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An arabinogalactan protein isolated from medium of cell suspension cultures of *Silybum marianum* (L.)Gaernt

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Abstract

Cell suspension cultures of *Silybum marianum* secreted polymers extracellularly containing 97% carbohydrates and 3% proteins. Fractionation of polysaccharides by anion-exchange chromatography yielded an unbound neutral fraction composed of glucose, xylose, galactose, arabinose and rhamnose and a bound fraction in which galactose and arabinose were predominantly found. The bound fraction specifically bind to Yariv phenylglycoside suggesting the presence of an arabinogalactan protein (AGP). Further purification of the AGP was done by precipitation of the culture medium with the Yariv reagent. The precipitated AGP eluated as single peak by gel permeation with an average molecular weight of 100. Eighteen aminoacids were detected, Ser, Gly, Glu, Asp, Thr and Hyp being the major ones. Linkage analysis showed terminal and 1,3-linked arabinose and almost all galactose was present in the 1,3-galactopyranoside form. The NMR spectral data revealed residues of galactopyranose and arabinofuranose as constituents of AGP. This study is the first examination of an AGP secreted by *S. marianum* cells in suspension culture.

Keywords: *Silybum marianum* (L.)Gaernt; Cell cultures; Arabinogalactan proteins; Yariv reagent

1. Introduction

The milk thistle *Silybum marianum* (L.)Gaernt is among the most ancient of all known herbal medicines. Various preparations of the plant, especially the fruits, have been used medicinally for over 2000 years to treat liver disorders. Silymarin is the pharmacological active principle of the fruit and is composed of an isomeric mixture of the flavonolignans silychristin, silydianin, and the diastereoisomers silybin and isosilybin (Morazzoni & Bombardelli, 1995). Besides the cytoprotective effects of silymarin due to by its antioxidative and radical-scavenging activities, new activities based on specific receptor interactions have been reported and there is a growing interest in its anticancer and chemopreventive effects, as well as in its hypocholesterolemic, cardioprotective, neuroactive and neuroprotective activities (Kren & Walterova, 2005). For this reason, tissue cultures derived from this species of the Asteraceae family could be an alternative for the production of flavonolignans. However, in approaches carried out by several authors, accumulation of silymarin in cultures was lower than in the fruit (Becker & Schrall, 1977; Cacho, Morán, Corchete, & Fernández-Tárrago, 1999; Sanchez-Sampedro, Fernández-Tárrago, & Corchete, 2005). Despite this failure, "in vitro" culture systems could still be useful for the production of a range of compounds with activities of biological interest. It is well known that plant cell walls are a source of polysaccharides with interesting laxative, hipoglucemic, immunostimulant, cosmetic and other biological properties (Hensel, Schnidgall, & Kresi, 1997) and in these sense plant cell cultures, which usually secrete high amounts of polysaccharides to the culture medium, could offer an alternative for their isolation and characterization. Among these products

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arabinogalactans (AGPs), a family of hydroxyproline-rich glycoproteins and proteoglycans, originally associated with plant gums and exudates, have been extensively studied due to their immunostimulant activities. Two well known examples are those of *Echinacea purpurea*, and *Larix* sp. (Allard & Tazi, 1993; Odonmazig, Ebringerova, Machova, & Alfoldi,1994). AGPs are predominantly secreted into the cell wall space, and thus they can be found in the medium of suspension-cultured plant cells. Several AGPs from culture media have been investigated, including those from ryegrass cells (Anderson, Clarke, Jermyn, Knox, & Stone, 1977), tobacco cells (Akiyama & Kato, 1981; Iraki, Bressan, & Carpita, 1989), blackberry cells (Cartier, Chambat, & Joseleau, 1987), sycamore cells (Aspinall, Molloy, & Craig, 1969) and carrot (Immerzeel, Schols, Voragen, & de Vries, 2004).

In this paper, we report on the kinetics of polysaccharide secretion in cell suspension cultures of milk thistle. Fractionation of sugars by anion-exchange chromatography and selective precipitation with the *P*-glucosyl Yariv reagent (Yariv, Raport, & Graf, 1962) allowed the identification of an AGP protein whose preliminary characterization is also included in this work.

2. Experimental

2.1. Materials

Silybum marianum L.(Gaernt) cell suspensions were established from 3-month-old undifferentiated hypocotyl callus in 2002 and maintained in darkness in Murashige & Skoog liquid medium (Murashige & Skoog, 1962) supplemented with 30 g/L sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L benzyl adenine at pH 5.6. Cultures were shaken at 90 rpm and routinely subcultured every two weeks. Cells were removed from the culture medium by filtration through four layers of gauze. The separated medium was then filtrated through a glass fibre filter and the filtrate was concentrated in a rotavapor (30 °C). The solution was dialyzed exhaustively against distilled water (molecular weight cut off 10 kDa), concentrated and freeze dried.

2.2. Methods

2.2.1. Fractionation of extracellular polysaccharides

Solutions of extracellular polysaccharides in 10 mM phosphate buffer, pH 7 (1 mg/mL) were applied to a column of DEAE–Sephadex (20 × 2.5 cm) equilibrated in the same buffer and eluted until no sugars could be detected in the colorimetric assay by the phenol sulphuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Material bound to the column was eluted with a linear gradient (0.01–1 M) of phosphate buffer over 300 mL. Fractions (5 mL) were assayed for total sugars. Appropriate fractions were pooled, concentrated, dialyzed extensively against water and freeze dried.

2.2.2. Isolation and quantification of AGPs

Freeze dried culture medium, (500 mg), prepared as above, was dissolved in 100 mL of distilled water and precipitated by addition of an equal volume of an aqueous solution of 1 mg/mL Yariv reagent and NaCl at a final concentration of 0.15 M as described (Kreuger & van Holst, 1993). Yariv phenylglycoside (1,3,5-tri[4-β-D-glucopyranosyl-oxyphenylazo]2,4,6-trihydroxybenzene) was prepared according to van Holst and Clarke (1985).

The amount of AGPs in culture medium was quantified by the single radial gel diffusion test as described (Van Holst & Clarke, 1985).

2.2.3. Molecular mass estimation

The molecular mass of the purified AGP was estimated by gel filtration chromatography on a GE Healthcare Superose 12 10/300 GL column at room temperature in 50 mM phosphate-buffered saline (pH 7.0) containing 0.15 M NaCl at 0.5 mL/min. Dextran sulfates with molecular masses in ranges 36–50, 400–600 and 1400 kDa (Sigma, USA) were employed for column calibration.

2.2.4. Linkage analysis

Methylation without a carboxyl reduction was performed according to the method of Ciucanu and Kerek (1984), modified by Needs and Selvendran (1993). The permethylated polysaccharide was converted into its corresponding partially *O*-acetylated, partially *O*-methylated alditols as described (York, Darvill, McNeil, Stevenson, & Albersheim, 1985). These products were separated on a SPB1 column (12 m × 0.2 mm i.d. × 0.3 μm film thickness) on a Hewlett–Packard 5890 Series II Finigan GC–MS.

2.2.5. NMR spectroscopy

AGP samples were dissolved in D_2O (50 mg/mL) and ^1H-, ^{13}C-, *J*-modulated spin-echo ^{13}C spectra, DQF-COSY and ROESY (650 ms mixing time) were recorded on a Bruker SY spectrometer at 400/100 MHz. Chemical shifts (δ) are given in ppm and peaks were assigned by comparison of published spectra (Gane et al., 1995; Samuelsen, Paulsen, Wold, Knutsen, & Yamada, 1998; Willför, Sjöholm, Laine, & Holmbom, 2002).

2.2.6. General techniques

Samples were assayed for total sugars by the phenol sulphuric method (Dubois et al., 1956) and uronic acids according to the procedure of Filisetti-Cozzi and Carpita (1991). Proteins were determined by the method of Bradford (1976). Neutral monosaccharides were analysed after acid hydrolysis (2 M trifluoroacetic acid containing myo-inositol as internal standard, 1 h, 121 °C) as their alditol acetates by gas chromatography (York et al., 1985). For amino acid analyses AGP was hydrolysed with 6 N HCl for 24 h at 110 °C. Samples were analysed on an amino acid analyser with cation-exchange column (Biochrom 20, Pharmacia LKB Biochrom Ltd.) using ninhydrin for post-column color development. Detection was carried at

570 nm except for Pro and Hyp which were detected at 440 nm.

3. Results and discussion

The increase of the viscosity in milk thistle culture medium was due to the accumulation of extracellular polysaccharides as the cell growth progressed. This made it difficult to separate the biomass by filtration.

The secretion of polysaccharides increased over the culture growth cycle and progressively stabilised when cells entered the stationary phase. As shown in Fig. 1, the secretion profile followed the growth pattern of the culture. The polymers secreted on day 14 were composed of ca. 97% carbohydrates and 3% protein; and an average of 4% uronic acids was detected colorimetrically.

Fractionation of extracellular polysaccharides by DEAE–Sephadex ion-exchange chromatography with a 10–500 mM gradient of phosphate buffer (pH 7) gave an unbound neutral fraction and a bound fraction which represented 24% of the total sugars loaded on the column. An average of 5% uronic acid was detected photometrically in this bound fraction.

The monosaccharide composition of the neutral fraction contained glucose, xylose, galactose and rhamnose, and that of the bound fraction was composed mainly of arabinose and galactose with minor amounts of rhamnose, glucose and xylose (Table 1). The uronic acid sugars were not identified.

The presence of galactose and arabinose as major sugars in the bound fraction indicated the presence of AGPs and this was confirmed by the positive reaction with Yariv antigen. Gel diffusion experiments with the Yariv's reagent allowed direct quantification of AGPs in the medium of suspensions during a growth cycle of culture. As shown in Fig. 3, the AGPs secretion into the culture medium accompanied growth with a trend similar to the release of total polysaccharides (Fig. 2).

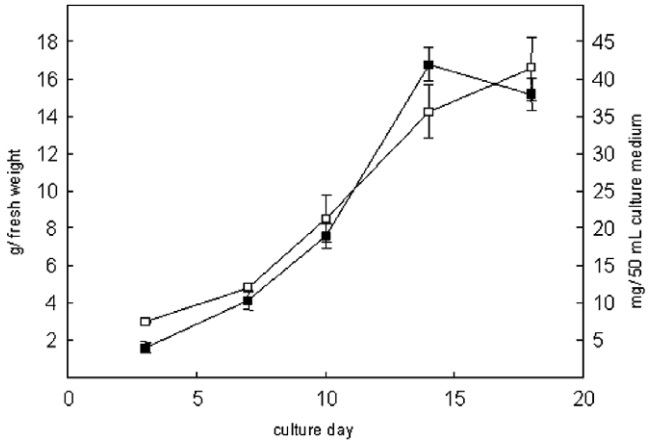

Fig. 1. Fresh weight (□) and extracellular polysaccharides (■) of cell suspension cultures of *Silybum marianum*.

Table 1
Neutral sugar composition (mol %) of DEAE–Sephacel fractions of the total polysaccharides isolated from the culture medium of 14-day-old cultures of *Silybum marianum*

Fraction	Glc	Gal	Ara	Xyl	Rham
Neutral	61.3	10.82	6.63	12.83	8.34
Anionic	9.5	49.1	32.6	6.1	2.7

AGPs were further characterized after isolation from 14-day-old culture medium by selective precipitation with the Yariv reagent.

The precipitated AGPs from the culture medium eluted from the gel permeation column as a single peak with an apparent molecular weight of 100,000. Amino acid analyses showed the presence of 18 amino acids, Ser, Glu, Gly and Ala were the major ones (Table 2). Hyp and Pro, which were separately analysed, were also present in samples in proportions of 5.2%, and 3.1%, respectively (see also Table 2). Aminoacid proportions in *Silybum* AGP were very similar to that of other AGPs described as those from rose, malva and carrot cells (Classen & Blaschek, 2002; Immerzeel et al., 2004; Komalavilas, Zhu, & Nothnagell, 1991).

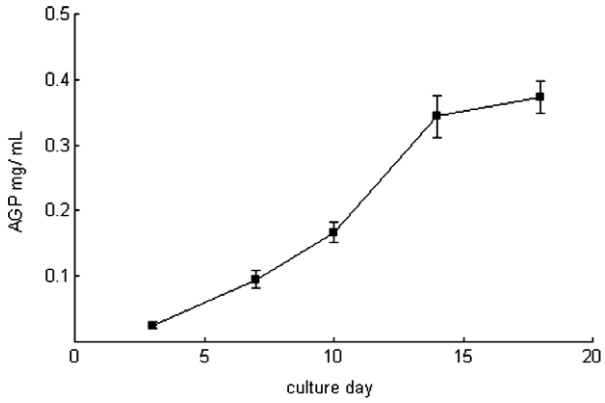

Fig. 2. Excretion of AGP into the medium of cell suspension cultures of *Silybum marianum*.

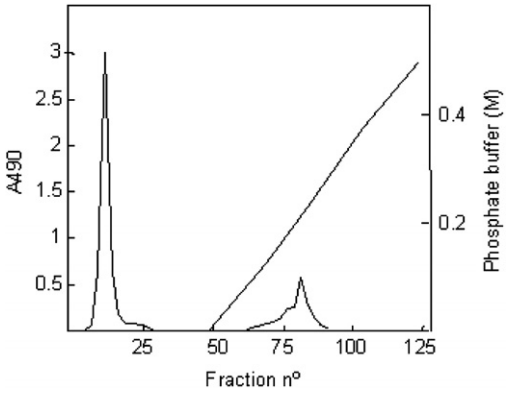

Fig. 3. Elution profile of extracellular polysaccharides isolated from 14-day-old cultures of *Silybum marianum* in anion-exchange chromatography on DEAE–Sephacel.

Table 2
Amino acid composition (as mol % of total aminoacids) of the AGP isolated from the culture medium of 14-day-old cultures of *Silybum marianum*

Aminoacids			
Asparragine	8.9	Leucine	3.9
Threonine	6.4	Tyrosine	1.2
Serine	18.2	Phenylalanine	2.8
Glutamine	10.26	Histidine	1.3
Glycine	14.8	Lysine	2.2
Alanine	4.5	Arginine	1.3
Valine	1.3	Proline	3.1
Methionine	2.2	Hydroxyproline	5.2
Isoleucine	3.87		

Table 3
Linkage analysis of the AGP isolated from the culture medium of 14-day-old cultures of *Silybum marianum*

Glycosyl residue	Methyl ether	Linkage	mol %[a]
Ara*f*	2,3,5-Ara[b]	Terminal	14.2
	2,5-Ara	1,3-	23.9
Gal*p*	2,3,4,6-Gal	Terminal	8.7
	2,3,6-Gal	1,4-	9.7
	2,4,6-Gal	1,3-	26.3
	2,3,4-Gal	1,6-	2.9
	2,4-Gal	1,3,6-	2.5
Others			12

[a] Average of triplicate determinations.
[b] 2,3,5-Ara = 1,4-di-*O*-acetyl-1-deuterio-2,3,5-tri-*O*-methylarabitol; etc.

Linkage analysis showed that arabinose was present in the furanose form and occurs as terminal sugar and 1,3-linked residues (Table 3). Galactose was mainly present as 1,3-linked residues; terminal, 1,4-, 1,6- and 1,3,6-linked galactose residues were also found. These sugar linkages were consistent with those of type II arabinogalactan isolated from cell suspensions of several species (Fincher, Stone, & Clarke, 1983; Hensel et al., 1997).

The NMR spectra of our AGP are similar to other similar AGPs and they are fully consistent with a polymer composed mainly of arabinose and galactose. In the ^{13}C NMR espectra of our AGP, a sharp resonance at 109.1 ppm was assigned to C-1 of Ara*f*, with accompanying C-4 and C-5 resonances at 83.7 and 61.1 ppm. Resonances corresponding to C-1 of Gal*p* appear at 103.0 ppm (Fig. 4). In the 1D ^{1}H NMR spectra, H-1 signals corresponding to the Ara*f* were detected at 5.15 ppm, and those corresponding to Gal*p* were detected at 4.3–4.5 ppm (Fig. 5). The 2D experiments showed correlation patterns that were consistent with these assignments and with the previous literature data.

This study represents the first examination of an AGP released by cells from *S. marianum* cultures. The secreted AGP can be easily purified by selective precipitation with the Yariv reagent making it readily available for future work aimed at determining its bioactivity. However, the similarities in composition shared by *S. marianum* AGP with AGP from other plant sources with known biological

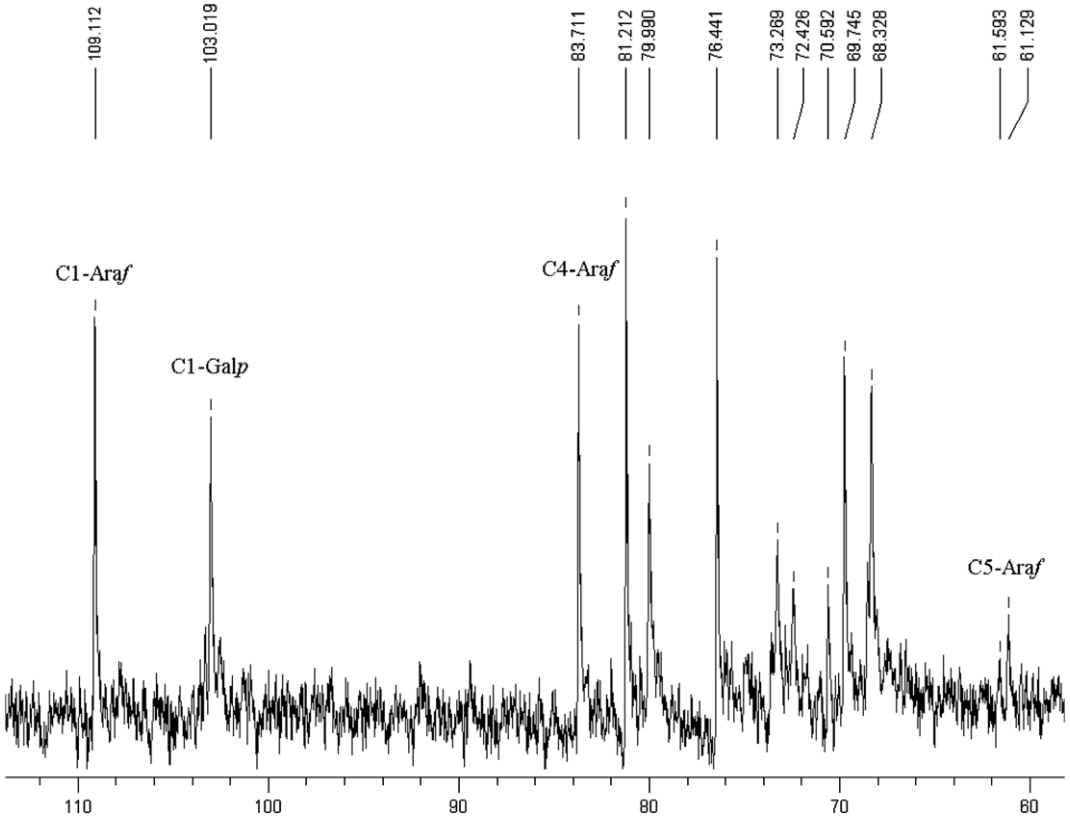

Fig. 4. Proton-decoupled ^{13}C NMR spectrum of the AGP isolated from 14-day-old-cultures of *Silybum marianum*.

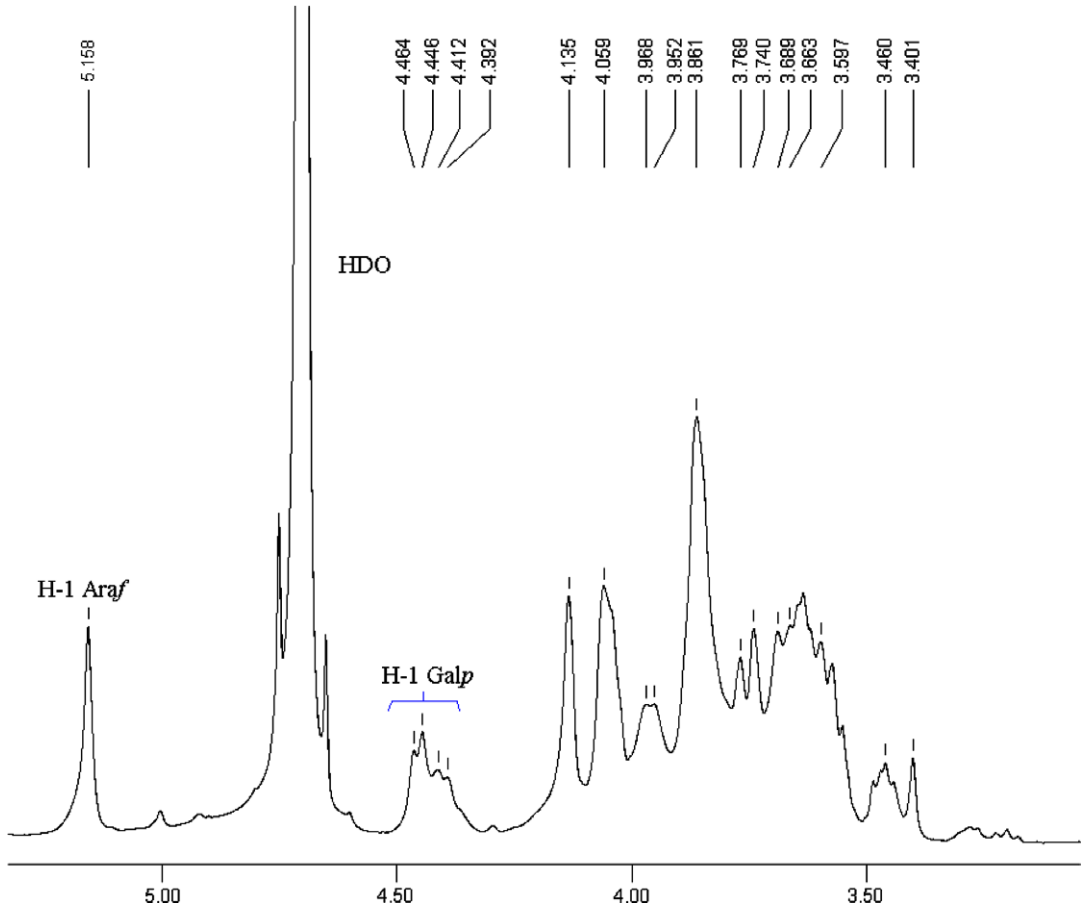

Fig. 5. ^{1}H NMR spectrum of the AGP isolated from 14-day-old cultures of *Silybum marianum*.

properties, should further extend the pharmacological uses of this plant.

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References

Akiyama, Y., & Kato, K. (1981). An extracellular arabinogalactan-protein from *Nicotiana tabacum*. *Phytochemistry, 20*, 2507–2510.

Allard, B., & Tazi, A. (1993). Influence of growth status on composition of extra-cellular polysaccharides from two *Chlamydomonas* species. *Phytochemistry, 32*, 41–45.

Anderson, R. L., Clarke, A. E., Jermyn, M. A., Knox, R. B., & Stone, B. A. (1977). A carbohydrate-binding arabinogalactan-protein from liquid suspension cultures of endosperm from *Lolium multiflorum*. *Australian Journal of Plant Physiology, 4*, 143–158.

Aspinall, G. O., Molloy, J. A., & Craig, J. W. T. (1969). Extracellular polysaccharides from suspension-cultured sycamore cells. *Canadian Journal of Biochemistry, 47*, 1063–1070.

Becker, H., & Schrall, R. (1977). Tissue and suspension cultures of *Silybum marianum*. I. Communication: isolation and growth of tissue and suspension cultures and studies on flavonoids. *Planta Medica, 31*, 185–192.

Bradford, M. M. (1976). A rapid sensitive method for the quantification of microgram quantities of proteins using the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Cacho, M., Morán, M., Corchete, P., & Fernández-Tárrago, J. (1999). Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L.) Gaertn. *Plant Science, 144*, 63–68.

Cartier, N., Chambat, G., & Joseleau, J. P. (1987). An arabinogalactan from the culture medium of *Rubus fruticosus* cells in suspension. *Carbohydrate Research, 168*, 275–283.

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

Classen, B., & Blaschek, W. (2002). An arabinogalactan-protein from cell culture of *Malva sylvestris*. *Planta Medica, 68*, 232–236.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of carbohydrates and related substances. *Analytical Chemistry, 28*, 350–356.

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

Fincher, G. B., Stone, B. A., & Clarke, A. E. (1983). Arabinogalactan-proteins: structure, biosynthesis, and function. *Annual Review of Plant Physiology, 34*, 47–70.

Gane, A. M., Craik, D., Munro, S. L. A., Howlett, G. J., Clarke, A. E., & Bacic, A. (1995). Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigma and styles of *Nicotiana alata*. *Carbohydrate Research, 277*, 67–85.

Hensel, A., Schnidgall, J., & Kresi, W. (1997). Extracellular polysaccharides produced by supension-cultures cells from *Digitalis lanata*. *Planta Medica, 63*, 441–445.

Immerzeel, P., Schols, H. A., Voragen, G. J., & de Vries, S. C. (2004). Different arabinogalactan proteins are present in carrot (*Daucus carota*) cell culture medium and in seeds. *Physiologia Plantarum, 122*, 181–189.

Iraki, N. M., Bressan, R. A., & Carpita, N. C. (1989). Extracellular polysaccharides and proteins of tobacco cell cultures and changes in composition associated with growth-limiting adaptation to water and saline stress. *Plant Physiology, 91*, 54–61.

Komalavilas, P. S., Zhu, J. K., & Nothnagell, E. A. (1991). Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells. *Journal of Biological Chemistry, 266*, 15956–15965.

Kren, V., & Walterova, D. (2005). Silybin and silymarin-new effects and applications. *Biomedical Papers, 149*, 29–41.

Kreuger, M., & van Holst, G. J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis in *Daucus carota* L. *Planta, 197*, 135–141.

Morazzoni, P., & Bombardelli, E. (1995). *Silybum marianum* (*Carduus marianus*). *Fitoterapia, 46*, 3–42.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473–497.

Needs, P. W., & Selvendran, R. R. (1993). Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide. *Carbohydrate Research, 245*, 1–10.

Odonmazig, P., Ebringerova, A., Machova, E., & Alfoldi, J. (1994). Structural and molecular properties of the arabinogalactan isolated from mongolian larchwood (*Larix dahurica* L.). *Carbohydrate Research, 252*, 317–324.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Knutsen, S. H., & Yamada, H. (1998). Characterization of a biologically active arabinogalactan from the leaves of *Plantago major* L. *Carbohydrate Polymers, 35*, 145–153.

Sanchez-Sampedro, M. A., Fernández-Tárrago, J., & Corchete, P. (2005). Enhanced silymarin accumulation is related to calcium deprivation in cell suspension cultures of *Silybum marianum* (L.) Gaernt. *Journal of Plant Physiology, 162*, 1177–1182.

Van Holst, G. J., & Clarke, A. E. (1985). Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion. *Analytical Biochemistry, 148*, 446–450.

Willför, S., Sjöholm, R., Laine, C., & Holmbom, B. (2002). Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood. *Wood Science and Technology, 36*, 101–110.

Yariv, J., Raport, M. M., & Graf, L. (1962). The interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycosides. *Biochemical Journal, 85*, 383–388.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1985). Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymology, 118*, 3–41.