Ceylon Journal of Science : Physical Sciences, 9(1), 110-115 (2002)

STRUCTURAL ANALYSIS OF A XYLAN FROM TEA STEMS AND XYLANASE ACTIVITY OF THE AMBROSIA FUNGUS.

NIVANDANA K. BANDARANAYAKE, THUSHARI BOMBUWALA, N. SAVITRI KUMAR* AND K.M. SWARNA WIMALASIRI

Department of Chemistry, Faculty of Science, University of Peradeniya, Sri Lanka

ABSTRACT

Alkali-soluble xylans were isolated from the pencil thick stem of tea (*Camellia sinensis* var. assamica) clones TRI 2023 and TRI 2025. Sugar and methylation analysis of the xylan from TRI 2023 indicated that the polysaccharide was composed mainly of xylose (90%). The main structural element was a β-(1→4) linked D-xylopyranosyl backbone with α-xylopyranosyl residues interspersed in the main chain and in the branches, forming a mixed configuration type homopolysaccharide. The presence of α-xylopyranosyl residues in the polysaccharide was supported by the ^{1}H and ^{13}C NMR spectral data of the native and degraded polysaccharides. The xylan was found to be hydrolyzed by xylanase enzyme extracts prepared from cultures of the ectosymbiotic fungus *Monacrosporium ambrosium* which is found in symbiotic association with the shot-hole borer, *Xyleborus fornicatus*, of tea.

Key Word: **Index Xylan,** *Camellia sinensis*, NMR data of xylan, tea, ambrosia fungus, *Monacrosporium ambrosium*, xylanase

1. INTRODUCTION

Camellia sinensis var. assamica (tea) is a major export crop of Sri Lanka. *Xyleborus fornicatus*, a wood-boring beetle known as the shot-hole-borer (SHB), attacks pencil-thick tea stems and causes serious damage to tea plantations in the mid-country tea plantation of Sri Lanka[1]. Studies carried out at the Tea Research Institute in Talawakelle, Sri Lanka, have shown that TRI 2023 and TRI 2025 are the least susceptible and the most vulnerable clones respectively, to SHB attack[2].

Some wood borers are able to break down cell wall components and use the resulting products as a source of nourishment. Insects such as SHB are unable to hydrolyse cell-wall material and rely for food upon cell contents or breakdown products of wood caused by the ambrosia fungus. Wood is a poor source of nutrients and the SHB beetle depends on the symbiotic ambrosia fungus, *Monacrosporium ambrosium*, for its nutrition[3]. The fungus has the capacity to transform simple sugars, amino acids and tea stem polysaccharides including lignocellulose into fungal biomass, containing all the nutrients required by the beetle[4].

The carbohydrate composition of the polysaccharides of tea plants was studied by Selvendran and Selvendran[5]. A higher percentage of xylose was found in the hemicellulosic fraction of wood compared with the bark. Glucan, galactan, araban, and xylan fractions in the hemicellulosic fractions were separated from the bark and wood of tea stems and roots. Changes in polysaccharides of tea plants after pruning have indicated that the hemicelluloses function only as structural material and do not appear to function as reserve food[5]. This paper describes the isolation of xylans from the alkali soluble fraction of pencil-thick tea stems of the two tea clones TRI 2023 and TRI 2025, and structural studies of the xylan isolated from TRI 2023.

There are reports that microogranisms pathogenic to plants produce enzymes that are capable of degrading cell wall polysaccharides[6]. It is likely

* nskumar@pdn.ac.lk

that the hemicellulosic fraction of tea stems, composed mainly of D-xylan, could be hydrolysed by hemicellulases such as xylanase. During this study, we also investigated the ability of the ambrosia fungus, which grows luxuriantly within SHB galleries, to produce xylanases, which would free xylose from the D-xylan.

2. EXPERIMENTAL

2.1 General methods

Concentrations were performed under diminished pressure at <40 °C. Blowing and co-distillations were performed by flushing air through stainless steel needles while the samples were maintained at a temperature <40 °C . GLC analysis was carried out with a Varian 3300 instrument equipped with flame ionisation detector. Alditol acetates and partially-*O*-methylated aldiotol acetates were analysed using DB-1 or DB-225 capillary columns. ^{1}H and ^{13}C NMR spectra were recorded in D_2O solutions using a JEOL GSX 270 instrument. The carbohydrate content were determined by the GLC method[16] using D-allose as the internal standard.

Sulphate [17] and phosphate[18] analyses were carried out according to the methods described by Terho *at al.* and Chen *et al.* respectively. The absolute configurations were determined according to Gerwig *et al.*[7]. Molecular size of the polysaccharides was determined by gel filtration on a column of Sephacryl S-400 (2.5 cm X 90 cm) with dextran samples of different molecular sizes as markers. Commercial xylan (birchwood) and sodium 2,2'-bicinchoninate were obtained from Sigma Chemicals Co.

2.2 Preparation of the polysaccharide samples

Pencil thick tea stems of the clones TRI 2023 and TRI 2025 were cut into small pieces freeze dried and powdered. The powdered samples (10g each) were blended with boiling ethanol (100ml), sonicated, (15 min) and centrifuged (10 min, 2000 rpm). The residue was washed successively with 100ml each of methanol: water (3:7), methanol, EtOAc, and diethyl ether. The residue was dried at 60°C to a constant weight to obtain the cell wall material (CWM). CWM (5g) from both clones were stirred with 1M aqueous NaOH (150 ml) under nitrogen atmosphere for 15 h and the supernatant was neutralized with dil. HCl The solution was then partitioned with ETOAc to remove polyphenols and freeze dried to give the crude polysaccharides, CPS_1 (200 mg, 4%) and CPS_2 (168 mg, 3.38%) from TRI 2023 and TRI 2025 respectively.

Contaminating polyphenols were removed from the crude polysaccharide fractions (100 mg each) by passage through a column of Biogel P-30 (2.5 cm X 70 cm) irrigated with 0.005% TCB. The polysaccharide fractions PPS_1 (80 mg, 80%) and PPS_2 (46 mg, 46%) respectively, which eluted at the void volume, were chromatographed separately on a column of DEAE Sephacel (2.5 cm X 20 cm) irrigated with distilled water, followed by a gradient of 0.0 X 0.5M-sodium chloride. In each case a neutral fraction {NSP_1, 4 mg, 5%) and NSP2 (1.3 mg, 2.8%) and an acidic fraction ACF_1 (65 mg, 81%) and ACF_2 (36 mg, 78%) respectively, were obtained.

2.3 Carbohydrate content, sugar and methylation analysis

The polysaccharide samples (ACF_1 and ACF_2) were treated with 2N TFA at 20° C for 1 h, reduced ($NaBH_4$ in IM NH_4OH) and acetylated with Ac_2O at 120° C for 30 min[16]. The alditol acetates were identified using GLC. The carbohydrate contents were determined by the same method using D–allose as the internal standard.

Methylation analysis was carried out according to a modified Hakamori procedure[19] and the partially *O*–methylated alditol acetates were analysed by GLC and GLC-MS. Identities of the partially *O*-methylated alditol acetates were confirmed by comparison with standard partially *O*–methylated xylitol acetate samples prepared in the same manner.

2.4 Methanolysis

The dried polysaccharide samples were treated with 4M methanolic HCl at 80°C for 8 h. The mixtures were neutralized with silver carbonate and

acetylated (Ac_2O, pyridine). The acetylated methyl glycosides were analysed by GLC and compared with those of the standard samples prepared in the same manner.

2.5 Partial acid hydrolysis

The polysaccharide sample ACF_1 (50 mg) was heated at 100°C (30 min) on a steam bath, in 0.1.N TFA solution. The partial hydrolysate was freeze dried, chromatographed on a column of Bio-Gel P-2 (2.5 x 60 cm) and the void volume fraction ACF_1/PH was separated. Sugar and glycosyl linkage composition of this fraction were determined.

2.6 Preparation of the xylanase enzyme extract

The xylan (Birchwood) was purified by the method described by Baker *et al*[11] and separated by precipitation into fractions I-V. The culture medium for the production of xylanase enzyme contained: NH_4NO_3 (500 mg), KH_2PO_4 (325 mg), $MgSO_4$ (90 mg), KCl (75 mg), $MnSO_4.H_2O$ (1.8 mg), $ZnSO_4.H_2O$ (1.7 mg), $FeCl_3.6H_2O$ (1.6 mg), $CuSO_4.5H2O$ (1.5 mg), yeast extract (Difco, 500 mg) and the xylan fraction IV (500 mg) in 500 ml of distilled water. The medium was stirred for 1 h to suspend the xylan sample and 100 ml portions were dispensed into two 200 ml Erlenmeyer flasks. The two flasks were autoclaved for 20 min [121°C, 1 atm (15 psi)], allowed to cool, and then inoculated with a spore suspension of a three day old culture of the ambrosia fungus isolated as described previously[20]. The cultures were incubated on an orbital shaker (100 rpm) for four days, filtered through four layers of cheesecloth and centrifuged at 3500 rpm for 30 min to remove cell debris. The supernatant was dialysed for 24 h against 20 mM sodium acetate buffer (pH 5.0) and concentrated by freeze drying to give the xylanase enzyme extract.

2.7 Xylanase assay:-

The purified xylan fraction V (1 ml, 1%) was mixed with enzyme extract (200 µl) and incubated at 30°C for 30 min. Xylanase activity was determined by measuring the release of reducing sugars using the sodium 2,2'-bicinchoninate (Sigma) reagent according to the method described by McFeeters[12]. The absorbance was measured after 30 min at 560 nm using a Shimadzu UV 160 spectrophotometer. Water was used as the blank. Six replicate determinations were made. The amount of the reducing sugar was determined by a calibration curve, constructed using D-xylose. Release of reducing sugars indicated that hydrolysis of the xylan has occurred. The effect of the xylanase extract on the tea stem xylan (1 ml, 1%) was determined using the same procedure.

3. RESULTS AND DISCUSSION

3.1 Separation of tea stem polysaccharides

Pencil thick tea stems of the tea clones TRI 2023 and TRI 2025 collected at the Tea Research Institute sub-station at Hantane, Kandy, were freeze dried, powdered and extracted with boiling ethanol. The residue from each extraction was washed successively with methanol:water, methanol, ethyl acetate and diethyl ether, and dried to obtain the crude cell wall material (CWM). CWM from each clone was separately stirred with 1M NaOH and each neutralized supernatant was partitioned with ethyl acetate to remove polyphenols. The aqueous layer was dialysed and freeze dried to give the crude polysaccharide fractions (CPS_1 and CPS_2) from TRI 2023 and TRI 2025 respectively.

Fractionation of the crude polysaccahrides on Bio-Gel P-30 led to the separation of polyphenol-free neutral fractions NPS_1 and NPS_2 from the respective crude polysaccharides CPS_1 and CPS_2. Further fractionation of NPS_1 and NPS_2 on DEAE-Sephacel yielded the acidic fractions ACF_1 and ACF_2 respectively. In this paper we describe the structural analysis of the acidic polysaccharide fraction ACF_1 isolated from the tea clone TRI 2023.

The purified acidic polysaccharide fraction ACF_1 (carbohydrate content 80%) was composed of arabinose, xylose and galactose in a molar ratio of 3:94:3. Methanolysis followed by acetylation of ACF_1, and analysis of the resulting acetylated methyl glycosides by GLC showed the presence of a small amount (< 1%) of galacturonic acid residues which accounted for the slight acidity of these two

polysaccharide fractions. The molecular sizes of the polysaccharides were found to be around 70,000 Da. The absolute configuration[7] of the xylosyl residues was found to be **D**. Peaks due to arabinose and galactose were not detected, probably due to the low concentrations of these residues in the polysaccharide. Sulphate and phosphate analyses revealed the absence of these substituent residues in the polysaccharide.

3.2 Structural analysis of the xylan sample ACF_1

The ^1H NMR spectrum of ACF_1 showed the absence of signals due to *O*-acetyl and *O*-methyl groups. Anomeric protons appeared as doublets at δ_{ppm} 5.26 (J 3.9 Hz), 4.65 (J 7.1 Hz) and 4.50 (J 7.4 Hz) in a molar ratio of 1:1:3.6. The doublets at δ_{ppm} 4.65 and 4.50 ppm were assigned to β-linked xylopyranosyl residues attached to glycosyl residues at different linkage positions[8]. The signal at δ 5.26 ppm was tentatively assigned to either α-arabinopyranosyl or α-xylopyranosyl residues[9]. But comparison of the NMR integral and the composition of ACF_1 (94%xylose) suggested that the signal at δ 5.26 ppm can only be due to α-xylopyranosyl residues. This signal however, is more downfield than has been reported earlier for unsubstituted α-linked xylopyranosyl residues[8]. The ^{13}C NMR spectrum showed anomeric carbon signals at δ_{ppm} 102.68 and 101.97, which were attributed to β-xylopyranosyl residues, while the signal at δ_{ppm} 98.57 was assigned to α-xylopyranosyl residues[9]. The C-5 signal of the xylosyl residues observed at δ_{ppm} 63.65 was split and this is consistent with a branched structure for the xylan[10].

GLC-MS of the partially-*O*-methylated alditol acetates of ACF_1 indicated the presence of 2,3,4-, tri-*O*-methyl, 2,3-di-*O*-methyl, 3-*O*-methyl and fully substituted pentoses in a molar ratio of 10:74:16:7. The component sugars were identified as 1,5-di-*O*-acetyl-2, 3,4-tri-*O*-methyl xylitol, 1,4,5-tri-*O*-acetyl 2,3-di-*O*-methylxylitol, 1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl-xylitol and unmethylated xylitol pentacacetate respectively, by comparing the relative retention time with standard samples of partially methylated xylitol acetates.

The polymeric fraction, ACF_1 was composed mainly of xylosyl residues (94%). The ^1H NMR spectrum of the partially hydrolysed sample ACF_1 /PH (99% xylose), was superimposable on that of ACF_1, suggesting that the signal at δ 5.26 ppm was due to α-xylopyranosyl residues and that both α- and β- xylopyranosyl residues are incorporated in the polysaccharide. The GLC-MS of per-*O*-methylated ACF_1/PH showed the presence of 2,3,4-tri-*O*-methyl xylitol, 2,3-di-*O*-methyl xylitol, 3-*O*-methyl xylitol and xylitol residues in a molar ratio of 8:80:3:9. Comparison of the GLC-MS data of ACF_1 and ACF_1 /PH indicated a decrease in branching at *O*-2 in ACF_1 /PH. The proportion of fully substituted xylosyl residues remained almost unchanged indicating that these residues are found in the main chain.

In a study of arabinoxyloglucans from Solanaceous plants York *et al.*[8] reported different chemical shifts for the anomeric protons of α-D-xylopyranosly residues in different environments. It was observed that the H-1of α- D-xylopyranosly residues having *O*-2 substitutions of β-D-glucopyranosyl residues, appeared between δ_{ppm} 5.09-5.15 (J 3.7 Hz), while the signal due to H-1 of terminal β-D-xylopyranosyl residues was at δ_{ppm} 4.95 (J 3.7 Hz)[8]. Analysis of the ^1H NMR spectra, together with the data from GLC-MS of ACF_1 and ACF_1 /PH, suggested that the H-1 signal of ACF_1 and ACF_2 at 5.26 is probably due to fully substituted α-D-xylopyranosyl residues.

Chemical and NMR spectroscopic studies suggest that the acidic polysaccharide fraction from tea stems is a homoxylan composed of a backbone of β-(1→4)–D-xylopyranosyl residues, with α-xylopyranosyl residues incorporated in the main chain or as side chains. NMR spectroscopy indicated a configurational ratio of 1:5 (α/ β).

3.3 Xylanase activty

The commercial sample of birchwood xylan (Sigma) was purified by the method described by Baker *et al.*[11] The enzyme was produced in a liquid medium by cultivating the ambrosia fungus in a medium containing 1 g per litre of the purified xylan fraction IV as substrate. The mixture was filtered through a soft cloth to remove fungal mycelia and the filtrate was concentrated by freeze drying to give the xylanase enzyme extract.

Activity of this enzyme extract was measured by mixing it with the purified xylan fraction V[11]. The mixture was incubated at 30°C for 30 min. Xylanase activity was assayed for the production of xylose using the 2,2'-bicinchoninate reagent[12] and was determined by measuring the increase in reducing sugars (as the increase in absorbance at 560 nm) in the reaction mixture. One unit of enzyme activity is defined as the amount enzyme releasing one µmol of the reducing sugar (xylose) per minute under the given conditions. (Table.1). Six replicate measurements were carried out and the mean increase in absorbance was correlated to the concentration of reducing sugars using a calibration graph. Distilled water to which the 2,2'-bicinchoninate was added was used as the absorbance blank. The amount of xylose released was found to be 0.330 µg and corresponded to an activity of 2.02×10^{-3} µmol/min.

The procedure was repeated using purified samples of tea stem xylan, instead of the xylan fraction V, in the xylanase assay, gave similar results. The amount of xylose released was found to be 0.320µg (2.01×10^{-3} µmol/min.) when a 1% solution of the tea stem xylan was treated with the enzyme extract. Release of reducing sugars indicated that the xylanase enzyme production has occurred, and suggests that the fungus *M. ambrosium* is capable of producing xylanases to hydrolyse the xylan present in the hemicellulosic fraction of tea stems.

Many microorganisms that are pathogenic to plants are known to produce enzymes capable of degrading polysaccharides found in the cell walls of higher plant. Bateman *et al.*[6] reported that when the cell walls from bean hypocotyl were used as the carbon source, *Rhizoctonia solani* produced the enzyme xylanase. Anderson[13] has reported that the fungal pathogen *Collectotrichum lindemuthianum* of bean, and the corn pathogen *Helminthosporium maydis* produced cell wall degrading enzymes such as xylanase, when grown on bean or corn cell wall cultures. *Aspergillus fumigatus* and *Aspergillus oryzae* produced xylanase when grown on an unsubstituted insoluble beech xylan[14] while *Erwinia chrysanthemi* produced xylanase when grown on xylan from corn[15]. Therefore it is possible that plant cell wall degradation may occur during shot-hole borer infestation of tea, and that the xylan fraction in tea stems may also function as a nutritional reserve.

4. ACKNOWLEDGEMENTS

The authors thank the Tea Research Institute (TRI) substation at Hantane for cooperation and assistance.

This investigation was supported by a research grant from the Swedish Agency for Research Cooperation SAREC (now Sida). Financial assistance from the International Program in Chemical Science, Uppsala University, Sweden, is gratefully acknowledged. We also thank Prof. Per-Eric Jansson of the Clinical Research Center, Huddinge Hospital, Sweden, for assistance with NMR spectroscopy.

Table1 Activity of xylanase extracts produced by the ambrosia fungus

Sample	Increase in absorbance									
	1	2	3	4	5	6	Mean	S. D.	Xylose (µg) released	*Activity µmol/min
Birch xylan	0.958	0.960	0.962	0.958	0.959	0.955	0.958	0.0023	0.330	2.02×10^{3}
Tea xylan	0.923	0.928	0.942	0.920	0.939	0.936	0.929	0.0089	0.320	2.01×10^{3}

* Amount of enzyme releasing one µmol of the reducing sugar (xylose) per minute under the given conditions. UV absorbance of bicinchoninate reagent in water was 0.125 at 560 nm.

REFERENCES

1. W.Danthanarayana, *Ent. Exp. & Appl.* **16** (1973) 305.
2. D.Calnaido, and P.Kanapathipillai, *Tea Q.* **38** (1967) 275.
3. C.H.Gadd, and C.A.Loos, *Trans. Br. Mycol. Soc.* **31** (1947) 13.
4. L.R.Batru, *Science* **153** (1966) 193.
5. R.R.Selvendran, and S.Selvendran, *Tea Quart* **43** (4) (1972) 168.
6. D.F.Bateman, H.D.Van Etten, P.D.English, D.J.Nevins, and P.Albersheim, *Plant Physiol.* **44** (1969) 641.
7. J.G.Gerwig, J.P.Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res* **77** (1979) 1.
8. W.S.York, V.S.Kumar kolli, R.Orlando, P.Albersheim, and A.G.Darville, *Carbohydr. Res* **285** (1996) 99.
9. K.Bock, and H.Thogersen, *Ann. Reports on NMR spectroscopy* **13** (1982) 1.
10. A.Ebringerova, Z.Hromadkova, E.Peterkova, and M.Hricovini, *Carbohydr. Res.* **198** (1990) 57.
11. C.J.Baker, C.H.Whalen, and D.F.Batemann, *Phytopathology* **67** (1977) 1250.
12. R.F.McFeeters, *Anal. Chem.* **103** (1980) 302.
13. A.J.Anderson, *Physiol. Biochem., Physiol. Biochem* **68** (1978) 1585.
14. M.J.Bailey, and L.Viikari, *World J. Microbiol. Biotechnol* **9** (1993) 80
15. E.J.Braun, and C.a.Rodrigues, *Phytopathol* **83** (3) (1993)332
16. P.Albersheim, D.J.Nevins, P.D.English, and A.Karr, *Carbohydr. Res* **5** (1967) 340.
17. T.T.Terho, and K.Hartiala, *Anal. Biochem* **41** (1971)471
18. Jr., P.S.Chen, T.Y.Toribara, and H.Warner, *Anal. Chem* **28** (1956) 1756.
19. P.J.Harris, R.L.Henry, S.B.Blackeney, and B.A.Stone, *Carbohydr. Res* **127** (1984) 59
20. N.S.Kumar, P.Hewavitharanage, and N.K.B.Adikaram, *Phytochemistry* **40** (1995) 1113.