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Carbohydrate Polymers 41 (2000) 55–60

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Cell wall polysaccharides from *Mandevilla velutina* (Apocynaceae) cultured cells: extraction and chemical structure

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Received 7 September 1998; received in revised form 17 February 1999; accepted 24 March 1999

Abstract

Mandevilla velutina, a native plant to Brazil, is a source for bioactive triterpenoids which selectively and in a competitive way inhibited bradykinin (BK)-induced contractions of isolated rat uterus and guinea-pig ileum. These compounds were also found in cell cultures with an anti-BK action about two orders of magnitude larger than those from tuber extracts. Biomass from cell cultures was used to determine the basic composition of the cell wall, pursuing in further studies whether that parameter might be used to distinguish between cell lines and between cells of different ages. Hemicellulosic fractions showed a predominant neutral sugar composition, with D-Glc, L-Rha and D-Xyl as the major components. The deoxy-sugar L-Fuc was also found as a minor constituent. ^{13}C NMR spectrum of the hemicellulose A (5 M KOH) revealed β and α conformations for D-Glc and L-Rha, respectively; the discrete signal for the carboxyl from the uronyl moiety (downfield), and the absence of (1-2) and (1-3) linkages. Spectroscopy also suggested a β (1-6) linking for a polyglucose backbone or block. A more detailed structural analysis for the presence of L-Fuc is in progress. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Bradykinin-induced contractions; Neutral sugar composition; Polyglucose backbone; *Mandevilla velutina*

1. Introduction

Mandevilla velutina (Apocynaceae) is a native plant to the central-western region of Brazil. Folk medicine prescribes the use of infusions or alcoholic extracts from the rhizomes for the treatment of inflammations, including those caused by bites of the snake *Bothrops jararaca*. Rocha, Beraldo & Rosenfeld (1949) showed bradykinin (BK) release from plasma globulin after snake bites and Ferreira (1965) demonstrated a BK-potentiating effect by *B. jararaca* venom. Recently, it was shown that pure non-peptide compounds, obtained in crystal form from crude extract of *M. velutina*, selectively and in a competitive way inhibit BK-induced contractions of isolated rat uterus and guinea-pig ileum (Calixto, Pizzolatti & Yunes, 1988). Given orally, crude extracts were also able to inhibit pleurisy and increase the vascular permeability caused by BK and *B. jararaca* venom in mice and rats, respectively (Nicolau et al., 1986).

These anti-BK compounds were chemically characterized as triterpenoids (Caetano, 1993). Differences among the compounds are on the glycosylation and/or hydroxylation level. More recently, the structure of one isolate, v*elutinol A*, was elucidated (Bento, Calixto, Hawkes, Pizzalatti, Sant'Ana & Yunes, 1996). However, only very small amounts (0.001–0.0001%) of these compounds are found in plant crude extracts. Large-scale biomass production of *M. velutina* by the conventional agricultural methods seems to be economically unfeasible. Thus, cell cultures of *M. velutina* could be an interesting potential source for these bioactive compounds. Ethyl acetate extracts of *M. velutina* cell cultures revealed an anti-BK action about two orders of magnitude larger than those from crude rhizome (Calixto et al., 1989). Studies focusing on the presence of the triterpenoids in the cell cultures have been carried out using

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PII: S0144-8617(99)00069-7

extracts obtained by a supercritical fluid extraction (Maraschin etal., in preparation).

The purpose of the present study was to determine the chemical composition of the cell wall hemicellulose fractions from *M. velutina* cell cultures. In connection with further studies, we are particularly interested in determining whether the cell wall composition might be used to distinguish between cell lines, and between cells of different ages. In this context, the basic composition of cell wall components of *M. velutina* is necessary to be known, and hemicelluloses were chosen as the target for this goal. In recent years, the knowledge about polysaccharides has greatly contributed to the identification and classification of bacteria, specially using an immunochemical approach (Bishop & Jennings, 1982). A significant variation in the spear grass cell wall composition, e.g. hemicellulose A/B, was found within the stages of growth (Blake, Murphy & Richards, 1971).

2. Material and methods

2.1. Cell cultures

Calli from stock cultures were subcultured on MS medium (Murashige & Skoog, 1962), supplemented with 2 mg/l 2,4-D (2,4 dichlorophenoxyacetic acid), 2 mg/l BAP (6-benzylaminopurine), 3 mg/l KIN (6-furfurylaminopurine) and 30 g/l sucrose for biomass production. The cultures were maintained at 24 ± 1°C, 14 h light, 1500 lux (Philips TLF 65W33). Subculturing was performed every three weeks.

2.2. Hemicellulose extraction

Cellular biomass (800 g, fresh weight) was first treated with three volumes of ethyl acetate for 3 days and the residue freeze dried. The dry biomass was shaken in three volumes of an aqueous 1.5% SDS (sodium dodecyl sulfate) solution (pH 5.0) for 12 h agitation at 4°C, centrifuged (10 krpm/15 min/4°C), and the cytoplasm release in the supernatant (e.g. nucleic acids and proteins) was monitored through spectrophotometry, at 260 and 280 nm, respectively (Selvendran, Stevens & O'Neill, 1985). The residual cell wall designed as SDS-CW (cell wall after detergent washing) was washed with three volumes of water, treated three times with 90% DMSO (dimethyl sulfoxide) for 12 h agitation at 25°C and centrifuged (10 000 rpm/15 min/25°C). The re-extraction of the residue was carried out under sonication (70 Kc/10 min/3 pulses), incubated for 12 h at 25°C and sonicated again. The combined filtrate extracts were freeze dried and the residue (101.69 g; 12.71%) was then treated with 0.5% ammonium oxalate (2 h/60°C) and 1% Na_2EDTA (1 h/60°C). The solubilized cell wall pectic fraction was filtered, dialyzed and freeze dried overnight. The hemicellulose extraction of the depectinized residue was then carried out with sequential treatments with 1 and 4 M KOH for 12 h at 25°C (Selvendran et al., 1985) in a rotatory shaker, with $NaBH_4$ (100 mg) added to the medium. Finally, a hot 5 M extraction with the same alkali was performed in a water-bath, inside a microwave oven, with three sequential incipient boilings.

After each treatment the suspensions were centrifuged (10 000 rpm/15 min/4°C), supernatants were collected, treated with AcOH till pH = 5.2 and centrifuged. Precipitates and supernatants were treated with an excess of EtOH (three volumes), yielding for each extraction the hemicelluloses **A** and **B**, respectively. Residual potassium acetate was washed off through dialysis against distilled water. The deeply pigmented fractions were then treated for 2 min in an ice-bath with an aqueous $NaClO_2$–AcOH (1:1), as described bySelvendran and O'Neill (1987), dialyzed and lyophilized.

A sample (5 g) of the depectinized residue was separately treated with 250 ml of 0.3 M LiSCN. The extraction was performed at room temperature, overnight. The extracted cell wall material was obtained by centrifuging (10 krpm/15 min/4°C) and precipitating the supernatant with EtOH (three volumes), followed by dialysis against distilled water, lyophilization and acid hydrolysis.

2.3. Hemicellulose hydrolysis

Hemicellulose fraction samples (20 mg) were hydrolyzed with 2 M TFA (trifluoracetic acid) for 6 h at 100°C, using sealed Kimax tubes. Most of the TFA were evaporated in open vessels overnight at 25°C. Residual TFA was removed in a Savant vacuum centrifuge whose outlet had a NaOH trap. Enzymatic hydrolyses of the fractions (20 mg sample) were separately carried out using a 4% solution of the hydrolytic complex from *Megalobulimus paranaguensis* gastric juice (Fontana, Gebara, Blumel, Schneider, MacKenzie & Johnson, 1988), 1% Celluclast®, 1% Celluzyme®, or 1% Mutanase® (the latter three enzymes from Novo Nordisk Ferment/Dittingen, Switzerland) in 50 mM acetate buffer (pH = 5.0) at 30°C for 48 h, with 10 μl toluene added to the medium as a bacteriostatic agent. Hydrolyses were stopped by adding three volumes of EtOH and the precipitates after centrifugation (10 krpm/22°C/10 min) were discarded. Each supernatant was freeze dried and resuspended in a minimum volume of water for thin layer chromatography (TLC) analysis.

2.4. Thin layer chromatography

TLC analysis was performed on Silica G 60 chromatoplaques (Merck), with 2-PrOH/EtOAc–Nitroethane–AcOH–H_2O (45:25:10:1:19) as a mobile phase. Free sugar detection was performed by spraying 0.5% orcinol in sulfuric acid:MeOH (5:95), followed by heating at 105°C for full development of different colors and hues for pentoses, hexoses, deoxi-sugars and uronic acids.

Table 1
Yield of polysaccharides (% and mg) of *M. velutina* cell wall fractions, obtained from alkaline extraction

Cell wall fraction	Yield[a] (%)	Yield[a] (mg)
1 M KOH		
Hemicellulose A	5.14	175.27
Hemicellulose B	7.68	261.89
4 M KOH		
Hemicellulose A	0.88	30.01
Hemicellulose B	2.54	86.61
5 M KOH (hot)		
Hemicellulose A	0.88	30.01
Hemicellulose B	2.78	94.80

[a] Calculated from 3.41 g of freeze-dried cell biomass, after extraction with three volumes of ethyl acetate, overnight.

2.5. GLC and GC–MS conditions

The reducing sugars were converted to their persilyl derivatives (Fontana et al., 1988) by the addition of 0.1 ml of pyridine with 0.1 ml of BSTFA [*N,O*-bis-(trimethylsilyl)-trifluoracetamide] and heating at 70°C for 20 min with an occasional mixing in a vortex mixer. Derivatization was carried out in a 1-ml glass vial sealed with Teflon-coated rubber cap in order to avoid moisture. GC analysis of the hydrolyzed samples (1 μl) was performed on a Varian 3400 gas chromatograph equipped with a glass packed column (2 m long, 0.2 mm i.d.) packed with 3.8% silicone SE-30 and a temperature program consisting of 2 min at 140°C followed by heating at 10°C/min to 260°C. Detection was by means of a flame ionization detector (FID) at 300°C. N_2 was used as a carrier gas (20 psi; 1.0 ml/min flow) and the injection split ratio was 1:50. Phenyl-β-D-galactopyranoside (5 mg/ml) was used as an internal standard. Alternatively, a 60 m capillary HP-5 (5% phenyl methyl siloxane column) was used keeping the same temperature program, except that the initial temperature was raised to 160°C.

The hydrolysis products from the LiSCN-cell wall material were derivatized to their alditol acetates (Albersheim, Nevins, English & Karr, 1967) and GLC analysis was performed on a Varian 3400 gas chromatograph equipped with an OV 225 capillary column (30 m long; 0.25 mm i.d.). The temperature program consisted of 2 min at 50°C followed by heating at 40°C/min to 220°C. Helium was used as a carrier gas (20 psi; 2.0 ml/min flow).

The hydrolyzed hemicellulose samples from the strong alkali procedures were solubilized in MeOH (10 μl), and the GC–MS analyses of the alditol acetate derivatives were carried out by injecting 1 μl of each sample. Data were obtained with a Finnigan MAT 700 spectrometer ITD (ITDS software, version 4.2) coupled with a Packard 438A gas chromatograph, equipped with a CP-Sil 5CB column [25 m, fused silica: 50 (2 min)– 270°C (10°C/min.); injector: 225°C], with a scan time of 1 s. The source operating conditions were: EI mode at 70 eV, multiplied at 1900 V, with a transfer chamber temperature of 250°C. Helium was used as a carrier gas (100 KPa).

2.6. Uronic acid analysis

Uronic acids were detected by paper electrophoresis according to Fontana et al. (1988). The colorimetric assay was performed following the protocol described by Blumenkrantz and Asboe-Hansen (1973), using D-glucuronic acid as a standard.

2.7. Protein analysis

Protein was estimated by the method of Lowry, Rosenbrough, Farr and Randall (1951) with bovine serum albumin as a standard.

2.8. Molecular weight estimate of the fractions

Following the GLC and GC–MS quantification analysis, the mean molecular weight (M_w) for each fraction was estimated using CARBO-MASS software (version 1.2).

2.9. ^{13}C NMR spectra

Hemicellulose **A** (237 mg) from the hot 5 M KOH extraction was diluted in 700 μl D_2O and the soluble fraction (~100 mg) was transferred to a 5 mm probe. The insoluble fraction (~130 mg, lyophilized) was resuspended in DMSO-d_4 and transferred to a 10 mm probe tube. The spectra were measured on a Bruker DRX400 spectrometer, operating overnight. All the spectra were obtained at 28°C, using tetramethylsilane as an internal reference. An exponential multiplication window function with a line broadening equalling 1.0 for D_2O soluble fraction and 15.0 for DMSO-d_4 fraction was applied to the FID before Fourier transformation.

3. Results and discussion

The yield of polysaccharides obtained from *M. velutina* cell wall fractions from the alkaline extractions is shown in Table 1. The data from the TLC analyses of that hemicellulosic material indicated its rather heterogeneous composition. Following D-glucose as the major component in all the six KOH-fractions herein obtained, L-rhamnose, D-xylose, D-mannose, and L-arabinose, accounted for most of the composition. The TFA hydrolyses actually used, even the 2 M condition, would not allow glucose release from cellulose, as the applied temperature was lowered from 120 to 100 ± 2°C. Uronic acid was barely seen by TLC, but paper electrophoresis, allowing sample overload, revealed its presence as glucuronic acid. It is possible that the stronger acid hydrolysis conditions have partially degraded this compound (e.g. decarboxylation, according to Kakehi & Honda, 1990), while in the milder ones glucuronyl residues

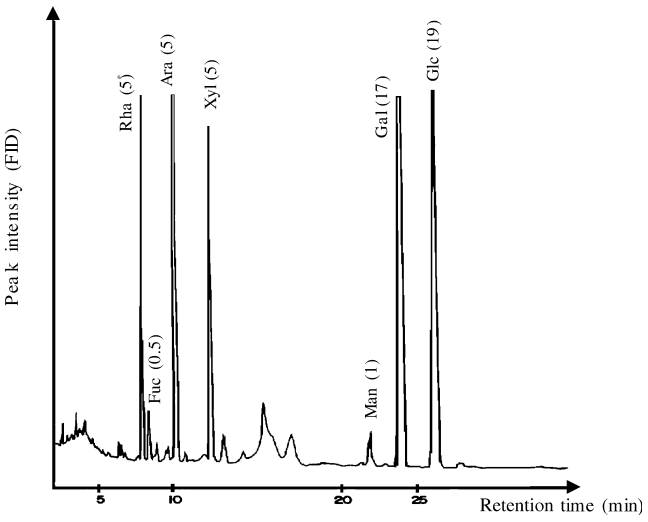

Fig. 1. The hemicellulose composition (absolute % of glycan) of *M. velutina* LISCN-cell wall isolate, according to GLC data from alditol acetate of the trifluoracetic hydrolyzates. Calculated from GLC patterns, considering the total area under the peaks as 100%.

are released preferentially as aldobiuronic acid or larger oligomers of the series (Fontana et al., 1988).

Surprisingly, a minimal but consistent presence of 6-deoxy-α-L-galactose (L-fucose) was found by GLC analysis and further confirmed by MS analyses of *M. velutina* hemicelluloses **A** and **B**. GLC analysis of the LiSCN-cell wall fraction also showed the presence of L-fucose, and a similar composition to that found for alkaline fractions as demonstrated in Fig. 1. The LiSCN-cell wall fraction contains more than 50% L-arabinose, and the next most abundant sugars being D-glucose and D-galactose. The predominance of arabinose in this fraction weakly associated to the cellulose backbone might characterize it as an arabinoglucan, or even an arabinogalactan, having the minor sugars probably as side-chain components. The efficiency of the alkaline chaotropic extractant (pH $>$ 11) on possible *Mandevilla* (OH) proline rich glycoproteins remains to be established.

The constituents for each cell wall fraction were quantified and the results are shown in Table 2. The sequential extraction performed revealed a meaningful decrease in the L-arabinose content for the polysaccharides more strongly associated to the cellulose, in particular for the strong alkali extractable hemicelluloses. D-glucose was the major component for all hemicellulose fractions **A** and **B** and L-rhamnose was the second major one. D-xylose appears as the third more abundant monosaccharide, confirming the TLC data. *M. velutina* has a neutral sugar composition in its KOH-cell wall polysaccharides, roughly in the molar (%) ratios glc:rha:xyl:man:ara:gal:fuc (100:60:65:45:75:45:5) and with an acidic portion $\geq$ 1.7%.

Carbon NMR spectroscopy was applied to hemicellulose A from the most drastic alkaline extraction, hot KOH 5 M, considering that it would be purer than the more easily extractable fractions. The ^{13}C NMR spectrum of the D_2O soluble sub-fraction (Fig. 2) does not differ from the DMSO-d_4 soluble sub-fraction in terms of conformation and type of linkages, revealing then the existence of polysaccharides with very similar composition and confirming the data previously found by GLC. Peaks in the C_2 and C_5 regions were not conclusively analyzed due to the fact that more than one monosaccharide shows quite similar chemical shifts, however, the signals found are related to the sugar composition showed by the GLC analysis.

Some structural characteristics could be deduced from the absence of signals for 1-2 and 1-3 linkages in both D_2O and DMSO-d_4 fractions. The C_1 signal of D-glucose occurs in the β conformation as expected and L-rhamnose was found only in the α conformation ($\delta = 99.4$: C_1 and $\delta = 18.7$: C_6). In both spectra, a small signal for D-glucose in the α conformation was found (Fig. 2), suggesting the occurrence of small quantities of another polysaccharide associated to the cell wall. It reacts positively with diluted iodine/iodide solution, which seems to indicate the presence of a starch-like polymer

Table 2
The hemicellulose composition (absolute % of glycan) of *M. velutina* cell wall fractions from the alkaline extraction, after acid hydrolysis

Component	1 M KOH (cold)		4 M KOH (cold)		5 M KOH (hot)		Mean total content (%)
	A	B	A	B	A	B	
L-arabinose	8[a]	7	10	12	12	5	15
L-rhamnose	14	10	14	20	18	9	12
L-fucose	1.6	1	1	1	1	1	1.0
D-xylose	14	13	11	12	11	16	13
D-galactose	11	9	9	6	11	4	9
D-glucose	16	25	22	20	24	20	20
D-mannose	17	17	5	11	7	7	9
Uronic acid[b]	1.8	1.8	1.0	1.8	2.2	1.6	1.7
Protein[c]	6.6	0.8	3.2	1.0	n.d[d]	1.2	2.1

[a] Calculated from GLC patterns, considering the total area under the peaks as 100%.
[b] Percent as GlcA.
[c] Determined by the method of Lowry et al. (1951).
[d] Not detected.

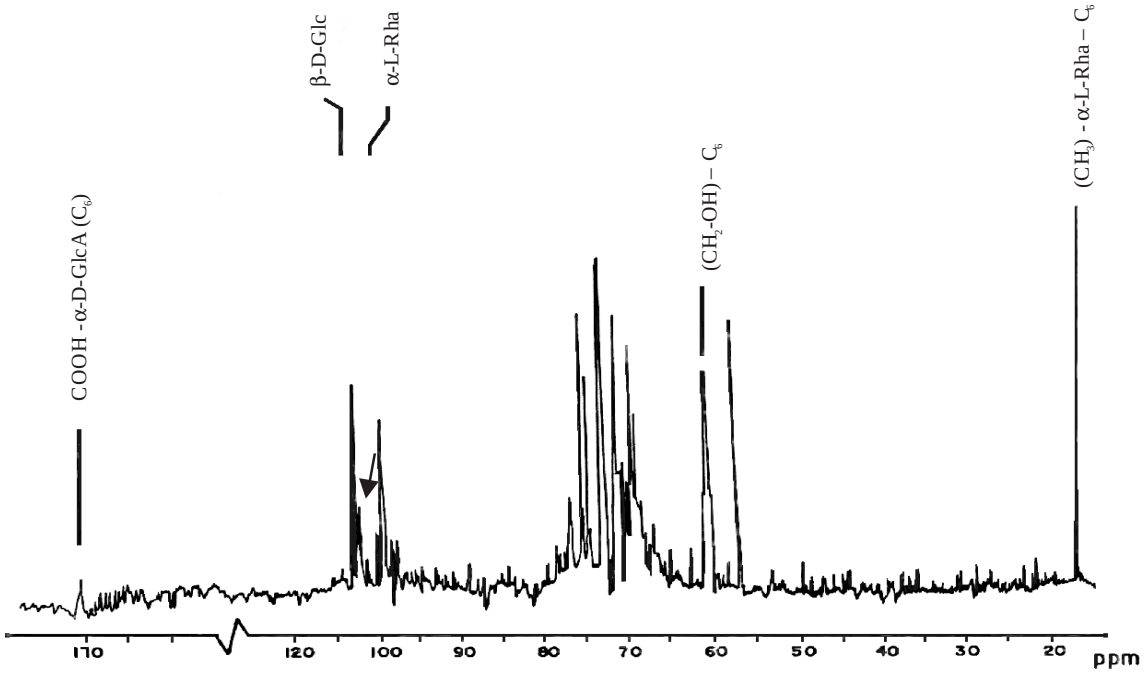

Fig. 1. ^{13}C NMR spectrum of hemicellulose A (5 M KOH/AcOH) from *M. velutina* cell wall, recorded for solution in D_2O at 28°C. The arrow (↓) indicates the signal for D-glucose in the α conformation and the region (76.626–69.744 ppm) is referred to 1 → 6 linkage. Chemical shifts for C_2, C_3, and C_4 linkages (∽ 78.511–96.527 ppm) were not detected.

bound to the cell wall. We are now performing enzymatic analyses to better understand its chemical structure, as xyloglucan, despite its β-linked gluco-backbone and irrespective to their galactosylated or fucosylated forms, is also reactive to iodine. Incidentally, all sugar units mentioned here do appear in the hemicellulosic hydrolyzates.

A typical chemical shift ($\delta = 60.4$) for the CH_2–OH group (C_6) was found in both spectra, as well as a signal for *N*-acetyl or *O*-Me group ($\delta = 57.1$). This signal could also be related to the NH_2 group from a protein component, but we have discarded this possibility as proteins were not detected in the analyzed fraction (see Table 2). It may also be considered that at least the last strong alkali step, due to the repeated boiling, would break the amide linkage of protein, polysaccharides being protected in all cases, through the action of $NaBH_4$. A chemical shift ($\delta = 170.4$), relating to the carboxyl group of the uronic acid was also found, which is in agreement with the electrophoresis data.

Molecular weight estimations of the KOH-isolate polysaccharides, based on monosaccharides (pentose/hexose), uronic acid and deoxyhexose contents, showed a range of 13 000–10 000 and point to a mean molecular weight for the cell-wall polysaccharides of about 11 500 and a hexose:pentose ratio 70:30. However, it is interesting to highlight the presence of two non-identified peaks in Fig. 1 chromatogram (Rt = 15.74 and 17.42). They were also detected in the GLC runs for silyl-derivatives with Rt_s 6.20 and 6.56. ^{13}C NMR spectra (Fig. 2) showed a characteristic signal for β-D-Glc-NAc, or *O*-Me compound ($\delta = 57.1$), which could be related to these unidentified compounds. The identification of these compounds is in progress.

The basic hemicellulose backbone might be glucan-like. It should be emphasized that cellulose is not hydrolyzed to any extent under the conditions employed here. From the ^{13}C NMR spectra data, we have tentatively assumed that the glucan main chain could be β (1 → 6) linked (Fig. 2).

The presence of the deoxy-sugar L-fucose in calli-derived hemicellulosic fractions, albeit the low amounts, is interesting because it is seldom found as part of the cell wall composition in higher plants. Tamarind seed xyloglucan is one example. Recently, a more branched and L-fucose containing xyloglucan from cell wall of *Arabidopsis* was isolated by Potter and Reiter (1997). The biological activity of oligosaccharides from cell walls of *Arabidopsis thaliana* mutant (*mur1)* was not altered when L-fucose was replaced by L-galactose, because the essential structural and the conformational features of the cell wall were retained (Zablackis et al., 1996).

This deoxy-sugar has been found in bacterial polysaccharidic antigens, specially as an end residue (Bishop & Jennings, 1982; Flowers, 1981), and also in human milk oligosaccharides (Malpress & Hytten, 1957). L-Fucose in α-glycosidic linkages has been related to blood-group-H(O) specificity in humans (Springer, Desai & Kolecki, 1964), and it is also related to recognition processes in viral infections (Müthing, 1996). These facts could be related to the strong immunological response found in previous studies for the *M. velutina* cellular envelope

(Maraschin, Carobez, Tofol & Fontana, 1995). Vreeland and Laetsch (1984) proved the intracellular synthesis of cell wall antigens containing L-fucose in *Fucus*. Osman, Fett and Hicks (1988) studying the lipopolysaccharide of *Pseudomonas syringae* pv. *phaseolicola* strain NPS 3121, found L-fucose, L-rhamnose, and D-glucose as structural components of its *O*-antigen. D-fucose, a rare sugar in nature, as furanosidic and terminal residue was found in the polysaccharide antigen of *Eubacterium saburreum* strain L. 452, as showed by Hoffman, Lindberg, Hofstad and Lygre (1977).

It is also worth mentioning the presence of L-fucose in the glyco-moiety of the MV 8612 compound, a glycosylated form of the *M. velutina* triterpenoid which has showed pharmacological activity similar to the parent compound of interest: velutinol *A*. Thus, the presence of the same deoxy-sugar in *M. velutina* cell wall seems to be related to structural traits, as a cell wall constituent, and also to a secondary metabolism. Needless to say, extensive SDS detergent pre-treatments of *M. velutina* calli led to cell wall preparations completely free of velutinol and their glycosylated forms.

The results obtained show a cell wall sugar composition whose feature is the predominance of common phytobiomass hexoses and pentoses, except for the occurrence of L-fucose. The occurrence of uronyl residues facilitates the chromatographic and electrophoretic subfractionation of the hemicellulosic material, thus allowing the determination for the anchoring site(s) for L-fucose. This approach is in progress and it will provide the fucose-enriched fraction which will allow us to continue to follow a species or cell line characterization/selection by an immunological approach (Maraschin et al., 1995).

References

Albersheim, P., Nevins, D. J., English, P. D., & Karr, A. (1967). A method for the analysis of sugars in plant cell-wall polysaccharides by gas–liquid chromatography. *Carbohydrate Research*, *5*, 340–345.

Bento, E. S., Calixto, J. B., Hawkes, G. E., Pizzolatti, M. G., Sant'Ana, A. E. G., & Yunes, R. A. (1996). The structure of velutinol A is (15R, 16R, 20S)-14, 16:15, 20:16, 21-triepoxy-15-16-seco-14β,17α-pregn-5-ene-3β,15-diol. A combined quantitative Overhauser effect and molecular modelling study. *Journal of Chemical Society, Perkin Transactions 2*, 1359–1366.

Bishop, C. T., & Jennings, J. (1982). Immunology of polysaccharides. In G. O. Aspinall (Ed.), (pp. 291). *The polysaccharides*, 1. New York: Academic Press.

Blake, J. D., Murphy, P. T., & Richards, G. N. (1971). Isolation and A/B classification of hemicelluloses. *Carbohydrate Research*, *16*, 49–57.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484–489.

Caetano, L.C. (1993). Estudo da formaÇão de metabólitos secundários em sistemas de cultura de tecidos vegetais—calos, suspensão de células, raízes normais, raízes transformadas via Agrobacterium rhizogenes, folíolos normais e folíolos transformados via A. tumefasciens (folRolo-mutante). In: I Encontro Brasileiro de Biotecnologia Vegetal. Brasília/DF. Proceedings, pp. 14–15.

Calixto, J. B., Pizzolatti, M., & Yunes, R. A. (1988). The competitive antagonistic effect of compounds from Mandevilla velutina on kinin-induced contractions of rat uterus and guinea-pig ileum in vitro. *Brazilian Journal of Pharmacology*, *94*, 1133–1142.

Calixto, J. B., Silva, A. L., Reis, M. S., Costa, R. M. B. F. L., Yunes, J. A., Cruz, A. B., & Yunes, R. A. (1989). Active anti-bradykinin compounds by callus culture of Mandevilla velutina. *Brazilian Journal of Medical and Biological Research*, *22*, 1275–1279.

Ferreira, S. H. (1965). A bradykinin potentiating factor (BPF) present in the venom of Bothrops jararaca. *Brazilian Journal of Pharmacology*, *24*, 163–169.

Flowers, H. M. (1981). Chemistry and Biochemistry of D- and L-Fucose. *Advances in Carbohydrate Chemistry and Biochemistry*, *39*, 279–345.

Fontana, J. D., Gebara, M., Blumel, M., Schneider, H., MacKenzie, R., & Johnson, K. G. (1988). The 4-O-methyl-α-D-glucuronidase component of xylanolytic complexes. *Methods in Enzymology*, *160*, 560–571.

Hoffman, J., Lindberg, B., Hofstad, T., & Lygre, H. (1977). Structural studies of the polysaccharide antigen of Eubacterium saburreum, strain L. 452. *Carbohydrate Research*, *58*, 439–442.

Kakehi, K., & Honda, S. (1990). Silyl ether of carbohydrates. In C. J. Biermann & G. D. McGinnis (Eds.), *Analysis of carbohydrates by GLC and MS*, (pp. 43). Boca Raton, FL: CRC Press.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Malpress, F. H., & Hytten, F. E. (1957). Oligosaccharides of human milk. *Nature*, *4596*, 1201.

Maraschin, M., Carobrez, S.G., Tofol, M., & Fontana, J.D. (1995). Polyclonal antibody production against *Mandevilla velutina* (Apocynaceae) cells. In: Proceedings of the Ninth International Congress of Immunology, San Francisco, CA, pp. 513.

Murashige, T., & Skoog, D. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology of Plants*, *15*, 473–497.

Müthing, J. (1996). Influenza A and Sendai viruses preferentially bind to fucosylated gangliosides with linear poly-N-acetyllactosaminyl chains from human granulocytes. *Carbohydrate Research*, *290*, 217–224.

Nicolau, M., Calixto, J.B., Bitencourt, M.L., Beins, M.N., Cordeiro, R.S.B., Henrique, M.G.O., Weg, V.B., & Yunes, R.A. (1986). Effects of Mandevilla velutina crude extracts in cutaneous rat vascular permeability and mouse carrageenin induced pleurisy. In: Proceedings of the French–Brazilian Symposium of Chemistry and Pharmacology of Natural Substances in Inflammation, Allergy and Thrombosis, Rio de Janeiro, pp. 233.

Osman, S. F., Fett, W. F., & Hicks, K. B. (1988). Structure of the O-antigen of Pseudomonas syringae pv. phaseolicola strain NPS 3121. *Carbohydrate Research*, *176*, 205–210.

Potter, I., & Reiter, W.D. (1997). Investigating the role of fucose in the plant cell wall, Proceedings of the Eighth International Meeting on Arabidopsis Research, Matson, pp. 8–21.

Rocha, S. M., Beraldo, W. T., & Rosenfeld, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma by snake venom's and by trypsin. *American Journal of Physiology*, *156*, 261–273.

Selvendran, R. R., Stevens, B. J. H., & O'Neill, M. A. (1985). Developments in the isolation and analysis of cell walls from edible plants. In C. T. Brett & J. R. Hillman (Eds.), *Biochemistry of the plant cell wall* (pp. 39). London: Cambridge University Press.

Selvendran, R. R., & O'Neill, M. A. (1987). Isolation and analysis of cell walls from plant material. *Methods in Biochemical Analysis*, *32*, 25–153.

Springer, G. F., Desai, P. R., & Kolecki, B. (1964). Synthesis and immunochemistry of fucose methyl ethers and their methylglycosides. *Biochemistry*, *3*, 1076–1085.

Vreeland, V., & Laetsch, V.M. (1984). Intracellular synthesis of Fucus cell wall antigens. Plant Physiol. (suppl.). In: Proceedings of the Annual Meeting of the American Society of Plant Physiologists, UCLA, pp. 350.

Zablackis, E., York, W. S., Pauly, M., Hantus, S., Reiter, W. D., Chapple, C. S., Albersheim, P., & Darvill, A. (1996). Substitution of L-fucose by L-galactose in cell walls of Arabidopsis mur 1. *Science*, *272*, 1808–1810.