Carbohydrate Polymers 94 (2013) 179–184

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structure and antiviral activity of arabinogalactan with (1→6)-β-D-galactan core from *Stevia rebaudiana* leaves

Arildo José Braz de Oliveira[a], Lucimara M.C. Cordeiro[b], Regina Aparecida Correia Gonçalves[a], Ligia Fernanda Ceole[c], Tania Ueda-Nakamura[c,d], Marcello Iacomini[b,*]

[a] Departamento de Farmácia, Universidade Estadual de Maringá, Avenida Colombo, 5790, CEP 87.020-900, Maringá, PR, Brazil
[b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19.046, CEP 81.531-980, Curitiba, PR, Brazil
[c] Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, PR, Brazil
[d] Departamento de Ciências Básicas da Saúde, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, PR, Brazil

ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form
30 November 2012
Accepted 27 December 2012
Available online 4 January 2013

Keywords:
Arabinogalactan
Stevia rebaudiana
Antiviral activity
Herpes simplex virus type-1

ABSTRACT

Cell wall polysaccharides from leaves of *Stevia rebaudiana* were extracted successively with water and with aq. 10% KOH. After the purification steps, homogeneous fractions (SFW-10RM and SSFK-10RM) were analyzed by sugar composition, HPSEC, methylation and ^{13}C NMR spectroscopy analysis. The results showed that SFW-10RM is a pectic arabinogalactan with an unusual β-(1→6)-linked D-Gal*p* residues forming the main chain. Approximately 38% of the β-D-Gal*p* units of the backbone carry branches on position *O*-3, consisting of single D-Gal*p* units or arabinan side chains. Arabinose residues were found to occupy mostly the terminal positions in both furanose and pyranose forms and as 2-, 5- and 3,5-linked residues in these side chains. Fraction SSFK-10RM is a similar arabinogalactan, differing mainly in the relative proportions of arabinans attached to the galactan core and in the content of D-Gal*p*A residues present in the pectic domain. The crude aqueous and alkaline extracts and homogeneous SSFK-10RM showed antiviral activity against Herpes Simplex Virus type-1 (HSV-1) *in vitro*.

1. Introduction

Stevia rebaudiana is a member of Asteraceae family, native to certain regions of South America (Paraguay and Brazil) and is used as a medicinal plant for a long time. It has been studied in depth because this plant is the source of several well-known sweet-tasting compounds. Stevioside, the main sweet component in their leaves tastes about 300 times sweeter than sucrose (0.4% solution). Interest in using *S. rebaudiana* as a commercial sweetener has led to extensive phytochemical investigations of the herb's constituents (Geuns, 2003; Kennelly, 2002). Thus, there are many studies describing the isolation and identification of various types of secondary metabolites from their leaves, including diterpenoid steviol-glycosides (Kinghorn & Soejarto, 1985), sesquiterpenes (Martelli, Frattini, & Chialva, 1985), bis-nor-diterpene, sterols (Oshima, Saito, & Hikino, 1986; Oshima, Saito, & Hikino, 1988; Sholichin, Yamasaki, Miyama, Yahara, & Tanaka, 1980) and flavonoids (Rajbohandari & Roberts, 1983).

Moreover, many biological activities, such as antihyperglycemic and anti-inflammatory, are related to these metabolites (Gregersen, Jeppesen, Holst, & Hermansen, 2004; Jeppesen, Gregersen, & Rolfsen, 2003; Lailerd, Saengsirisuwan, Sloniger, Toskulkao, & Henriksen, 2004; Nabaweya, Ibrahim, Motawe, & Riad, 2007).

Despite the extensive investigations about the secondary metabolites present in *S. rebaudiana* leaves, little attention was paid to the study of primary metabolites, mainly polysaccharides. Takahashi et al. (2001) described that hot-water extract of *S. rebaudiana* leaves (SE) had inhibitory activity against the replication of Human rotavirus (HRV). These authors showed that the anti-HRV components of SE were heterogeneous anionic polysaccharides with different ion charges. Analyses of sugar residues suggested uronic acid(s) as sugar components. It did not contain amino and neutral sugars and sulfate residues. Recently, Oliveira et al. (2011) described the isolation of fructooligosaccharides form *S. rebaudiana* leaves.

In this work we describe the chemical composition, structural features and antiviral activity against Herpes simplex virus type-1 (HSV-1) of two fractions containing arabinogalactans with unusual (1→6)-β-D-galactan main chain isolated from leaves of *S. rebaudiana*.

* Corresponding author. Tel.: +55 41 33611655; fax: +55 41 32662042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.12.068

2. Materials and methods

2.1. Plant material

Dried and powdered leaves of *S. rebaudiana* (Bert.) Bertoni were purchased from SteviaFarma, Maringá, Paraná State, Brazil. Voucher specimens are deposited in the Herbarium of the Department of Biology at the Maringá State University (identification number 14301-HUEM).

2.2. General analytical methods

Fractions were carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving products with the —COOH groups of its uronic acid residues reduced to $-CH_2OH$.

2.3. Extraction and purification of polysaccharides

The extraction and purification of polysaccharides from leaves of *S. rebaudiana* was processed according to Fig. 1A. Ground leaves (100 g) were extracted with refluxing acetone for 2 h (×3, 1 L each), in order to remove lipids, pigments and other hydrophobic material. The polysaccharides were extracted from the residue with water at 100 °C for 3 h (×3, 1 L each). The aqueous extracts were obtained by centrifugation (3860 × *g*, 20 min at 25 °C), joined and concentrated under reduced pressure. The polysaccharides were precipitated with EtOH (3 vol.) and freeze–dried, giving fraction FW (4.85 g). The remaining residue I was then extracted with aq. 10% KOH, at 100 °C for 2 h (×3, 1 L each), in the presence of a trace of $NaBH_4$, and the alkaline extracts were neutralized with acetic acid, dialyzed for 48 h with tap water (Spectra Por®, MWCO 6000–8000), concentrated under reduced pressure and freeze–dried, originating fraction FK (4.98 g).

Fractions FW and FK were dissolved in water (100 mL), and the solutions were treated with 10% aqueous TCA (100 mL) to precipitate protein (Cipriani, Mellinger, Souza, Baggio, & Freitas, 2006). After centrifugation, the supernatants were neutralized with aq. NaOH, dialyzed and freeze–dried. Then, a freeze–thaw treatment (Iacomini, Gorin, & Baron, 1988) was applied in these fractions, to give cold-water soluble fractions SFW (2.68 g) and SFK (0.93 g). In this procedure, the sample was frozen and then thaw at room temperature. Insoluble polysaccharides were recovered by centrifugation.

Fraction SFK was solubilized in water and treated with Fehling solution, and the resulting insoluble Cu2+ complex isolated by centrifugation. Both the supernatant and the insoluble complex were neutralized with HOAc, dialyzed, deionized with H+ form cation-exchange resin and lyophilized, yielding the Fehling supernatant (Fraction SSFK, 0.66 g yield).

Solutions of 500 mg of fractions SFW and SSFK in 100 mL of NaOAc/AcOH buffer, pH 4.5, were incubated with endo-inulinase (INU, 230 U/mL, MEGAZYME). Hydrolysis was conducted up to exhaustive degradations of polysaccharides. The reactions were stopped by heating at 100 °C for 5 min, dialyzed against water, and freeze–dried, yield 292.3 mg of SFW and 385.0 mg of SSFK after endo-inulinase treatment.

The polysaccharides present in fractions SFW and SSFK were purified by sequential ultrafiltration through membranes (Fig. 1B) with cut-offs of 50 kDa, 30 kDa and 10 kDa (Ultracel, Millipore).

The yields were expressed on the initial weight of leaves that were submitted to extraction (100 g).

2.4. Sugar composition

Neutral monosaccharide components of the polysaccharides and their ratio were determined by hydrolysis with 2 M TFA for 8 h at 100 °C, followed by conversion to alditol acetates by successive $NaBH_4$ or $NaBD_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v, 1 mL) at room temperature for 14 h, and the resulting alditol acetates extracted with $CHCl_3$. These were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model (ITD 800) mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, hold at 50 °C during injection for 1 min, then programmed at 40 °C/min to 220 °C and hold at this constant temperature for 19.75 min was used for the quantitative analysis.

Uronic acid contents were determined using the m-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991).

2.5. Determination of homogeneity of polysaccharides and molecular weight of components

The homogeneity and average molar mass (M_w) of soluble polysaccharides were determined by high performance steric exclusion chromatography (HPSEC), using a differential refractometer (Waters) as detection equipment. Four columns were used in series, with exclusion sizes of 7×10^6 Da (Ultrahydrogel 2000, Waters), 4×10^5 Da (Ultrahydrogel 500, Waters), 8×10^4 Da (Ultrahydrogel 250, Waters) and 5×10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min. The sample, previously filtered through a membrane (0.22 μm, Millipore), was injected (250 μL loop) at a concentration of 1 mg/mL. The specific refractive index increment (dn/dc) was determined and the results were processed with software ASTRA provided by the manufacturer (Wyatt Technologies).

2.6. Methylation analysis of polysaccharide

The purified polysaccharides were *O*-methylated according to the method of Ciucanu and Kerek (1984), using powdered NaOH in DMSO-MeI. The per-*O*-methylated derivatives were hydrolyzed with 45% formic acid for 18 h at 100 °C, and the resulting mixture of partially *O*-methylated monosaccharides was successively reduced with $NaBD_4$ and acetylated with Ac_2O-pyridine. The products (partially *O*-methylated alditol acetates) were examined by capillary GC–MS. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection for 1 min, then programmed at 40 °C/min to 210 °C and held at this temperature for 31 min was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005a; Sassaki, Iacomini, & Gorin, 2005b).

2.7. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR spectra and DEPT-135 experiment (Distortionless Enhancement by Polarization Transfer) were obtained with a Bruker DRX 400 MHz AVANCE III NMR spectrometer (Bruker Daltonics, Germany), according to standard Bruker procedures. Analyses were performed with a 5 mm inverse gradient probe, at 50 °C, the water soluble samples being dissolved in D_2O. Chemical shifts are expressed as δ PPM, using the resonances of CH_3 groups of acetone internal standard (δ 30.2). The spectra were handled using the computer program Topspin® (Bruker).

2.8. Cytotoxicity assay and antiviral activity

Vero cells were maintained in DMEM (Dulbecco's Modified Eagle Medium – Gibco®) supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL gentamicin in a humid atmosphere of 5% CO_2 at 37 °C. For cytotoxicity assays, a density of 2.5×10^4 of cells/well were seeded into 96-well plates and incubated for

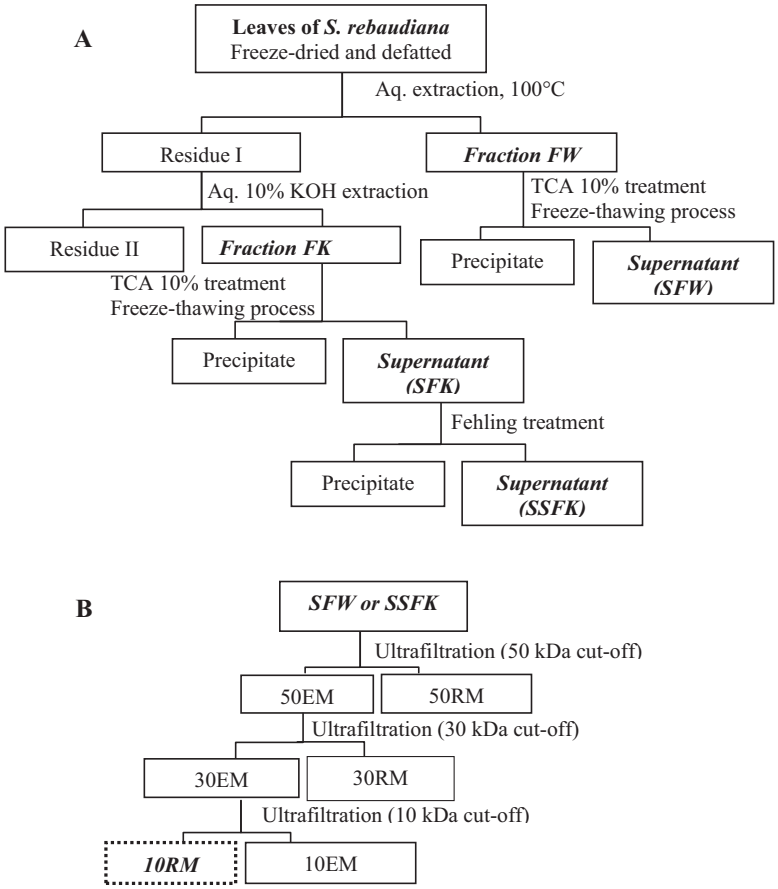

Fig. 1. Scheme of extraction (A) and fractionation (B) of polysaccharides from leaves of *Stevia rebaudiana*. **R** indicates that the fraction was retained, while **E** indicates that the fraction was eluted in the ultrafiltration membrane.

24 h at 37 °C to obtain a confluent monolayer. The medium was removed and fresh DMEM containing different concentrations of polysaccharides were added onto cells. After 72 h of incubation, the medium was replaced by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution and incubated for 4 h (Mosmann, 1983). Then, formazan crystals were solubilized in DMSO and the absorbance was read at 562 nm in a microplate reader (Power Wave XS, Bio-Tek).

In order to investigate the anti *Herpes simplex* virus type-1 (HSV-1) activity, confluent cell monolayers, non-infected or infected ($TCID_{50}$) with HSV-1 clinical isolate were treated with different concentrations of polysaccharides prepared in DMEM, and incubated during 72 h, at 37 °C, 5% CO_2. Cell viability was monitored by the MTT colorimetric method as described above and percentage of cellular protection was determined.

3. Results and discussion

Leaves of *S. rebaudiana* were extracted by reflux with boiling H_2O, and the extract was treated with excess EtOH, to obtain a crude precipitate of polysaccharides. It was deproteinized and submitted to a freezing-thawing process. The latter furnished a supernatant component (SFW, 2.68% yield), which was heterogeneous when analyzed by high-performance size exclusion chromatography (HPSEC). The neutral monosaccharide analysis indicated the presence of Ara (26%), Gal (28%), Rha (13%), Glc (22%) and Man (3%). The glucose and mannose content could arise from the presence of fructose, as products of its reduction with $NaBH_4$ during derivatization for GC–MS analysis. Moreover, Oliveira et al. (2011) demonstrated the presence of fructose oligomers in the leaves of *S. rebaudiana*, indicating the presence of inulin. Thus, fraction SFW was submitted to endo-inulinase treatment, and after this, the content of glucose of fraction SFW was reduced to 1%. Then, it was submitted to purification by sequential ultrafiltration through membranes (Fig. 1B) with cut-offs of 50 kDa, 30 kDa and 10 kDa (Millipore). HPSEC analysis (Fig. 2) showed that the retained material on 10 kDa membrane contained a homogeneous polysaccharide (SFW-10RM) with an average molar mass (*Mw*) of 10450 g/mol ($dn/dc = 0.136$). It contained arabinose, galactose, uronic acid, rhamnose in a 38:20:37:5 molar ratio, indicating the presence of a pectic arabinogalactan.

In order to achieve the linkages present in the pectic arabinogalactan, fraction SFW-10RM was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972) and submitted to methylation analysis (Table 1). The presence of 2,3,6-Me_3-galactitol

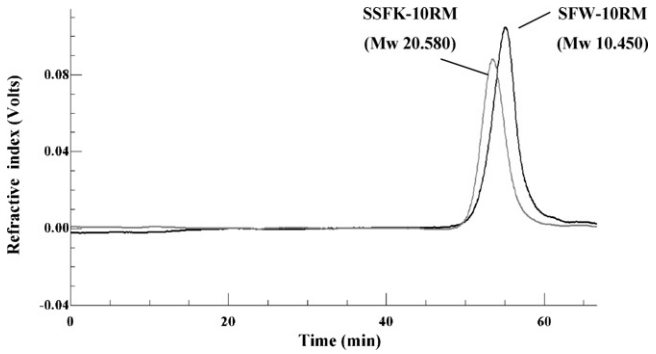

Fig. 2. HPSEC elution profile of purified fractions SFW-10RM and SSFK-10RM. Refractive index detector.

Table 1
Linkage types based on analysis of partially *O*-methyl alditol acetates obtained from methylated fractions of *Stevia rebaudiana*.

Partially *O*-methylalditol acetate	SFW-10RM[b]	SSFK-10RM[b]	Linkage type[c]
Panel A: Pectic saccharides			
3,4-Me_2-Rha	6.0	10.0	→2)-Rha*p*-(1→
3-Me-Rha	1.0	2.0	→2,4)-Rha*p*-(1→
2,3,6-Me_3-Gal[d]	32.0	11.0	→4)-Gal*p*-(1→
Panel B: Arabinogalactan saccharides			
2,3,4-Me_3-Ara[a]	3.0	1.0	Ara*p*-(1→
3,4-Me_2-Ara	1.0	1.0	→2)-Ara*p*-(1→
2,3,5-Me_3-Ara	12.0	13.0	Ara*f*-(1→
2,3-Me_2-Ara	14.0	30.0	→5)-Ara*f*-(1→
2-Me-Ara	9.0	11.0	→3,5)-Ara*f*-(1→
2,3,4,6-Me_4-Gal	6.0	6.0	Gal*p*-(1→
2,3,4-Me_3-Gal	10.0	11.0	→6)-Gal*p*-(1→
2,4-Me_2-Gal	6.0	4.0	→3,6)-Gal*p*-(1→

[a] 2,3,4-Me_3-Ara = 2,3,4-tri-*O*-Methylarabinitolacetate, *etc.*
[b] % of peak area of *O*-methylalditol acetates relative to total area, determined by GC–MS. Samples were carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), prior to methylation analysis.
[c] Based on derived *O*-methylalditol acetates.
[d] Arises from Gal*p*A.

acetate (Table 1, panel A) and its absence in the methylation analysis of native polymer (data not shown) indicated that the galacturonic acid must have been present in a (1→4)-linkage. The detection of derivatives of 2,4-di-*O*- and 4-*O*-linked rhamnopyranose (Table 1, panel A) suggests that in SFW-10RM, like in other rhamnogalacturonans, small blocks of (1→4)-linked D-GalA are interspersed by rhamnopyranosyl units carrying most of the side chains *via O*-4. These units correspond to approximately 14% of total rhamnose units present. Due to the presence of higher amounts of D-GalA than that of L-Rha units, the presence of some longer uninterrupted D-GalA chains (characteristic of homogalacturonans) could not be fully discarded.

Unlike other arabinogalactans (so-called arabino-3,6-galactans or type II), the galactan core of SFW-10RM is built up by (1→6)-linked galactopyranosyl units, due to the prevalence of 6- and 3,6-linked galactopyranose residues. The absence of 2,4,6-Me_3-galactitol acetate in the methylation analysis (Table 1, panel B), demonstrated that (1→3)-linked galactopyranosyl units, typical of the main chain of type II arabinogalactans are not present in SFW-10RM. Approximately 38% of the D-Gal*p* units of the backbone of SFW-10RM must have branches on position *O*-3, consisting of single D-Gal*p* units or arabinan chains. Arabinose residues were found to occupy mostly the terminal positions in both furanose and pyranose forms and as 2-, 5- and 3,5-linked residues in the side chains (Table 1, panel B).

The ^{13}C NMR spectrum (Fig. 3A) of SFW-10RM showed signals at δ 109.2, 107.4 and 107.1 due to C-1 resonances of terminal, 5- and 3,5-linked α-L-arabinofuranosyl residues, respectively. The low-field chemical shifts indicated L-Ara adopted α configuration and the furanose form. Two signals that appeared close together at δ 103.4 corresponded to C-1 of 6- and 3,6-linked β-D-Gal*p* units (Wagner & Jordan, 1988; Thude & Classen, 2005). The weaker signal δ 101.6 was assigned to anomeric carbon of β-arabinopyranosyl units (Agrawal, 1992; Gorin & Mazurek, 1975). The signals of pectic saccharides appeared at δ 98.9, 98.4 and 97.5 and are corresponding to C-1 of →4)-Gal*p*A-(1→4)-Gal*p*A, →4)-Gal*p*A-(1→2)-Rha*p* and →4)-Gal*p*A-(1→2)-Rha*p* units, respectively, while their C-6 signals were seen at δ 175.3 (α-D-Gal*p*A) and 16.6 (α-L-Rha*p* units).

The signals at δ 78.0, 69.0 and 68.3 corresponding to C-4, C-3 and C-2 of (1→4)-linked D-Gal*p*A residues (Simas-Tosin et al., 2012; Vriesmann, Silveira, & Petkowicz, 2009). The resonances at 76.6 and 79.3–84.0 derived from C2–C4 of α-L-Ara*f* units. The C-5 of non-reducing end and 5-*O*-substituted α-L-Ara*f* units appeared at δ 61.2 and 66.0–66.8 (four signals inverted in DEPT-135 experiment), respectively (Thude & Classen, 2005; Wagner & Jordan, 1988). Moreover, the DEPT-135 experiment also showed inverted signals at δ 69.1 and 69.2, corresponding to substituted C-6 of the β-D-Gal*p* units. Due to the complexity of the spectra, the other signals were difficult to assign.

Finally, the results suggest that SFW-10RM is a pectic arabinogalactan with an unusual β-(1→6)-linked D-Gal*p* residues forming the main chain carrying single residues of β-D-Gal*p* and arabinosyl side chains at *O*-3.

The residue of water extraction was extracted with 10% aqueous KOH in the presence of a trace of $NaBH_4$, The alkaline extract was neutralized with HOAc, dialyzed against water, and freeze–dried, to obtain a crude precipitate of polysaccharides (Fraction FK). It was deproteinized and submitted to a freezing–thawing process. The cold water soluble fraction (Fraction SFK, Fig. 1A) was treated with Fehling solution, furnishing a supernatant component (Fraction SSFK, 0.66% yield), which was heterogeneous when analyzed by high-performance size exclusion chromatography (HPSEC) and monosaccharide analysis indicated the presence of Ara (29%), Gal (18%), Rha (12%), Glc (28%), Man (2.0%) and Xyl (11%). As seen above for fraction SFW, the glucose content could arise from $NaBH_4$ reduction of fructose present in the inulin. After endo-inulinase treatment, the content of glucose of fraction SSFK reduced to <1%. Then, it was submitted to purification by sequential ultrafiltration through membranes (Fig. 1B) with cut-offs of 50 kDa, 30 kDa and 10 kDa (Millipore). HPSEC analysis (Fig. 2) showed that, as for fraction SFW, the retained material on 10 kDa membrane contained a homogeneous polysaccharide (SSFK-10RM) with an average molar mass (*M*w) of 20.580 g/mol (dn/dc = 0.136). It contained arabinose, galactose, uronic acid, rhamnose in a 55:21:12:12 molar ratio. When compared with SFW-10RM, it contains higher amounts of Ara residues and less of uronic acids. The methylation analysis of the carboxy-reduced SSFK-10RM (Table 1) yielded the same derivatives present in the SFW-10RM arabinogalactan. However, quantitative comparison showed that SSFK-10RM has higher % of 2,3-Me_2-arabinitol acetate, indicating the presence of longer (1→5)-arabinan side chains and lower amounts of 2,3,6-Me_3-Gal, from (1→4)-linked D-Gal*p*A residues, which were in agreement with monosaccharide analysis. This was also observed in the ^{13}C NMR spectrum (Fig. 3B) of SSFK-10RM, which showed an increase in the intensity of signals of arabinosyl moieties (anomeric signals at δ 109.2, 107.4 and 107.1, and skeletal C2-C4 signals at δ 76.6 and 79.3–84.0) and a decrease in the signals at δ 98.9, 78.0, 69.0 and 68.3, corresponding to C1, C-4, C-3 and C-2 of (1→4)-linked D-Gal*p*A residues, respectively.

Thus, as seen from methylation analysis and ^{13}C NMR spectroscopy SFW-10RM and SSFK-10RM differ mainly in the relative proportions of arabinans attached to the galactan core and in the content of D-Gal*p*A residues present in the pectic domain.

Most plant arabinogalactans are reported to have a 1,3-linked galactopyranan backbone with branch points of 6-linked β-D-Gal*p* (arabino-3,6-galactans). The occurrence of arabinogalactans with the β-1,6-galactan core branched at C-3 by α-L-Ara*f* side chains has been described only in few plants to date, as for example, in leaves of *Tridax procumbens* (Raju & Davidson, 1994), *Nerium indicum* (Dong & Fang, 2001) and *Salvia offininalis* (Capek, 2008), in 'berries' of *Viscum album* (Wagner & Jordan, 1988), in pollen of *Phleum pratense* (Brecker et al., 2005) and in roots of *Angelica acutiloba* (Kiyohara, Yamada, & Otsuka, 1987).

Concerning biological activity, like others arabino-3,6-galactans, the arabinogalactans with β-1,6-galactan core were also immunologically active and displayed anti-complementary activity *in vitro* (Kiyohara et al., 1987; Wagner & Jordan, 1988). We now show that the arabinogalactans from *S. rebaudiana* leaves have antiviral activity against Herpes Simplex Virus type-1.

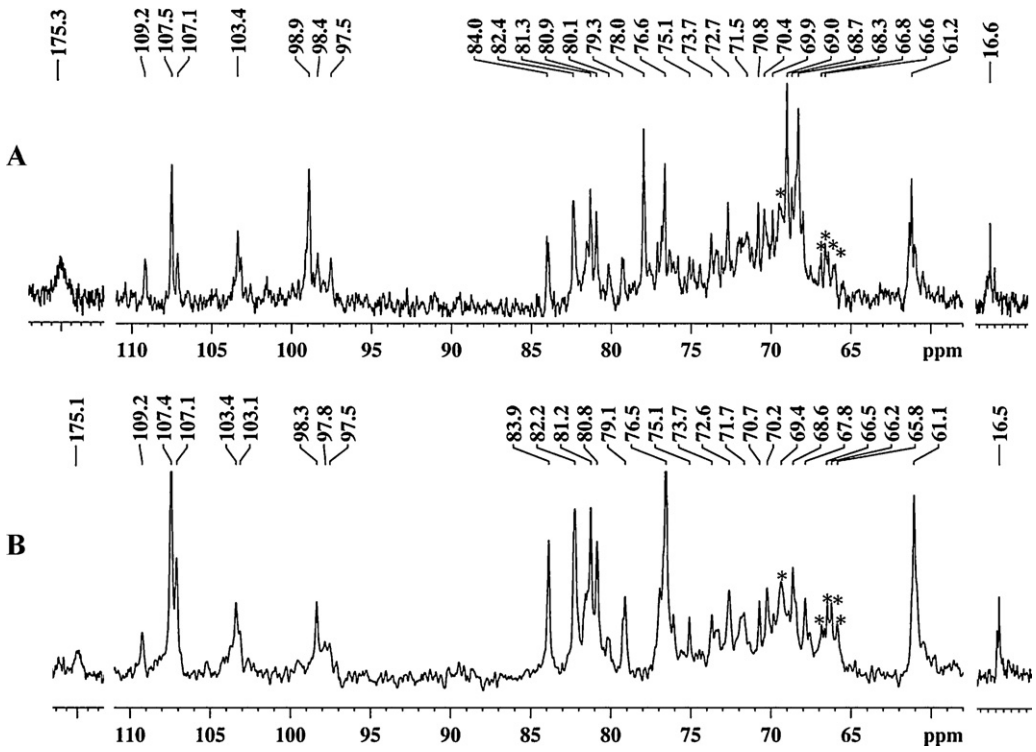

Fig. 3. ^{13}C NMR spectra of (A) fraction SFW-10RM and, (B) fraction SSFK-10RM, in D_2O at 50 °C, (chemical shifts are expressed as δ PPM) obtained from leaves of *Stevia rebaudiana*. Inverted signals in DEPT-135 experiment are marked with asterisk.

Considering the possibility of practical applications of polysaccharides obtained from leaves of *S. rebaudiana*, as a steviosides extraction sub products, we choose initially to use crude polysaccharides in the antiviral assay. The two crude polysaccharides, SFW and SSFK, obtained from leaves of *S. rebaudiana* demonstrated activity against HSV-1. 50% cytotoxic concentration (CC_{50}) of polysaccharide SFW was 275.0 μg/mL and higher than 1000 μg/mL for SSFK. The mean 50% effective concentration (EC_{50}) values were 0.3 for SFW and 18.8 μg/mL for SSFK, respectively. The CC_{50} and EC_{50} values were calculated by linear regression analysis. The selectivity index (SI) of each sample was calculated as CC_{50}/EC_{50}, and results are SI > 53 for SSFK and for SFW SI = 917.

As SSFK showed a low cytotoxicity, then we evaluated the antiviral activity of homogenous polysaccharide obtained from this extract, SSFK-10RM. The CC_{50} of SSRK-10RM was higher than 1000 μg/mL, EC_{50} value was 0.9 μg/mL and its SI > 1000 μg/mL.

Among the three polysaccharides tested for anti-HSV-1, when we take in account the SI values, the SSRK-10RM showed the best SI, that was higher than 1000, however, the antiviral activity of SSFK reaches almost 100% of protection against HSV-1 as the concentration increases. Inversely, for SSRK-10RM and SFW, although the EC_{50} values were lower when compared to SSFK the cell protection against HSV-1 infection decreases as drugs concentration increases. This result can be explained by the fact that higher concentrations of SFW seem to be toxic to cells.

However, anti-HSV-1 activity was observed in both crude arabinogalactan (SFW and SSFK) and homogeneous arabinogalactan (SSFK-10RM). The SFW showed an antiviral activity suggesting that the dominant Homogalacturonan (HG) region might correlate with the exhibition of anti-HSV-1 activity. Supporting this outcome, another pectin-type polysaccharide (named RP) isolated from *P. oleracea* L., which was composed of predominant HG regions but not pectic regions (named AP), showed potential anti-HSV-2 activity (Dong, Hayashi, Lee, & Hayashi, 2010). As seen from a structural point of view, the highly charged HG regions might contribute to the interference with electrostatic interactions between positive-charged regions of viral glycoproteins and negative-charged heparin sulfate chains of the cell-surface glycoprotein receptor, while the high branching characteristic of RG-I regions might shield the electrostatic interactions.

The increase in antiviral activity of SSFK-10RM compared to crude SSFK can be explained by the higher degree of purity of the polysaccharide and cytotoxicity reduction of this is probably related to its shorter highly charged HG region.

Control of infections caused by HSV-1 has been a challenge, because of their peculiar ability to avoid immune detection and establish latency in significant patient population (up to 80% human adults). Nucleoside analogs, especially acyclovir, have been employed in the treatment of HSV-1 infection (cold sores), but it is not able to eliminate the virus permanently. During the reactivation of the infection the virus particles are spread among hosts. Although it does not result in severe illness in health individuals, it can cause life-threatening diseases in immunocompromised individuals, including newborns, patients with HIV or patients undergoing immunosuppressive treatment (Whitley & Roizman, 2001). Therefore, the search for new candidate compounds with alternative mode of action to HSV-1 is crucial not only against it, but to other related viral diseases.

4. Conclusions

Two pectic arabinogalactans were purified from the leaves of *Stevia rebaudiana*: fraction SFW-10RM contains unusual β-(1→6)-linked D-Gal*p* residues forming the main chain. Approximately 38% of the β-D-Gal*p* units of the backbone carry branches on position *O*-3, consisting of single D-Gal*p* units or arabinan side chains. Fraction SSFK-10RM is a similar arabinogalactan, differing mainly in the relative proportions of arabinans attached to the galactan core and in the content of D-Gal*p*A residues present in the pectic domain. The crude aqueous and alkaline extracts

and homogeneous SSFK-10RM showed antiviral activity against Herpes simplex virus type-1 (HSV-1) *in vitro*.

Acknowledgements

This research was supported by CNPq foundation and by PRONEX-Carboidratos.

References

Agrawal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, *31*, 3307–3330.

Brecker, L., Wicklein, D., Moll, H., Fuchs, E. C., Becker, W. M., & Petersen, A. (2005). Structural and immunological properties of arabinogalactan polysaccharides from pollen of timothy grass (*Phleum pratense* L.). *Carbohydrate Research*, *340*, 657–663.

Capek, P. (2008). An arabinogalactan containing 3-O-methyl-D-galactose residues isolated from the aerial parts of *Salvia officinalis* L. *Carbohydrate Research*, *343*, 1390–1393.

Cipriani, T. R., Mellinger, C. G., Souza, L. M., Baggio, C. H., & Freitas, C. S. (2006). A polysaccharide from a tea (infusion) of *Maytenus ilicifolia* leaves with anti-ulcer protective effects. *Journal of Natural Products*, *69*, 1018–1021.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.

Dong, Q., & Fang, J. (2001). Structural elucidation of a new arabinogalactan from the leaves of *Nerium indicum*. *Carbohydrate Research*, *332*, 109–114.

Dong, C. X., Hayashi, K., Lee, J. B., & Hayashi, T. (2010). Characterization of structures and antiviral effects of polysaccharides from *Portulaca oleracea* L. *Chemical and Pharmaceutical Bulletin*, *58*, 507–510.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

Geuns, J. M. C. (2003). Stevioside. *Phytochemistry*, *64*, 913–921.

Gorin, P. A. J., & Mazurek, M. (1975). Further studies on the assignment of signals in 13C magnetic resonance spectra of aldoses and derived methyl glycosides. *Canadian Journal of Chemistry*, *53*, 1212–1222.

Gregersen, S., Jeppesen, P. B., Holst, J. J., & Hermansen, K. (2004). Antihyperglycemic effects of stevioside in type 2 diabetic subjects. *Metabolism*, *53*, 73–76.

Iacomini, M., Gorin, P. A. J., & Baron, M. (1988). Novel D-glucans obtained by dimethyl sulfoxide extraction of the lichens *Letharia vulpina*, *Actinogyra muehlenbergii*, and an *Usnea* sp. *Carbohydrate Research*, *176*, 117–126.

Jeppesen, P. B., Gregersen, S., & Rolfsen, S. E. (2003). Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. *Metabolism*, *52*, 372–378.

Kennelly, E. (2002). Sweet and non-sweet constituents of *Stevia rebaudiana*. In A. D. Kinghorn (Ed.), *Stevia, the genus Stevia. Medicinal and aromatic plants–industrial profiles* (pp. 68–85). London and New York: Taylor and Francis.

Kinghorn, A. D., & Soejarto, D. D. (1985). Current status of stevioside as a sweetening agent for human use. In H. Wagner, H. Hikino, & N. R. Farnsworth (Eds.), *Economic and medicinal plant research* (pp. 1–52). New York: Academic Press.

Kiyohara, H., Yamada, H., & Otsuka, Y. (1987). Unit structure of the anti-complementary arabinogalactan from *Angelica acutiloba* Kitagawa. *Carbohydrate Research*, *167*, 221–237.

Lailerd, N., Saengsirisuwan, V., Sloniger, J. A., Toskulkao, C., & Henriksen, E. J. (2004). Effects of stevioside on glucose transport activity in insulin sensitive and insulin resistant rat skeletal muscle. *Metabolism: Clinical and Experimental*, *53*, 101–107.

Martelli, A., Frattini, C., & Chialva, F. (1985). Unusual essential oils with aromatic properties, I. Volatile components of *Stevia rebaudiana* Bertoni. *Flavour Fragrance Journal*, *1*, 3–7.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal Immunology Methods*, *65*, 55–63.

Nabaweya, A., Ibrahim, S. E., Motawe, H., & Riad, S. A. (2007). Phytochemical and biological investigation of *Stevia rebaudiana* Bertoni; 1-labdane-type diterpene. *European Food Research Technology*, *224*, 483–488.

Oliveira, A. J. B., Gonçalves, R. A. C., Chierrito, T. P. C., Santos, M. M., Souza, L. M., Gorin, P. A. J., et al. (2011). Structure and degree of polymerisation of fructooligosaccharides present in roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni. *Food Chemistry*, *129*, 305–311.

Oshima, Y., Saito, J. I., & Hikino, H. (1986). Sterebins A, B, C and D, bisnorditerpenoids of *Stevia rebaudiana* leaves. *Tetrahedron*, *23*, 6443–6446.

Oshima, Y., Saito, J. I., & Hikino, H. (1988). Sterebins E, F, G and H, diterpenes of *Stevia rebaudiana* leaves. *Phytochemistry*, *27*, 624–626.

Rajbohandari, A., & Roberts, M. F. (1983). The flavonoids of *Stevia rebaudiana*. *Journal of Natural Products*, *46*, 194–195.

Raju, T. S., & Davidson, E. A. (1994). Structural features of water-soluble novel polysaccharide components from the leaves of *Tridax procumbens* Linn. *Carbohydrate Research*, *258*, 243–254.

Sassaki, G. L., Gorin, P. A. J., Souza, L. M., Czelusniak, P. J., & Iacomini, M. (2005). Rapid synthesis of partially O-methylated alditol acetate standards for GC–MS: some relative activities of hydroxyl groups of methyl glycopyranosides on Purdie methylation. *Carbohydrate Research*, *340*, 731–739.

Sassaki, G. L., Iacomini, M., & Gorin, P. A. J. (2005). Methylation–GC–MS analysis of arabinofuranose- and galactofuranose-containing structures: rapid synthesis of partially O-methylated alditol acetate standards. *Annals of the Brazilian Academy of Sciences*, *77*, 223–234.

Sholichin, M., Yamasaki, K., Miyama, R., Yahara, S. S., & Tanaka, O. (1980). Labdane-type diterpenes from *Stevia rebaudiana*. *Phytochemistry*, *19*, 326–327.

Simas-Tosin, F. F., Abud, A. P. R., De Oliveira, C. C., Gorin, P. A. J., Sassaki, G. L., Bucchi, D. F., et al. (2012). Polysaccharides from peach pulp: structure and effects on mouse peritoneal macrophages. *Food Chemistry*, *134*, 2257–2260.

Taylor, R. L., & Conrad, H. E. (1972). Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. *Biochemistry*, *11*, 1383–1388.

Takahashi, K., Matsuda, M., Ohashi, K., Taniguchi, K., Nakagomi, O., Yurika, A., et al. (2001). Analysis of anti-rotavirus activity of *Stevia rebaudiana*. *Antiviral Research*, *49*, 15–24.

Thude, S., & Classen, B. (2005). High molecular weight constituents from roots of *Echinacea pallida*: an arabinogalactan-protein and an arabinan. *Phytochemistry*, *66*, 1026–1032.

Wagner, H., & Jordan, E. (1988). An immunologically active arabinogalactan from *Viscum album* 'berries'. *Phytochemistry*, *27*, 2511–2517.

Vriesmann, L. C., Silveira, J. L. M., & Petkowicz, C. L. O. (2009). Chemical and rheological properties of a starch-rich fraction from the pulp of the fruit cupuassu (*Theobroma grandiflorum*). *Materials Science and Engineering: C*, *29*, 651–656.

Whitley, R. J., & Roizman, B. (2001). Herpes simplex virus infections. *Lancet*, *357*, 1513–1518.