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Structural analysis of the polysaccharides from *Echinacea angustifolia* radix

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Abstract

In this paper we report the characterization by monosaccharide and linkage analyses and by NMR spectroscopy and size exclusion chromatography of the carbohydrate fraction extracted from *Echinacea angustifolia* radix. In addition, the products of *endo*-pectin lyase, *endo*-pectate lyase, *endo*-polygalacturonase, *endo*-galactanase, and *endo*-arabinase digestion were characterized by MALDI-TOF mass spectrometry. The data obtained during this study showed that the carbohydrate fraction extracted from *E. angustifolia* radix is constituted by two polysaccharides with molecular weight of about 128,000 and 4500 Da. The low molecular weight polysaccharide corresponds to inulin while the high molecular weight component is a high metoxy pectin in which the backbone structure of the smooth region is constituted by α-(1–4)-polygalacturonan partially methyl esterified (60%) and acetylated (9%) and with the hairy regions containing 2-*O*- and 2,4-*O*-rhamnopyranose, 5-*O*- and 3,5-*O*-arabinofuranose, 3,6-galactopyranose, and terminal rhamnopyranose, arabinofuranose, arabinopyranose, galactopyranose, and galacturonopyranose. Mass spectrometry data of a galactanase treated sample showed evidence of a novel structure in the pectin hairy region, namely a galactose–galacturonic acid alternating sequence.

Keywords: *Echinacea*; Immunostimolation; Structure; Pectin

1. Introduction

Echinacea is a hardy perennial plant indigenous to North America, which belongs to the Asteraceae or Compositae plant family and includes nine different species (McGregor, 1968). Of these species *Echinacea purpurea*, *Echinacea angustifolia*, and *Echinacea pallida* have medical properties (Schulthess, Ginger, & Baumann, 1991), and are commercially traded as medicinal plants. Native Americans used *Echinacea* extensively for a variety of medical purposes (Foster, 1991; Hobbs, 1989; Kindscher, 1989). European settlers first used *Echinacea* around the beginning of 19th century. By 1921, *Echinacea* preparations were among the most widely sold medicines extracted from an American plant (Flannery, 1998).

The various *Echinacea* species are often misidentified. Much of the early research on *E. angustifolia* and *E. purpurea* was probably actually conducted on *E. pallida*, and studies published prior to 1987 must be viewed with suspicion in terms of the actual species being evaluated (Bauer, Khan, & Wagner, 1988; Bradley, 1992; Schumacher & Friedberg, 1991). In products made from *E. pallida* and *E. angustifolia*, the roots are typically used, whereas for *E. purpurea*, juices from the fresh leaves, stems, and flowers are most often used, though roots are sometimes included (Schulz, Hansel, & Tyler, 1997).

From the 1930s to 1970s, antibiotic development resulted in a sharp decline in *Echinacea* use, but due to a

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subsequent disenchantment with the medical establishment, an herbal medicine "renaissance" in the 1980s led to renewed interest in *Echinacea*'s benefits.

From the research conducted during the last 20 years *Echinacea* appears to have strong anti-inflammatory activity, wound-healing action, stimulates the immune system and may be effective against some viral and bacterial infections (Bauer, Reminger, & Alstat, 1990; Burger, Torre, & Warren, 1997; Cox, 1998; Schulthess et al., 1991). Scaglione and Lund (1995) observed that an anti-cold remedy including *E. purpurea* was effective for the treatment of common cold and it shortened the healing time. *Echinacea* products are medically prescribed in Germany and more than 200 pharmaceutical preparations are made from *Echinacea* plant (Blumenthal, 1998; Hobbs, 1989; Leslie, 1995). In the US, many health remedies or dietary supplements include *Echinacea* (mainly *E. purpurea* and *E. angustifolia*) (Leung & Forster, 1996; Li, 1998).

Chemical analysis of plants in the genus *Echinacea* has identified seven groups of medically important components including polysaccharides, flavonoids, caffeic acid derivatives, essential oils, polyacetylenes, alkylamides, and miscellaneous chemicals. A growing collection of scientific evidence supports also *Echinacea*'s important contribution to stimulating the immune system (Percival, 2000). Generally, *Echinacea* is thought to create activity in the immune system by stimulating T-cell production, phagocytosis, lymphocytic activity, cellular respiration, activity against tumour cell (thought its application is debatable), and inhibiting hyaluronidase enzyme secretion (Brauning, Dom, Linburg, & Knick, 1992). Some experts believe that the polysaccharides are primary active ingredients for immune modulating effects (Tubaro, Tragni, Del Negro, Galli, & Della Loggia, 1987; Wagner, Stuppner, Schafer, & Zenk, 1988). It appears that the immune-stimulating effects of *Echinacea* result from polysaccharides surrounding tissue cells and thereby providing protection from bacterial and pathogenic invasion (Newall, Anderson, & Phillipson, 1996). The polysaccharide components have also been shown to promote tissue regeneration by stimulating fibroblasts and inhibiting the enzyme hyaluronidase, which breaks down the intracellular cement called hyaluronic acid (Enbergs & Woestmann, 1986).

Barman has reported about the HIV killing activity induced by *E. angustifolia* (Barman, See, See, Justis, & Tilles, 1998). Because the agents used for cancer chemotherapy and for treating the patients with depressed cellular immunity (AIDS) are known to be highly toxic towards normal host cells, possible immune-enhancing activity by herbal compounds is looked with interest and more research is needed in this area.

Numerous clinical studies of *Echinacea* have been conducted over the last 20–30 years that overwhelmingly demonstrate its therapeutic benefit and safety, even in patients with autoimmune disorders (Jurcic, Melchart, Holzmann, & Martin, 1989; Parnham, 1996; Scaglione & Lund, 1995; Tyler, 1993; Weiss, 1988). The British Herbal Pharmacopoeia (1983), The British Herbal Compendium (1992), and The Encyclopaedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics (Leung & Forster, 1996) list no contraindications for *Echinacea* so it has been possible to conclude that the use of this plant is safe.

Despite its popularity, the scientific understanding of how *Echinacea* extracts work on the immune system is incomplete and so is also the knowledge of the active components. Three different polysaccharides with immune-stimulating properties were isolated from *E. purpurea* and characterized: two neutral fucogalactoxyloglucans with mean molecular mass (M_r) of 10,000 and 25,000 and an acidic arabinogalactan with a mean M_r of 75,000 (Wagner & Proksch, 1987; Wagner et al., 1988), while the structure of polysaccharides from *E. angustifolia* is still unknown, even though the immune-stimulating properties of the polysaccharidic fractions is known since 1987 (Tubaro et al., 1987). In this paper, we report the characterization of the polysaccharides extracted from *E. angustifolia* radix, by monosaccharide and linkage analyses and by NMR spectroscopy and size exclusion chromatography. In addition, polysaccharidic fractions obtained by enzymatic degradations were characterized by MALDI-TOF mass spectrometry. The data obtained permitted to determine the distribution of methyl groups and degree of esterification over the homogalacturonan segments, and some structural features of the hairy region.

2. Materials and methods

2.1. Samples preparation

2.1.1. Extraction of E. angustifolia roots (steps a and b)

Six hundred grams of ground roots of cultivated *E. angustifolia* is extracted under reflux for 4 h with 2.5 L of 90% (v/v) ethanol. The percolate is collected and further seven extractions with the same solvent are carried out. The resulting extract is discarded. The roots are then extracted seven times at 70 °C with 15% (v/v) ethanol, each extraction lasting 4 h. The combined percolates are filtered and concentrated under vacuum. The concentrate is diluted with water to a total volume of 700 ml and 1.13 L of ethanol are then added. The mixture is stirred for about 1 h, the precipitate is decanted for about 20 min, filtered, washed with 850 ml of 66.5% (v/v) ethanol, and dried at 55 °C under vacuum for 48 h, affording 140 g of a hazel-color solid. The solid is taken up with 2.1 L of water and the resulting suspension is left under stirring for 20 min, then separated from the insoluble residue (which is discarded). The aqueous solution, enriched in the polysaccharide, gives a residue after drying of 38.7 g (carbohydrate fraction from *E. angustifolia* radix).

2.1.2. Recovery of the polysaccharide by pre-purification with solvents and anion exchange chromatography

One hundred and seventy grams of whole polysaccharides extract is dissolved in 570 ml of water and 1.13 L of

ethanol are then added. The mixture is stirred for about 1 h, the precipitate is decanted for about 20 min, filtered, washed with 850 ml of 66.5% (v/v) ethanol, and dried at 55 °C under vacuum for 48 h, affording 140 g of a hazel-color solid. The solid is taken up with 2.1 L of water and the resulting suspension is left under stirring for 20 min, then separated from the insoluble residue (which is discarded).

The aqueous solution, enriched in the polysaccharide, gives a dry residue of 38.7 g. The solution is loaded on a chromatographic column containing 0.9 L of Diaion HPA 25 resin conditioned with AcOH/$AcNH_4$ buffer at pH 6.1. The resin is washed with 5.4 L of AcOH/$AcNH_4$ buffer at pH 6.1, and the fraction containing the polysaccharide is eluted with 5.4 L of a 0.5 M $AcNH_4$ aqueous solution. The eluate is concentrated to 0.3 L under reduced pressure. The concentrated solution is subjected to dialysis by tangential ultrafiltration with 6 L of purified water, using a 10,000 Da cutoff spiral-wound membrane in polyethersulfone. The retentate is recovered and concentrated under reduced pressure and the residue is heat-dried under vacuum, affording 7.1 g of an ivory-colour powder (polysaccharide HPLC titre: 96%).

2.2. Monosaccharide and linkage analysis

Analysis of monosaccharides by liquid chromatography was carried out by acid hydrolysis with 0.5 ml of 2 M trifluoroacetic acid (TFA) on 200 μg samples of polysaccharide at 120 °C for 3 h. TFA was then removed at 40 °C with a stream of dry air and successive co-evaporation with isopropyl alcohol. The high performance anion exchange (HPAE) apparatus consisted of a gradient pump equipped with a CarboPac PA-1 column (0.4 × 25 cm) and a pulsed amperometric detector (PAD) all from Dionex (Sunnyvale, CA, USA). Elution was 20 min with NaOH 16 mM, during which the neutral sugars were eluted, and then by a NaOH and sodium acetate gradient from 16 to 200 mM and from 0 to 400 mM in 25 min, respectively. Sugar analysis by gas chromatography was performed using the trimethylsilyl ether derivatives, as described by York, Darvill, McNeil, Stevenson, and Albersheim (1985). GC analyses were run on a Dani 6800 gas chromatography equipped with a flame ionization detector and a DB1 column (30 m × 0.32 mm i.d., J&W Scientific, Folsom, CA, USA) using helium as the carrier gas. Injections were made in the split mode with a 1:20 split ratio. Monosaccharide amounts were calculated as a mean of three measures.

Methylation was carried out by the Hakomori method as described by York et al. (1985). Briefly, 0.1 ml of dimethylsulfinyl carbanion was added to 5 mg of sample dissolved in DMSO, and the reaction mixture stirred for 1 h at room temperature. Then methyl iodide (0.035 ml) was added and the reaction mixture stirred for 1 h at room temperature. The resulting per-O-methylated polysaccharide was purified by C18 solid phase extraction (SPE) (Sep-Pak) and methyl carboxylate groups reduced with 1 ml of $Li(Et)_3BD$ 1 M in THF for 1 h at room temperature, the reducing agent destroyed with AcOH and removed as borate methyl ester. The resulting polysaccharide was purified by C18 SPE and again methylated repeating twice the addition of base and CH_3I. The permethylated polysaccharide, after purification on Sep Pak cartridges, was hydrolyzed with 1.2 ml of CF_3COOH 2 M at 120 °C for 3 h, the resulting monosaccharides deuterium reduced at C-1 by addition of $NaBD_4$ (1 ml of 10 mg/ml in 1 M NH_4OH) and finally, after removal of borate, acetylated with 1 ml pyridine/acetic anhydride 1:1 at 120 °C for 20 min. The acylation mixture was carefully evaporated at RT after addition of toluene and dissolved in chloroform for analysis by GC–MS.

GC–MS analyses were run on a TRIO 1 GC–MS (Carlo Erba, Milano, Italy) systems equipped with a SP 2330 column (30 m × 0.32 mm i.d. Supelco, Milano, Italy) with helium as the carrier gas. The quadrupole analyser was set to scan from 30 to 400 m/z with 0.9 s scan time and 0.1 inter-scan delay. Temperature program: from 80 to 170 °C at 30 °C/min and from 170 to 240 °C at 4 °C/min.

2.3. Enzymatic hydrolysis

endo-Pectate lyase (PCTL) (600 U/ml), *endo*-inulinase (INU) (230 U/ml), *exo*-inulinase (INU) (2000 U/ml), *endo*-polygalacturonase (PG) (1.7 U/ml), endo-arabinase (ARA) (140 U/ml) and *endo*-galactanase (GALC) (940 U/ml) were purchased from MEGAZYME (Bray, Co. Wicklow, Ireland), whereas *endo*-pectin lyase (PL) was obtained from Sigma (St. Louis, Missouri, USA) with an activity of 620 U/ml.

Solutions of 5 mg of polysaccharide in 1 ml of NaOAc/AcOH buffer, pH 4.5, were incubated with INU, PL, PG, GALC, and ARA. The degradation with PCTL was performed in NH_4HCO_3/NH_4OH buffer, pH 8. Hydrolysis were conducted up to exhaustive degradations of polysaccharides (analyzing the digests by SEC). The reactions were stopped by heating at 100 °C for 5 min.

2.4. ^1H and ^{13}C NMR spectra

Nuclear magnetic resonance spectra were obtained on a VARIAN UNITY INOVA (Palo Alto, CA, USA) spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C) equipped with a SUN SPARC station 5 Data System and VNMR software rev. 6.1B. A 5 mm inverse detection probe was used for ^1H spectra at 27 °C and ^{13}C spectra at 50 °C; for the ^{13}C spectrum at 70 °C a broadband direct probe was used. Samples were exchanged twice with 99.9% D_2O by lyophilization and finally dissolved in 0.7 ml of 99.96% D_2O. Chemical shifts were referenced to the HOD signal (4.76 ppm at 27 °C) for ^1H and to the C2 signal of fructose of Inulin set at 104.08 ppm for ^{13}C. Wilmad 535-PP tubes 7 in. × 5 mm were used.

2.5. SEC-LALLS

A low-angle laser light scattering (LALLS) detector connected in series with the chromatographic column and a

differential refractive index detector (DRI) were used for MW determination. Both the LALLS and DRI responses may be monitored with a data acquisition system. The experimental set-up included as: injection and pumping unit: Rheodyne 9125 injector (100 μl loop), Jasco PU 880 pump; separation system: a serial arrangement of TSK PW_{XL} G6000 + G5000 + G3000 with the columns thermostated at 40 °C; LALLS detector; TSP-Chromatix CMX-100 equipped with a He–Ne laser ($\lambda = 632.8$ nm); data processing and acquisition software: TSP-Chromatix PC-LALLS™.

Sample was weighed in a 10 ml volumetric flask and initially 5 ml of MQ water were added. The flask was then placed on an automatic shaker overnight. Soon afterward the flask was topped off with NaCl 0.03 M the final solution looked very cloudy. Heating the solution at 50 °C for about 30 min, it was possible to obtain the complete solubilization of the sample.

2.6. MALDI MS

MALDI-TOF mass spectrometry experiments were carried out on a Voyager STR (PerseptiveBiosystems, Framingham, MA, USA) equipped with delayed extraction technology operating in linear or reflectron mode. Ions formed by a pulsed UV laser beam (nitrogen laser, 337 nm) were accelerated through 20 kV. Mass spectra reported are the result of 128 laser shots. The matrices used were 2,5-dihydroxybenzoic acid (DHB) and 2,4,6-trihydroxyacetophenone (THAP), both from Aldrich (Gillingham, Dorset, UK).

The dried analytes were dissolved in H_2O + 0.1% TFA at a concentration of about 25 pmol. DHB was used dissolved in H_2O + ACN (1:1 v/v) at a concentration of 25 g/L. The sample–matrix solution (1:3 v/v) was deposited onto stainless steel gold plated 100-sample MALDI probe tip and dried at room temperature.

THAP was dissolved in methanol to a concentration of 200 μg/μl. Nitrocellulose was dissolved in acetone to a concentration of 30 μg/μl and diluted with propan-2-ol to a final concentration of 15 μg/μl. THAP and nitrocellulose solutions were mixed in the ratio 4:1. A 0.2 μl volume of matrix solution was placed on the target. The solution spread out fast, forming a thin layer of homogeneous, very fine crystals. This crystal layer can be washed with water to remove salt contaminants. A 0.2 μl volume of 20 mM dibasic ammonium citrate solution and 0.2 μl of sample solution were placed on top of the matrix layer and were allowed to dry in air (Korner, Limberg, Mikkelsen, & Roepstorff, 1998).

3. Results and discussion

SEC analysis of the carbohydrate fraction extracted from *E. angustifolia* radix showed that it is constituted by two polysaccharides with molecular weight (MW) of about 128,000 and 4500 Da. Ultrafiltration with a cut-off of 30,000 of this mixture yielded two fractions, one containing the low molecular weight polysaccharide and the second mostly containing the high MW polymer.

NMR and MALDI-TOF MS characterization of the low molecular weight fraction (data not shown) allowed to identify this sample as inulin. In fact ^{1}H NMR and MALDI-TOF MS spectra of a commercial sample of inulin were performed and appeared identical to those obtained from this fraction.

Fig. 1 presents the size-exclusion chromatogram of the high MW fraction. Two main peaks are evident. On the ground of the refractive index signal, the amount of the

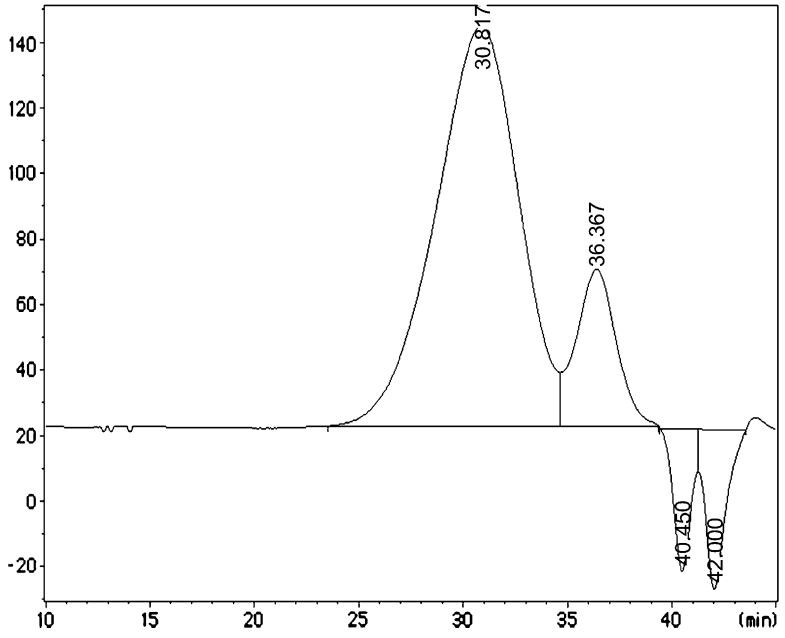

Fig. 1. SEC chromatogram of the high MW fraction.

first polysaccharide is 84% with a molecular weight based on column calibration of 128,000, while the amount of the second polysaccharide is 16%. For the first polymer the LALLS signal was good enough and the absolute estimation of the MW was 128,000 Da. After incubation with a mixture of *endo*-inulinase and *exo*-inulinase the signal of the low molecular weight polysaccharide disappeared and a new peak due to fructose was present. Fructose was eliminated by dialysis so it was possible to conduct most of the analyses on the high MW polysaccharides as a purified sample.

Monosaccharide analysis, conducted on the high MW polysaccharide and performed both by HPAEC and GC showed that the monosaccharides present are GalA, Gal, Ara, and Rha with a molar ratio of 20.5, 3.5, 5.0, and 1.0, respectively. Linkage analysis was conducted according to Lindberg (1972) and the results are reported in Table 1.

In the 500 MHz ^{1}H spectrum of the polysaccharide, reported in Fig. 2, the methyl group of Rha is visible as a broad multiplet at about 1.3 ppm. Comparing the area of this multiplet with that of the H4 of GalA (a broad resonance at 4.46 ppm) an abundance of 5% of Rha respect to GalA can be calculated, confirming the sugar analysis results. At 2.17 and 2.06 ppm there are two singlets that can be assigned to acetyl groups. The acetylation degree calculated respect to the H4 of GalA is 9%. In the spectrum of Fig. 2 there is also an intense singlet at 3.80 ppm which suggests the presence of $–OCH_3$ groups belonging to the esterified GalA.

In the ^{13}C spectrum shown in Fig. 3, two signals at 100.19 and 100.93 can be readily assigned to the anomeric carbons of GalA and galacturonic acid methyl ester (GalAMe), respectively.

The presence of GalAMe is confirmed by a resonance at 53.66 ppm, characteristic of methyl ester groups. In the carbonyl region of the spectrum of Fig. 3 the signals at 174.0 and 171.4 correspond to C6 of GalA and GalAMe, respectively. From the integration of the areas related to anomeric and carbonyl resonances it can be calculated the same degree of esterification of 60%.

Table 1
Linkage analysis results

Partially methylated alditolacetates
Terminal rhamnopyranosyl
2-*O*-Rhamnopyranosyl
Terminal arabinofuranosyl
Terminal arabinopyranosyl
5-*O*-Arabinofuranosyl
3,5-*O*-Arabinofuranosyl
Terminal galactopyranosyl
3-*O*-Galactopyranosyl
6-*O*-Galactopyranosyl
3,6-*O*-Galactopyranosyl
Terminal galacturonosyl
4-*O*-Galacturonosyl

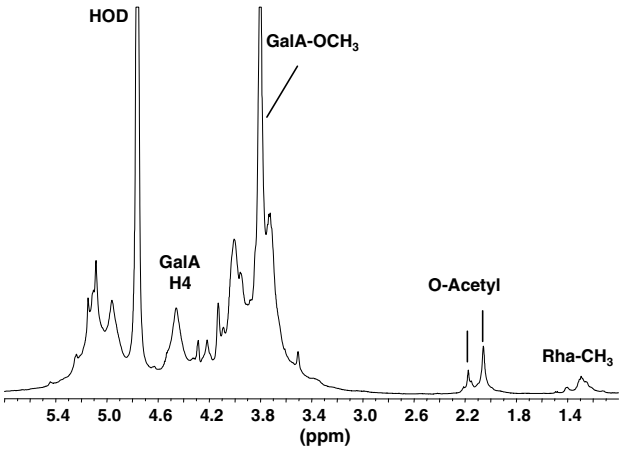

Fig. 2. ^{1}H NMR spectrum of the high MW polysaccharide after hydrolysis with a mix of *endo*- and *exo*-inulinase.

Treating the polysaccharide with 0.1 M NaOD at room temperature for 24 h and recording again the ^{1}H and ^{13}C spectra, after neutralization of the base with DCl, the results reported above can be confirmed.[1] In particular, the proton spectrum, presented in Fig. 4, now appears simplified, as the heterogeneity due to partial methylation is absent. Consequently, the resonances of GalA from H1 through H5 can be assigned. Furthermore, the intense singlet at 3.80 ppm due the $–OCH_3$ of GalAMe present in the spectrum of the native polysaccharide is now absent, and in its place a singlet is found at 3.34 ppm. This peak is due to methanol formed after the saponification of the ester, and from its area the degree of esterification of 60% can be verified. The two *O*-acetyl signals of the native polysaccharide have been substituted by one singlet at 2.06 ppm that can be assigned to AcONa. Indeed, this chemical shift coincides with one of the two acetyl resonances in the spectrum of the native polysaccharide, but recording the NMR spectrum after saponification and before neutralization this signal goes to 1.90 ppm. In fact, at lower pH AcONa is partially converted to AcOH, bringing the methyl chemical shift to lower field. Again the area calculation of this signal confirms a degree of acetylation of 9%. In the ^{13}C spectrum of the saponified polysaccharide, shown in Fig. 5, a signal at 49.08 ppm indicates the presence of methanol deriving from hydrolysis of GalAMe. The resonances of C1 and C6 of GalAMe at 100.93 and 171.4 found in the spectrum of native sample are now absent, remaining only the corresponding signals of GalA.

From the data illustrated above it is possible to assert that the polysaccharide is a high methoxy (HM) pectin in which the backbone structure of the smooth region is constituted by α-(1–4)-polygalacturonan partially carboxymethylated (60%) and acetylated (9%) and with the hairy regions containing 2-*O*- and 2,4-*O*-rhamnopyranose,

[1] The sample used in this experiment was not previously digested with inulinase; the saponification and the subsequent neutralization reactions were conducted in the NMR tube.

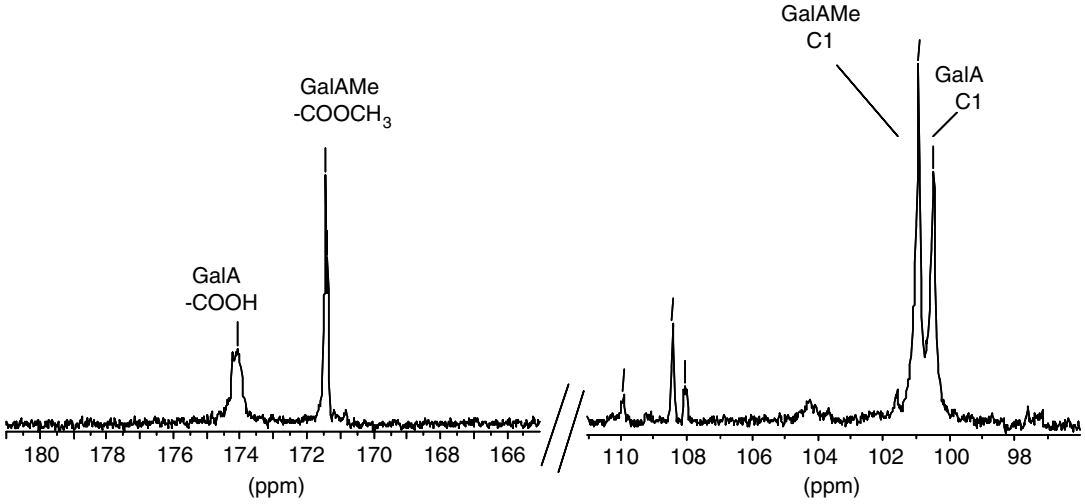

Fig. 3. ^{13}C NMR spectrum of the high MW polysaccharide after hydrolysis with a mix of *endo*- and *exo*-inulinase.

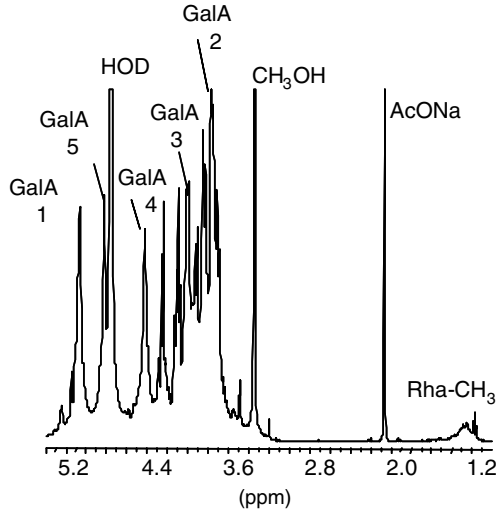

Fig. 4. ^{1}H NMR spectrum of the high MW polysaccharide after hydrolysis with a mix of *endo*- and *exo*-inulinase and incubation with NaOD.

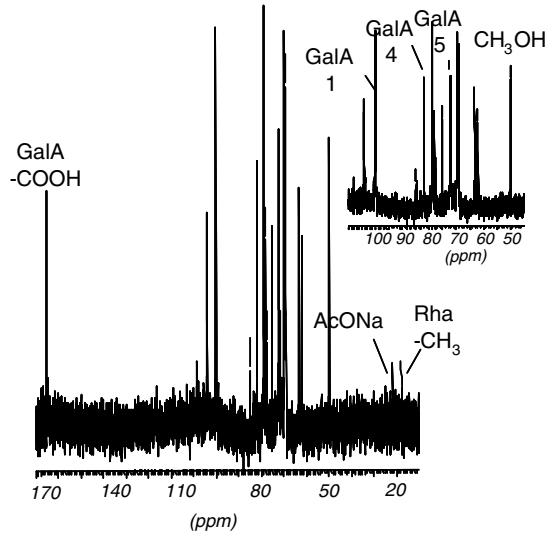

Fig. 5. ^{13}C NMR spectrum of the high MW polysaccharide after hydrolysis with a mix of *endo*- and *exo*-inulinase and incubation with NaOD.

5-*O*- and 3,5-*O*-arabinofuranose, and 3,6-galactopyranose and terminal rhamnose, arabinofuranose, arabinopyranose, galactopyranose, and galacturonopyranose.

In order to have some information about the GalAMe distribution along the homogalacturonan backbone, the polysaccharide was subjected to enzymatic degradations utilizing *endo*-pectin lyase and *endo*-pectate lyase and analyzing the oligomers produced by MALDI MS. Obtaining MALDI mass spectra from complex high molecular mass biological samples, like polysaccharides, requires reduction of the system complexity by SEC separation, enzymatic degradation or other chemical reactions adapted to generate oligomers with a mass minor of 5000 Da (Garozzo, Impallomeni, Spina, Sturiale, & Zanetti, 1995; Garozzo, 1997; Garozzo, Impallomeni, Spina, & Sturiale, 1998; Garozzo, Spina, Cozzolino, Cescutti, & Fett, 2000; Montaudo, Garozzo, Montaudo, Puglisi, & Samperi, 1995).

Fig. 6 reports the MALDI mass spectrum of the digest obtained from the degradation of the sample with *endo*-pectin lyase. In this spectrum, which extends up to 2000 Da, there are several clusters of peaks which differ by 176 Da, that corresponds to a GalA unit. Within each cluster, peaks can differ by n 14 or n 42 Da, matching the mass of methyl or acetyl groups, respectively. These data signify that each peak belonging to a cluster is related to an oligomeric distribution of GalA units with a different methyl and acetyl groups content. For example, the peak at *m*/*z* 894 is related to an oligomer with 5 GalA unit containing one methyl and no acetyl group, so in the spectrum it is indicated as $^{1}5_{0}$. The number 5 designates the number of GalA residues constituting the oligomer, while the apex and the subscript indicate, respectively, the amount of methyl and acetyl groups present in the species. According to this, the oligomers designated in the spectrum with the symbols $^{2}5_{0}$ (*m*/*z* 908) and $^{3}5_{0}$ (*m*/*z* 922) indicate, respectively, a pentamer of GalA with 2 and 3 methyl groups, while the species denominated with $^{1}5_{1}$ (*m*/*z* 936) and $^{1}5_{2}$ (*m*/*z* 978) represent pentamers with 1 methyl and 1 or 2

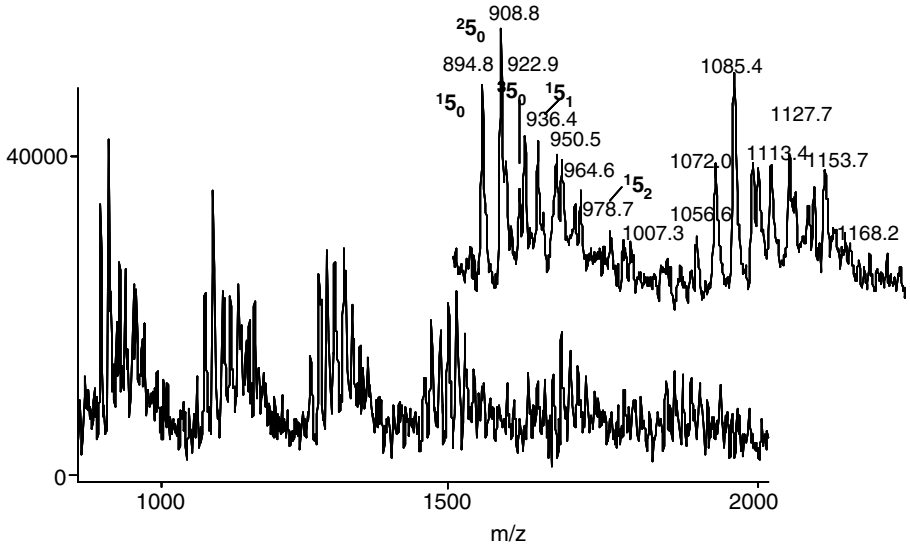

Fig. 6. MALDI mass spectrum of the high MW polysaccharide hydrolyzed with pectin lyase.

acetyl groups, respectively. In particular, in Fig. 6 peaks starting from pentamer to 13-mer and more can be clearly distinguished.

In parallel, the pectin was treated with *endo*-pectate lyase and the MALDI mass spectrum obtained is shown in Fig. 7. Also in this case it is possible to observe a distribution of partially methylated and acetylated oligogalacturonic acids very similar to that of Fig. 6, the only difference being a decrease of the methyl groups. This diversity can be easily explained considering the degradation mechanisms of pectin lyase and pectate lyase. Because, pectin lyase cleaves glycosidic linkage between two GalAMe units, the oligomers related to MALDI mass spectrum of Fig. 6 contain at least two methyl ester groups on both terminal and reducing ends. On the other hands, pectate lyase cuts glycosidic linkage between two GalA, so the degradation products formed by this enzyme, recorded in Fig. 7, possess two GalA monomers on both terminal ends.

MALDI mass spectra of Figs. 6 and 7 are very similar meaning that there are not substantial differences between reaction products originating from enzymatic hydrolysis with pectin lyase and pectate lyase. This results indicates that the methyl ester groups are randomly distributed over the homogalacturonan backbone.

In order to confirm this result, statistical calculation have been performed. Regarding this, Fig. 8 presents the MALDI mass spectrum of a high molecular weight fraction obtained from isopropanol precipitation of the products derived from degradation with *endo*-pectate lyase. This spectrum, containing oligomers of GalA units up to 20 and more, is particular suitable for statistical estimate.

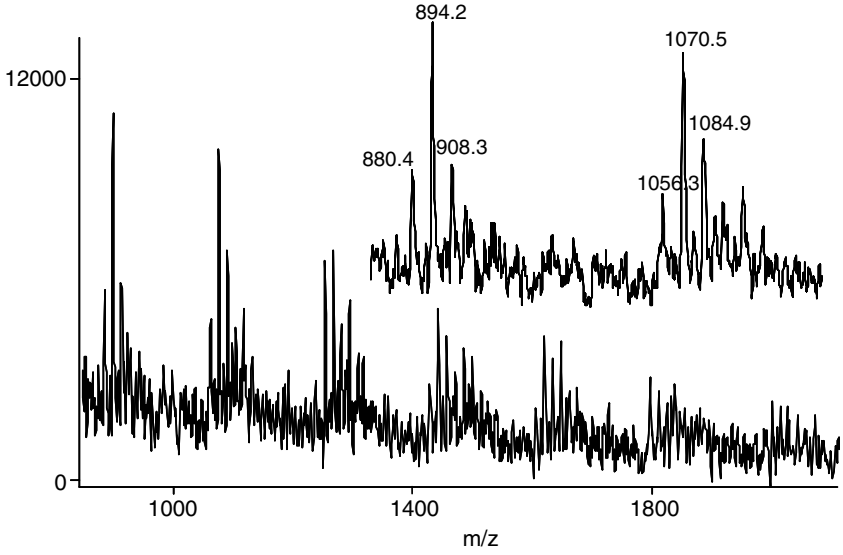

Fig. 7. MALDI mass spectrum of the high MW polysaccharide hydrolyzed with pectate lyase.

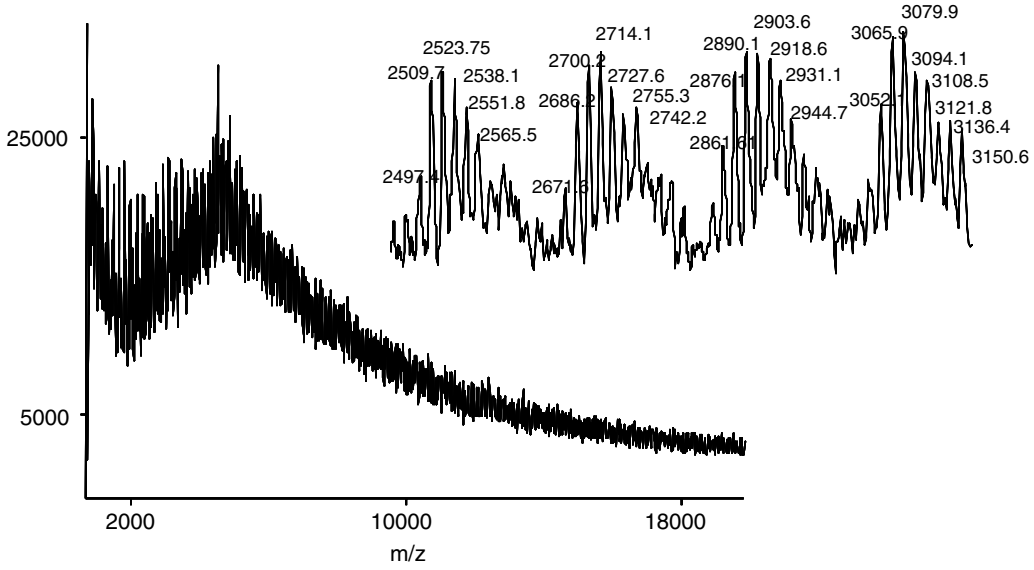

Fig. 8. MALDI mass spectrum of the high MW fraction obtained from isopropanol precipitation of the products derived from degradation with *endo*-pectate lyase. The polygalacturonic acid can be considered a copolymer of GalA and GalAMe units and assuming a random (Bernoullian) distribution of the monomers, the statistical probability ($P_{x,y}$) of finding a given (GalA)x(GalAMe)y oligomeric sequence can be calculated by the following equation: $P_{x,y} = \frac{(x+y)!}{x!y!} Pa^x Pb^y$ where Pa and Pb are the molar fractions of GalA and GalAMe in the copolymer and the binomial coefficient $\frac{(x+y)!}{x!y!}$ represents the number of possible sequence arrangements of the AxBy oligomers.

By comparing theoretical spectra obtained using statistical calculation (Abate et al., 1993) with the MALDI mass spectrum presented in Fig. 8, it was possible to verify a random distribution of the methyl ester groups according to a Bernoullian law and to calculate a methylation degree about 60% confirming the datum reported above.

Successively, the polysaccharide was subjected to enzymatic degradation with *endo*-α-(1–4)-polygalacturonase (PG). From the comparison of the SEC analysis of the sample after the hydrolysis (data not reported) with the chromatogram of Fig. 1, it resulted that the enzymatic reaction caused a lowering of the molecular weight of the HMW polysaccharide. Negative MALDI mass spectrum of the reaction mixture, reported in Fig. 9, shows a series of peaks the mass difference of which permit to understand that they correspond to oligomers of $(GalA\text{-}Rha)_n$. Mass signals up to pentamer (m/z 1629) can be observed while there is no side chains linked to the species and no acetyl groups (mass differences of 42 Da are absent).

The MALDI mass spectrum of *E. angustifolia* pectin treated with *endo*-arabinase, data not shown, gave an oligomer distribution from 1500 to 7000 Da demonstrating that very long regions of arabinan are present in the polysaccharide. Pectin hairy regions are characterized also by sequences of galactans, which, in particular, have never been studied by MALDI MS. Fig. 10 reports the MALDI spectrum of the *E. angustifolia* pectin degraded with galactanase. As it can bee readily seen, the signals in the spectrum differ by 338 Da, which corresponds to Gal-GalA sequences. This result suggests that among the galactan chains there are GalA residues. To our knowledge this structure has never been, up to now, reported in literature for a pectin hairy region.

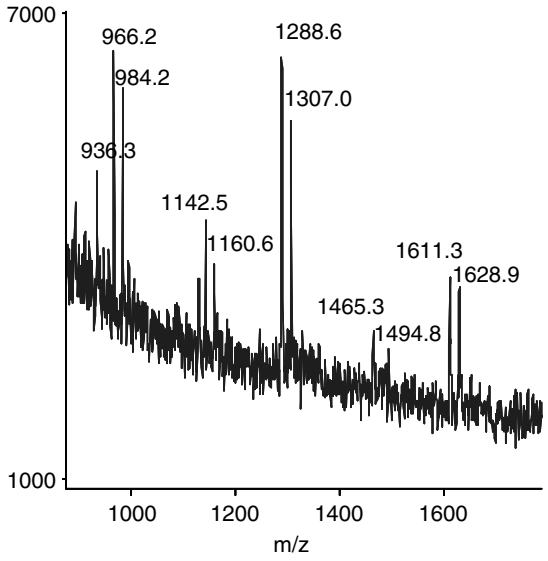

Fig. 9. Negative MALDI mass spectrum of the high MW polysaccharide treated with *endo*-polygalacturonase.

Finally, the data obtained during this study allow to conclude that *E. angustifolia* radix contains two polysaccharides: inulin, and a high molecular weight polysaccharide corresponding to high methoxy (HM) pectin in which the backbone structure of the smooth region is constituted by α-(1–4)-polygalacturonan partially carboxymethylated (60%) and acetylated (9%) and with the hairy regions containing 2-*O*- and 2,4-*O*-rhamnopyranosyl, 5-*O*- and 3,5-*O*-arabinofuranosil, 3,6-galactopyranosyl, and terminal rhamnosyl.

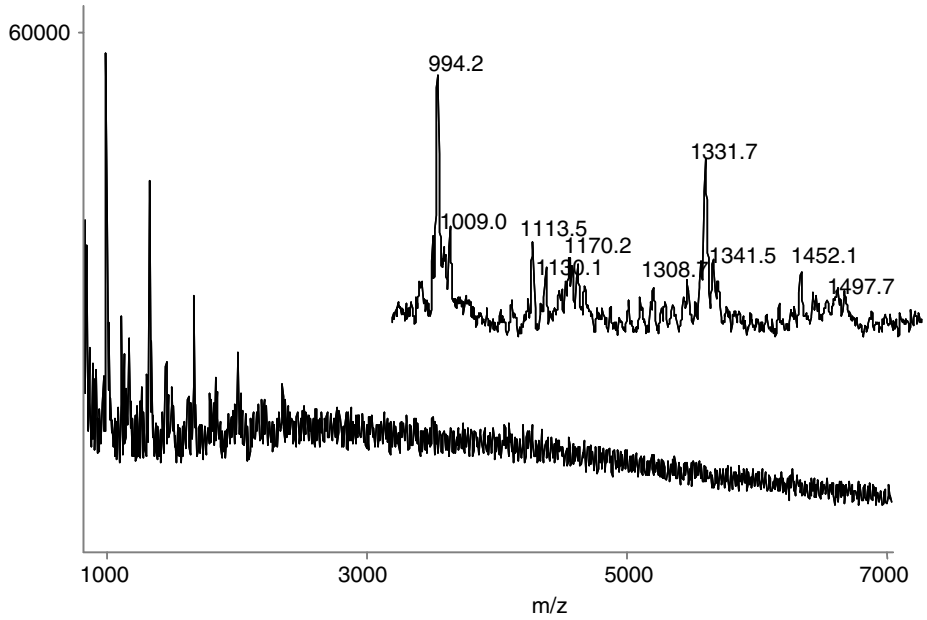

Fig. 10. MALDI mass spectrum of the high MW polysaccharide degraded with galactanase.

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