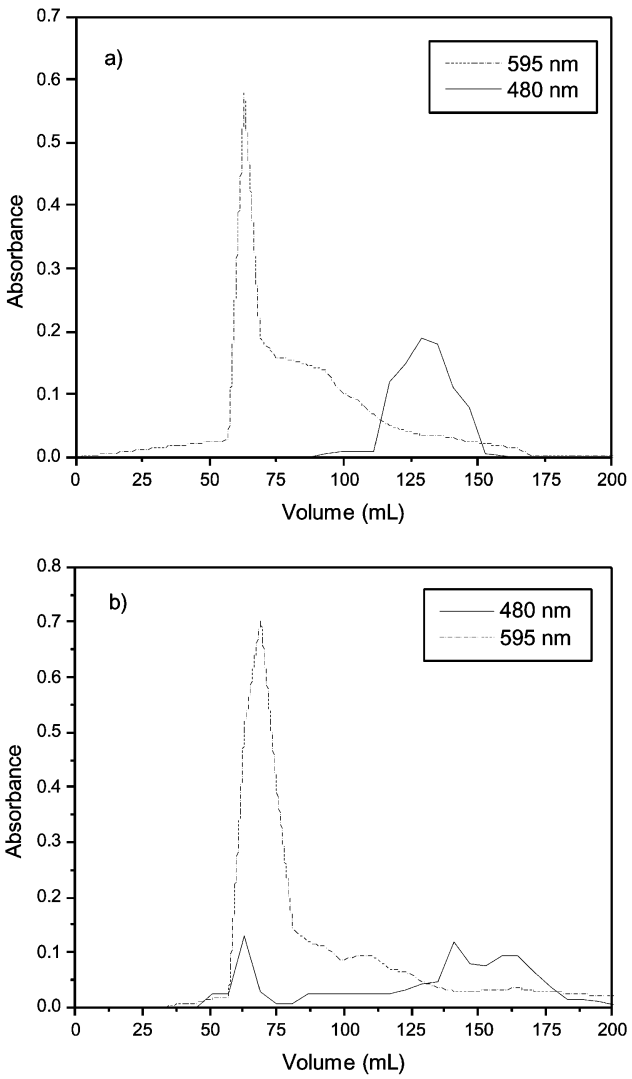

Fig. 10. Elution profiles of the chromatography on Bio-Gel A 0.5: (a) tetanus toxoid and the modified polysaccharide–caprolactam derivative and (b) the conjugation product. The protein content was monitored by the Bradford reaction (absorbance at 595 nm) and the polysaccharide content was monitored by the phenol–sulfuric acid reaction (480 nm).

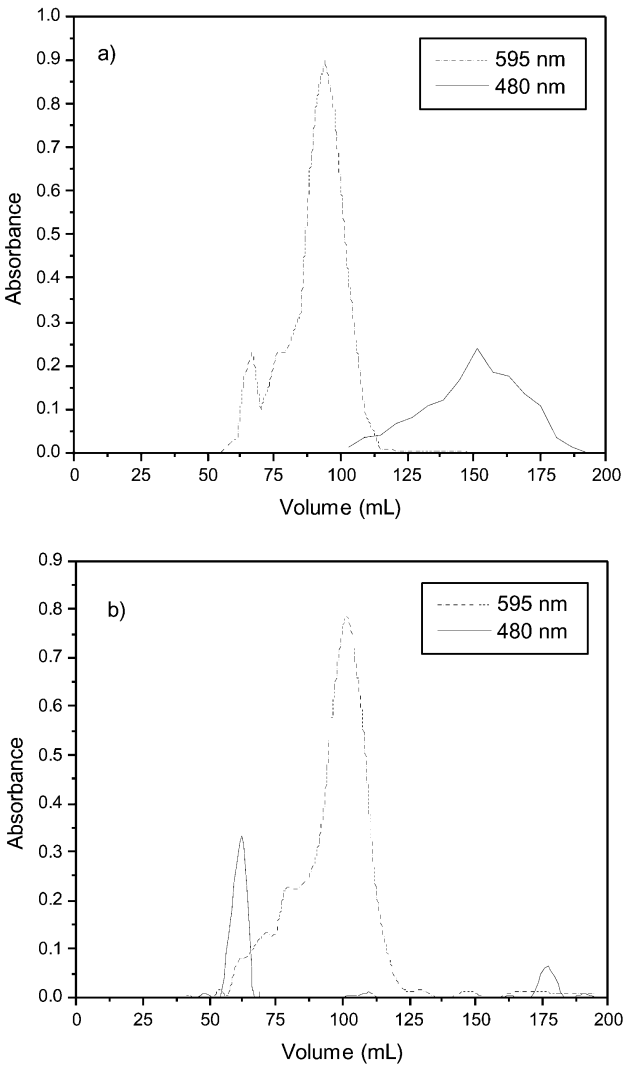

Fig. 11. Elution profiles on Bio-Gel A 0.5 of: (a) BSA and the modified polysaccharide–caprolactam derivative and (b) the conjugation product. The protein content was monitored by the Bradford reaction (absorbance at 595 nm) and the polysaccharide content was monitored by the phenol–sulfuric acid reaction (480 nm).

stretching of CH_2 groups) (Fig. 7(a)). In the second-derivative spectrum (Fig. 7(b)), the strong signal at 1619.5 cm^{-1} is resolved in four signals. The signal at 1660.7 cm^{-1} was assigned to the C=O stretching of an amide, the signal at 1604.5 cm^{-1} to the C=O stretching of carboxylate, and the signal at 1562 cm^{-1} to the amide II band. The signal at 1630.0 cm^{-1} could not be assigned. The second-derivative spectrum shows also, two signals at 1491.6 and 1479.5 cm^{-1} assigned to the deformation of the methylene groups of linker. The ^1H NMR spectrum (Fig. 8) shows signals at 5.34 ppm (H_1), 3.35 ppm (H_6), 2.31 ppm (CH_3), and, at 3.16 ppm, assigned to the methylene protons linked to the amide ($-CH_2-NH-CO-$) group (Silverstein, Bassler, & Morrill, 1991) and no well resolved signals around 1.8 and 1.5 ppm which are indicative of the presence of an aliphatic side chain. The ^{13}C NMR spectrum (Fig. 9) corroborates the amidation of the carboxyl group and the introduction of the side chain.

Amidation reaction of the carboxyl residue of the modified polysaccharide–caprolactam derivative with the amine group of the tetanus toxoid in the presence of a carbodiimide (EDC) at pH 7.0 was analysed by gel-permeation chromatography. Fig. 10 shows the elution profile of the reactants and, of the reaction product. The elution profile of the conjugation product presents a new band of high-molecular weight assigned to the conjugate. The reaction was conducted at pH 7.0, since below this value precipitation of tetanus toxoid occurred. According to the amount of recovered material that solely reacted with the phenol–sulfuric acid reagent, an incorporation of 40% of the modified poly-α-D-galactosamine in the conjugate was accomplished. Similar result was previously obtained in this

laboratory in the coupling of alginic acid–caprolactam derivative with tetanus toxoid (Jerez, Matsuhiro, Urzúa, & Zúñiga, 1999).

Conjugation of the polysaccharide–caprolactam derivative to BSA was assayed at pH 5.6, since the optimal pH of EDC-mediated amidation reaction is around 5.0 (Kossaczka et al., 1997). The elution profiles of BSA and of the modified polysaccharide–caprolactam derivative are shown in Fig. 11(a). Gel permeation profile of the reaction mixture on Bio-Gel presents a new band of high-molecular weight assigned to the conjugate polysaccharide derivate—BSA (Fig. 11(b)). The elution profile did not show a fraction of free polysaccharide.

The results obtained in this work indicate that caprolactam is an effective new linker for the coupling of acidic polysaccharides to proteins. This linker may be applied for the conjugation of Vi polysaccharide and other polyuronides of biological interest.

Acknowledgments

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