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Chemical modifications of the xylan from *Palmaria decipiens*

J.R. Jerez, B. Matsuhiro & C.C. Urzúa*

Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 5659, Santiago, 2, Chile

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The neutral fraction isolated from the aqueous extract of the red seaweed *Palmaria decipiens* (Palmariales) was characterised as a D-xylan with β-(1→4) and β-(1→3) linkages. Oxidation with bromine introduced the formation of carbonyl groups at C-2 of the xylopyranosyl residues. Coupling of the oxidised xylan in heterogeneous medium with p-chloroaniline gave a stable Schiff base in a better yield than in aqueous solutions. Conjugation of the bromine-oxidised xylan with bovine serum albumin was achieved by reductive amination. © 1997 Elsevier Science Ltd

INTRODUCTION

The main water-soluble polysaccharides extracted from red seaweeds of the Palmariales and Nemaliales are neutral xylans. Björndal *et al.* (1965) studied two fractions of the xylan isolated from *Palmaria palmata* (synonym: *Rhodymenia palmata*) and showed that both fractions contained β-(1→3) and β-(1→4) linked xylopyranosyl residues, albeit in different proportions. ^{13}C-NMR spectroscopy has been used to characterise xylans from *Palmaria stenogona* (synonym: *Rhodymenia stenogona*), *Halosaccion glandiforme, Nemalion vermiculare* and *Galaxaura squalida* (Usov, 1984; Usov *et al.*, 1978). Matulewicz *et al.* (1992) applied ^{1}H- and ^{13}C-NMR to three fractions obtained by stepwise precipitation of the xylan from *Nothogenia fastigiata* (Nemaliales) and confirmed that the β-(1→3) linkages are distributed throughout the polymer rather than contiguously.

The most important applications of soluble polysaccharides utilise their ability to modify the properties of aqueous solutions. Selective enzymic and chemical modifications have facilitated the preparation of derivatives and conjugates with novel applications (Yalpani, 1985). Treatment of polysaccharides with oxidants such as bromine and periodate may introduce ketone or aldehyde functions, respectively. Agarose, dextran and cellulose were oxidised with aqueous bromine and then coupled with amines by reductive amination to give conjugates with potential biological applications (Larm & Schölander, 1977). Larm *et al.* (1979) obtained a heparin analogue by bromine oxidation of a partially reduced alginic acid and subsequent reductive amination. Salomonsson & Theander (1992) conjugated bromine-oxidised starch with 1-aminododecane and obtained a product with emulsifying properties. Recently, Zhang & Marchant (1994) prepared conjugates with surface-active properties by oxidation of dextrans with iodine to give uronic acid residues, which were then coupled with hydrophobic hexylamines.

Xylans represent an interesting source of raw material for the preparation of modified polysaccharides with novel properties. Having no primary alcoholic groups, they should form fewer side products on oxidation than cellulose or starch.

Ishak & Painter (1971) studied the oxidation of pectates, C_6-oxycellulose, C_6-oxyamylose, guaran and the xylan of *Rhodymenia palmata* with aqueous sodium periodate. They found that the oxidation proceeded in two stages: an initial, rapid stage in which the aldehyde groups form hemiacetal rings with vicinal hydroxyl groups; and a slow, terminal stage, in which the remaining unoxidised units are oxidised. Reduction of the hemiacetals with sodium borohydride allowed the rapid consumption of further periodate, to give the oxidation limit.

Palmaria decipiens Ricker [synonym: *Leptosomia simplex* (A. and E.S. Gepp) Kylin] has been described as one of the most abundant members of the Rhodophyta in the Chilean Antarctic (Etcheverry, 1983; Ramírez, 1982; Ricker, 1987; Westermeier *et al.*, 1992). Adams *et al.* (1988) isolated a neutral xylan from *Leptosarca simplex* from New Zealand.

This paper describes the characterisation of the xylan from *P. decipiens* and its chemical modification by oxidation and coupling with amines.

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Palmaria decipiens was collected in Collins bay (62°10′S, 58°30′W) during the XXV Chilean Scientific Antarctic Expedition and identified by Dr M.E. Ramírez at the Museo Nacional de Historia Natural, Santiago de Chile, where a specimen is held.

Analytical paper chromatography (PC) was carried out on Whatman N°. 1 and preparative PC on Whatman 3M paper. The solvent systems used were (A) n-butyl alcohol–ethanol–water (4:1:1) and (B) ethyl acetate–acetic acid–formic acid–water (18:3:1:4). Gas–liquid chromatography (GLC) of the alditol acetates was carried out in a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detector using a fused silica capillary column (15 m×0.25 mm) coated with SP-2330. GLC was performed with an initial 5 min hold at 150°C and then at 5°C/min to 210°C for 10 min. The helium flow rate was 20 ml/min and the detector and injector temperature was 220°C. The identities of all derivatives were determined by comparison with authentic standards.

Optical rotations were measured in a Perkin-Elmer 241 spectropolarimeter. NMR spectra of D_2O:H_2O (1:1 v/v) solutions were obtained with a Bruker 200 spectrometer. FT-IR spectra were recorded with KBr pellets of polysaccharides in a Bruker FT-IR 66 v infrared spectrometer. Microanalyses were determined at the Facultad de Química y Farmacia, Universidad de Chile.

The extraction and fractionation of the water-soluble polysaccharides from *Palmaria decipiens* have been described elsewhere (Matsuhiro & Urzúa, 1996). The neutral polysaccharide was isolated from the supernatant of the cetrimide fractionation by addition of 10% aqueous KI solution, and removal of the precipitated cetrimide iodide by centrifugation. The solution was dialysed against running tap water for 1 day, followed by distilled water, concentrated *in vacuo* and freeze-dried. The solid was purified by dissolution in water and precipitation in 2-propanol.

Total hydrolysis

The neutral fraction (0.100 g) was heated at 95°C with 2 M trifluoroacetic acid for 16 h. The excess of acid was removed by repeated evaporations *in vacuo* and the resulting syrup was analysed by PC in systems A and B. An aliquot was reduced with $NaBH_4$, acetylated and analysed by GLC.

Treatment with amyloglucosidase

The extract (0.500 g) was dissolved in 100 ml sodium citrate buffer (pH 5.0) and incubated with SIGMA amyloglucosidase (10 mg) at 40°C for 24 h. The solution was boiled for 5 min, cooled and extracted with toluene. The aqueous layer was dialysed against distilled water and freeze-dried. An aliquot of the dried solid was hydrolysed and analysed by PC.

Preparation of di-*O*-benzylidene-xylose dimethyl acetal

The extract purified by amylolysis was subjected to hydrolysis and the resulting syrup was purified by preparative paper chromatography in system A. The main fraction (80.5 mg) with the same mobility of xylose was dissolved in 1.7 ml of a solution of freshly distilled benzaldehyde (40 ml) and acetyl chloride (3.4 ml) in anhydrous methanol (140 ml). The resulting solution was left for 1 week at room temperature, cooled and the precipitate was removed by filtration and recrystallised from anhydrous methanol giving needles of m.p. 203–204°C, $[\alpha]_D = -6.39°$(c, 0.97, chloroform), lit. m.p. 201–203°C (Cerezo *et al.*, 1971); m.p. 211–212°C, $[\alpha]_D = -7.0$ (chloroform) (Whistler & BeMiller, 1962).

Oxidation with sodium periodate

The xylan (0.500 g) was dissolved in 20 ml of water and treated with 100 ml of a 0.077 M sodium periodate solution in the dark. After 48 h the reaction was stopped by the addition of 5 ml of ethane-1,2-diol. Aliquots of the sample were removed and analysed for periodate consumption by the iodometric method (Hay *et al.*, 1965). The solution was dialysed against distilled water, concentrated *in vacuo* and freeze-dried. It was then dissolved in 20 ml of distilled water, sodium borohydride (80 mg) was added and the mixture was stirred for 24 h. The resulting solution was dialysed against distilled water, freeze-dried, dissolved in 20 ml of water and oxidised with sodium periodate as before. The product of the second oxidation was hydrolysed with 2 M trifluoroacetic acid and the hydrolysis products were analysed by GLC as alditol acetates.

Bromine oxidation of xylan

Xylan (0.300 g) was dissolved in 10 ml of distilled water and 2.5 ml of 0.1 M aqueous bromine was added. The solution was adjusted to pH 7.0 by the addition of 0.5 M NaOH and stirred for 36 h at 30°C. It was then dialysed against tap water, followed by distilled water, concentrated *in vacuo* and poured into 5 vol. of ethyl alcohol.

Reduction and hydrolysis of the bromine-oxidised xylan

An aliquot of the bromine-oxidised xylan was reduced overnight with sodium borohydride, treated with Zeokarb 225 cation exchange resin, filtered and dialysed against distilled water. The resulting solution was concentrated *in vacuo* and freeze-dried. The dry material was subjected to total acid hydrolysis and analysed by GLC as alditol acetates.

Schiff base formation

(1) The bromine-oxidised xylan (0.050 g) was dissolved in 5 ml of distilled water and treated with 0. 075 g of *p*-chloroaniline (Fluka) in 5 ml of ethyl alcohol. The solution was left for 24 h at room temperature and then, poured into acetone (4 vol.), centrifuged, washed with fresh acetone and dried *in vacuo*. A light yellow solid (18.3% yield) with a nitrogen content of 0.256% was obtained.

(2) The bromine-oxidised xylan (0.050 g) was dispersed in 5 ml of methyl alcohol and a solution of 0.120 g of *p*-chloroaniline in 10 ml of methyl alcohol was added. The mixture was stirred for 16 h at room temperature, after which it was centrifuged. The product was washed with several portions of methyl alcohol, then with diethyl ether and dried *in vacuo* giving a yellow solid with 60.0% of yield. Anal. calcd. for $[(C_5H_8O_4)_{0.95}(C_{11}H_{10}O_3NCl)_{0.05}\cdot H_2O]_n$: C, 40.93; H, 5,21; N, 0.386. Found C, 40.56; H, 5,28; N 0.384.

Reductive amination

With p-*chloroaniline*

A solution of bromine-oxidised xylan (0.050) in water (5 ml) was adjusted to pH 6.0 by the addition of acetic acid and 0.075 g of *p*-chloroaniline in 5 ml of ethyl alcohol, and 25 mg of recrystallised sodium cyanoborohydride (Sigma) were added. The solution was stirred for 48 h at 40°C. The pH was then adjusted to 4.0 and the reaction mixture was stirred for two more hours at room temperature. It was then poured into ethyl alcohol (5 vol.). The precipitate was collected by centrifugation and dried *in vacuo*, giving a light brown solid (20.5%) with 0.701% of nitrogen.

With bovine serum albumin

To an aqueous solution of the bromine-oxidised xylan (0.080 g), bovine serum albumin (Sigma) (0.160 g) in 10 ml of phosphate buffer (pH 7.0) and 160 mg of recrystallised sodium cyanoborohydride were added. The resulting solution was stirred for 60 h at room temperature and then dialysed, just against distilled water for 48 h, and then against phosphate buffer (pH 7.0) for 48 h. The dialysate was subjected to gel-permeation chromatography on a Sepharose column (70×1.5 cm) equilibrated in phosphate buffer (pH 7.0). The dead volume (37.4 ml) was determined with a 0.2% Blue Dextran 2000 solution. Elution was carried out with the same buffer and fractions of 4.5 ml were collected. Elution was monitored spectrophotometrically at 280 nm for proteins and with the phenol–sulphuric acid reagent (Chaplin & Kennedy, 1986) for neutral polysaccharide. The presence of protein was also followed with the Bradford reagent (Boyer, 1993). The fractions that gave positive tests for carbohydrates and proteins were pooled, dialysed against distilled water and freeze-dried to give 95.3 mg of a white powder.

RESULTS AND DISCUSSION

The crude polysaccharide (21.6% yield) obtained by hot water extraction of *Palmaria dicipiens* was fractionated with cetrimide. An acidic polysaccharide was removed by precipitation (Matsuhiro & Urzúa, 1995) leaving a solution that gave, after removal of the cetrimide iodide, the neutral fraction (24.6% yield). Total acid hydrolysis followed by paper chromatography showed the presence of xylose and minor amounts of glucose. The polysaccharide recovered after treatment with amyloglucosidase was shown, by acid hydrolysis followed by GLC analysis, to contain only xylose. This was characterised as the crystalline dibenzylidene dimethyl acetal. The polysaccharide showed a specific optical rotation of $-106.0°$ (c, 0.20, water) that it is in close agreement with the reported value for β-D-xylans (Cerezo, 1972). The FT-IR spectrum showed two characteristic bands, at 1465 cm^{-1} assigned to CH_2 deformation vibration and at 896.2 cm^{-1} due to C_1-H deformation of β-linked residues (Mathlouthi & Koenig, 1986). Its ^{13}C-NMR spectrum showed five major signals assigned to β-linked 4-O-substituted units and was very similar to that reported by Adams *et al.* (1988) for the xylan extracted from *Leptosarca simplex*. No signals due to the presence of α-(1→4)-glucans were present. Assignments are shown in Table 1.

The xylan was oxidised in two steps with periodate, hydrolysed and analysed by GLC as polyalcohol acet-

Table 1. Chemical shift assignments oxidised derivative for the ^{13}C-NMR spectra of the xylan and its bromine-oxidised derivative

β-D-Xylopyranosyl residue	Chemical shift (ppm.)						
	C-1 1→3	1→4	C-2 1→3	1→4	C-3	C-4	C-5
Xylan							
3-O-substituted	104.08	102.50	73.39		84.39	68.61	65.78
4-O-substituted	104.08	102.50	74.02	73.64	74.62	77.29	63.80
Bromine-oxidised derivative							
3-O-substituted	104.05	102.48	73.56		84.28	68.51	65.75
4-O-substituted	104.05	102.48	74.15		74.54	77.22	63.80
CO	203.73						

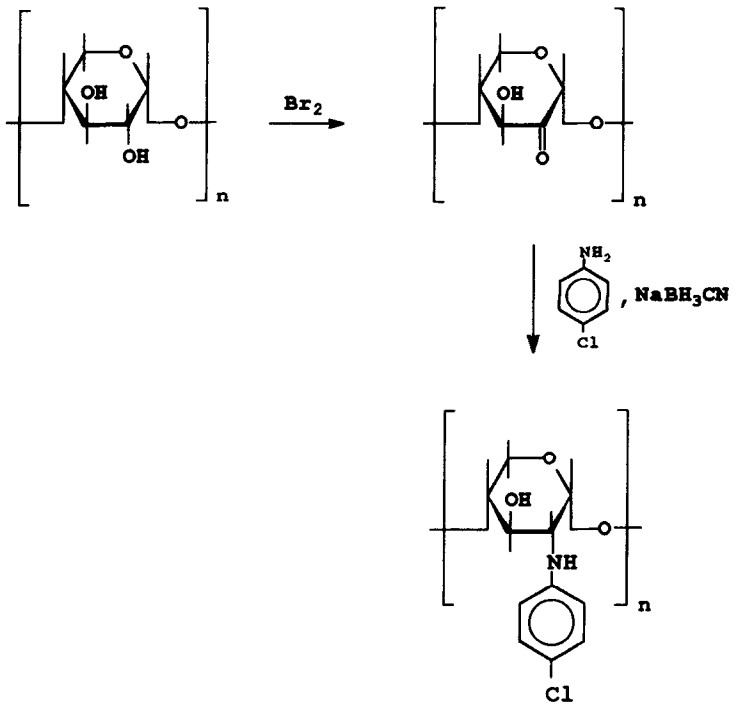

Fig. 1. Reductive amination of the bromine-oxidised xylan.

ates. The detection of the derived xylitol (22.6%) and glycerol (61.4%) indicates that the amount of 3-linked residues is lower than in the xylan from *Leptosarca simplex* (Adams *et al.*, *loc. cit.*).

Oxidation of the xylan with bromine afforded a water-soluble, white solid in 66.2% yield. Its FT-IR spectrum showed a new band at 1741.1 cm^{-1} assigned to the C-O stretching of the carbonyl group of ketones. A signal at 203.73 ppm in the ^{13}C-NMR spectrum (Table 1) of the modified xylan confirmed the introduction of a keto function.

Salomonsson *et al.* (1991) found that when starch is oxidised with bromine the proportion of the keto group was larger in position 2 than in position 3. When a bromine-oxidised glycan is reacted with sodium borohydride, the keto–glycosyl units are reduced to the parent sugar and to its epimer. In the case of the bromine-oxidised xylan, the GLC analysis of the alditol acetates of its hydrolysis product showed the presence of xylitol peracetate and lyxitol peracetate in the molar ratio 1:0.05. As ribitol peracetate was not found, it seems that the oxidation did not occur significantly on C-3 of the β-(1→4) xylosyl residues.

The presence of the carbonyl group was confirmed by reaction with *p*-chloroaniline. When the reaction was carried out with the modified xylan suspended in methyl alcohol, the yield (60.0%) was better than in aqueous solution (18.3%), in which the reverse (hydrolysis) reaction might also occur. The FT-IR spectrum of the reaction product showed new characteristic bands due to the C=N stretching absorption band at 1651.7 cm^{-1}, the vibrations due to the aromatic ring and the C—Cl stretching vibration at 741.5 cm^{-1}. Microanalysis indicated that all the carbonyl groups reacted in the heterogeneous medium giving the Schiff base.

When the reaction with the aromatic amine was conducted under reducing conditions, a white solid, insoluble in water, was obtained (Fig. 1). Its FT-IR spectrum showed the characteristic bands at 1630 (C=C), 1585 (N–H deformation), 1497 (C=C), 1077 (C-N), 895.8 (β-anomeric), 798.9 (1,4-aromatic substitution) and 778.4 cm^{-1} (C–Cl) of a secondary amine.

This reductive amination reaction was applied to the conjugation of the bromine-oxidised xylan to BSA. All the carbohydrate-rich fractions gave a band at 280 nm in the UV indicating that the conjugation of the modified polysaccharide to the protein was achieved.

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