Pharmaceutical Institute, Department of Pharmaceutical Biology, Christian-Albrechts-University of Kiel, Germany

Characterization of a Yariv precipitated arabinogalactan-protein from fruits of rye (*Secale cereale* L.)

E. M. GOELLNER, W. BLASCHEK, B. CLASSEN

Received November 28, 2012, accepted December 13, 2012

Dr. Esther Marie Göllner, Pharmaceutical Institute, Department of Pharmaceutical Biology, Christian-Albrechts-University of Kiel, Gutenbergstraße 76, 24118 Kiel, Germany
mgoellner@pharmazie.uni-kiel.de

Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

Pharmazie 68: 631–635 (2013) *doi: 10.1691/ph.2013.6519*

An arabinogalactan-protein (AGP) with a molecular mass of 110 kDa was isolated from whole grain of rye (*Secale cereale* L.) by double precipitation with $(\beta\text{-D-Glc})_3$-Yariv-phenylglycoside (βGlcY) and its structure was analyzed. The AGP consists of a hydroxyproline-rich protein backbone of about 7 % and an arabinogalactan moiety of about 93%. By alkaline hydrolysis, hydroxyproline was identified as the main amino acid responsible for the binding between the protein and the carbohydrate subunits via an O-glycosidic linkage. The arabinogalactan moieties are highly branched consisting of 1,3-linked Gal*p* residues, some of them linked in position 6 to 1,6-Gal*p* side chains, terminating in Ara*f* residues. With regard to its structure, the rye AGP is comparable to other cereal AGPs like those from oat or wheat grain.

1. Introduction

In Europe rye is cultivated widely and serves as one of the main bread cereals. Together with a great variety of other food, this leads to a healthy, balanced nutrition. In contrast, there are regions in the world where people rely on grains as their primary food source and therefore suffer from malnutrition of micronutrients like iron, tin and copper. Arabinogalactan-proteins (AGPs) as components of the plant apoplast can have an increased binding affinity to these ions and are therefore an interesting target to improve the nutrition value of cereals (Aizat et al. 2011).

AGPs are macromolecular glycoproteins consisting of a big carbohydrate part rich in arabinose and galactose and a smaller protein part that is normally rich in the amino acid hydroxyproline. AGPs have been found in many different plants and also plant derived products like gums (Qi et al. 1991) or honey (Steinhorn et al. 2011). The glycoproteins occur in the cell wall area of plant cells and some of them can be linked via a glucosylphosphatidylinositol-anchor to the plasma membrane. The exact functions of AGPs in the plant cell are not fully understood, but functions reach from an influence on cell growth and cell differentiation to an involvement in processes like programmed cell death (Seifert and Roberts 2007).

The easiest way to isolate AGPs from plant material is to precipitate AGPs from a high molecular weight fraction of aqueous extracts using an artificial carbohydrate antigen, the $(\beta\text{-D-Glc})_3$-Yariv-phenylglycoside (Yariv et al. 1962), called βGlcY. In the past it has been neglected that this is possible for AGPs from *Triticum spec*. and also from *Avena sativa* (Jermyn et al. 1975). Up to now it has therefore been common practice to isolate cereal AGPs without βGlcY (van den Bulck et al. 2005; Tryfona et al. 2010), which might result to contamination of AGP preparations with accompanying polysaccharides or proteins. The aim of our work was the isolation of pure AGPs from rye grain and structural characterization of these interesting constituents of our daily food.

2. Investigations, results and discussion

From a high molecular fraction of aqueous extract of rye grain we isolated a crude AGP preparation by precipitation with βGlcY. Isolation of purified rye AGP by second precipitation with βGlcY was possible only in low amounts (0.001%), comparable to the yield of purified AGPs from oat and wheat grain (Göllner et al. 2010, 2011a).

With the help of a gel diffusion assay, it is possible to show binding of βGlcY to AGPs. The sharp precipitation line between the cavities with βGlcY and the AGP preparations of wheat, oat and also of rye indicated a characteristic positive reaction (Fig. 1). Our investigations showed for the first time that AGPs from grains of rye could be isolated by precipitation with βGlcY. The neutral monosaccharides (Table 1) of the high molecular weight compounds of an aqueous extract of rye grain were mainly xylose (48.7%), arabinose (31.2%) and glucose (13.7%) and smaller amounts of galactose and mannose (4.3% and 2.1%). The presence of soluble arabinoxylans and β-glucans in addition to the desired arabinogalactan-proteins in this high molecular weight fraction might be possible. After a first precipitation with βGlcY, the crude AGP preparation was rich in the monosaccharides galactose (43.9%) and arabinose (32.1%), but still showed also considerable amounts of xylose (13.5%) and glucose (8.1%). Mannose (2.1%) and fucose (0.3%) were only present in traces. Thus after the first Yariv precipitation small amounts of accompanying β-glucans and arabinoxylans remained attached to the AGP. These contaminants could be removed by a subsequent second precipitation with βGlcY resulting in a purified AGP with galactose and arabinose as main components (60.1% and 37.2%, resp.) in an AGP-typical ratio

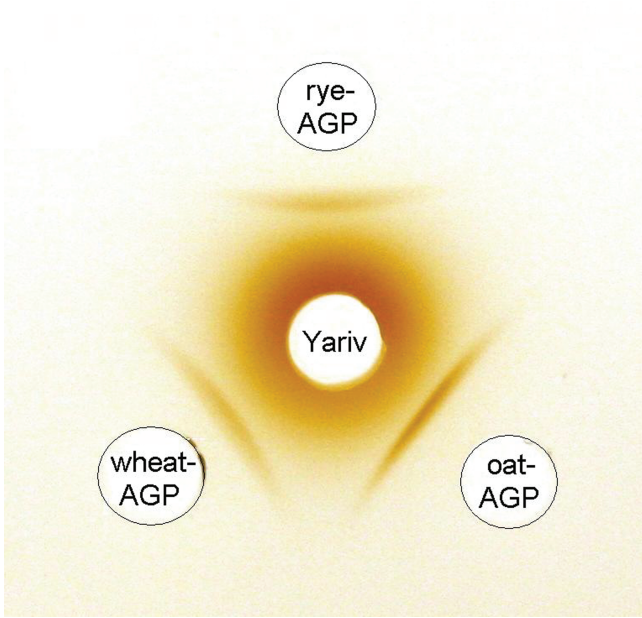

Fig. 1: Gel diffusion assay with βGlcY and AGPs isolated from grains of oat, wheat and rye.

of Ara to Gal (1 : 1.6). Accompanying monosaccharides in only trace amounts were glucose (1.6%), fucose (0.6%) and xylose (0.6%). No uronic acids were detectable.

The analysis of the linkage types of the purified rye AGP (Table 2) revealed that arabinose was present as terminal furanosidic arabinose and 1,5-linked arabinofuranose (32.1% and 7.7%). The galactose containing part of the AGP mainly consisted of galactose in its 1,3,6-linked (36.2%) and 1,3-linked pyranosidic form (14.3%) but there could also be detected terminal and 1,6-linked galactose linkage types (3.3% and 6.4%, resp.).

After partial acid hydrolysis, which cleaved preferentially labile bonds between furanosidic arabinose residues (Fig. 2), the content of the arabinose linkage types decreased strongly. 1,5-linked arabinose was not detectable any more, and only a small amount of 5.8% terminal arabinose remained.

This loss of arabinose resulted in a strong increase of the relative proportion of 1,6-Gal*p* and a decrease in 1,3,6-Gal*p* residues. An increase in 1-Gal*p* residues and the missing increase in 1,3-Gal*p* indicated the most likely structural model of the carbohydrate moiety described in Fig. 2.

The results of linkage analysis indicated that the carbohydrate moieties of rye AGP can be classified as a type III arabinogalactan (AG), typified by *Lolium* AGP (Clarke et al. 1979). Its carbohydrate part consists of a backbone of β-1,3-linked galactose residues frequently branched at position 6 to galactose containing side chains terminating in α-glycosidic bound arabinose residues.

Like in the AGP from rye also in the *Lolium* AGP there were found no uronic acids. Interestingly, the rye AGP has 1,5-linked arabinose residues, not present in the *Lolium* AGP model, but found in other AGPs, e.g. from *Echinacea* (Classen et al. 2000) or *Physcomitrella* (Lee et al. 2005).

The protein part of rye AGP made up about 7 % and the amino acid hydroxyproline about 1.1 % of the whole AGP molecule. The quantitative analysis of amino acids by HPLC after acid hydrolysis showed a hydroxyproline content of 15.1 mol% of the protein part (Fig. 3). Other main amino acids were alanine (16.6 mol%), serine (9.5 mol%), glycine (9.4 mol%), threonine (6.0 mol%), valine (5.3 mol%), proline (5.0 mol%) and leucine (4.7 mol%), which reflected a typical amino acid composition of classical AGPs (Showalter 2001). Furthermore, quite high amounts of glutamine/glutamic acid (7.1 mol%) and asparagine/aspartic acid (5.9 mol%) have been detected, which is in good correspondence with data for other AGPs, e.g. from cereals like *Avena sativa* (Göllner et al. 2011a), *Triticum aestivum* (Göllner et al. 2010), but also from dicots like *Echinacea purpurea* (Volk et al. 2007) or *Baptisia tinctoria* (Wack et al. 2005).

In order to get information on the type of polysaccharide-protein linkage, alkaline treatment of the AGP was carried out, which should lead to complete degradation of the protein part, resulting in carbohydrate units attached only to one (the binding) amino acid. In the case of rye AGP, hydroxyproline (24.0 mol%), glutamine/ glutamic acid (12.7 mol%) and asparagine/ aspartic acid (10.7 mol%) increased (Fig. 3), indicating that these amino acids might be involved in linkages between protein and polysaccharide moieties. Linkage of hydroxyproline to AG moieties via an O-glycosidic bond to galactose is a well-known feature of AGPs (Seifert and Roberts 2007) and is also most likely for rye AGP. The detection of residual amounts of all other amino acids could possibly be due to incomplete alkaline hydrolysis of the protein moiety. Asparagine/ aspartic acid and glutamine/ glutamic acid could be located next to hydroxyproline and remained attached to the AG-hydroxyproline residues in the case of incomplete alkaline hydrolysis (Volk et al. 2007). This would explain their increased appearance in the degraded AGP. In the amino acid sequence of *Lolium*, also member of the Poaceae family like rye, glutamic acid is located next to hydroxyproline (Gleeson et al. 1989). A N-glycosidic linkage type has been found only in chimeric AGPs with the conserved amino acid sequence Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except proline (Ellis et al. 2010). Under the alkaline conditions of this degradation method also possible linkages between glycan and protein would be broken if serine and threonine would be the amino acids responsible for binding (β-elimination), but O-glycosidic linkages of the AG part to serine or threonine in AGPs have only been described rarely (Tsumuraya et al. 1987).

Table 1: Neutral monosaccharide composition of high molecular weight compounds of an aqueous extract, crude AGP preparation and purified AGP of rye

	Relative amount (mol%)		
Monosaccharide	High molecular weight compounds of an aqueous extract of rye	Crude rye AGP preparation	Purified rye AGP
Gal	4.3 ± 0.3	43.9 ± 7.2	60.1
Ara	31.2 ± 0.8	32.1 ± 6.1	37.2
Glc	13.7 ± 1.1	8.1 ± 6.5	1.5
Fuc	n.d.*	0.3 ± 0.7	0.6
Xyl	48.7 ± 0.8	13.5 ± 2.7	0.6
Man	2.1 ± 1.5	2.1 ± 4.2	n.d*.
Ara : Gal	1/0.1	1/1.4	1/1.6
Sample size	n = 2	n = 7	n = 1

* not detectable

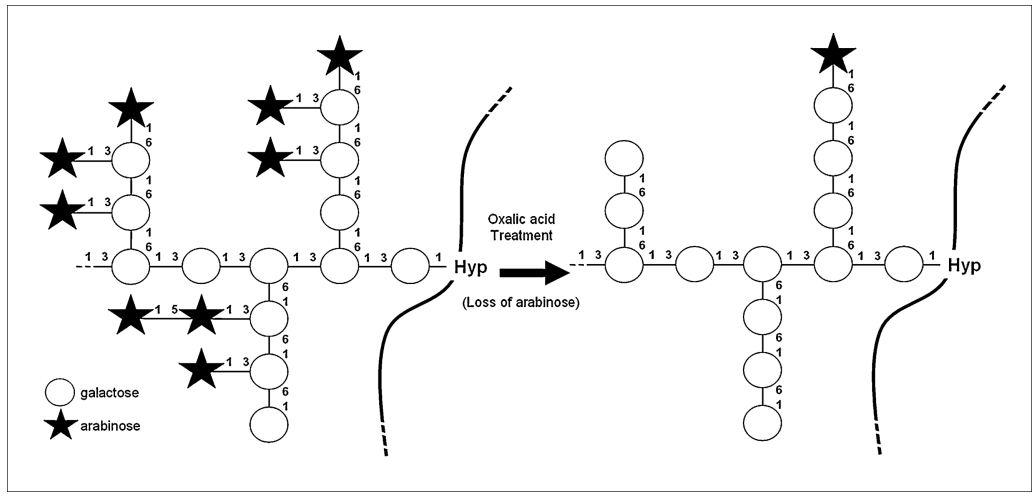

Fig. 2: Structural model of the carbohydrate moiety of rye AGP and illustration of degradation under mild acidic conditions.

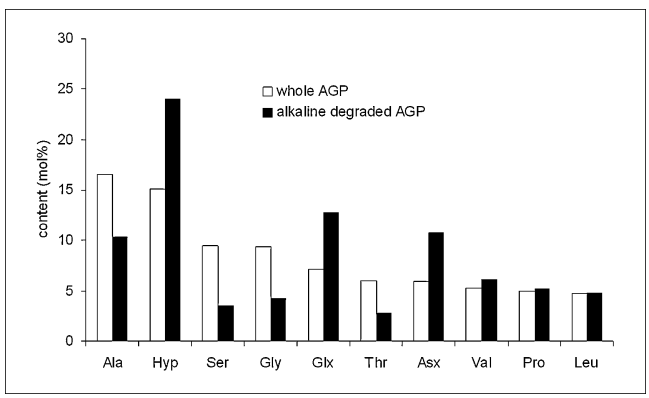

Fig. 3: Amino acid composition of purified AGP before and after alkaline degradation of the protein part. Only the ten amino acids of the highest concentrations are shown.

Characterization of the carbohydrate part of AGPs is normally complicated by the difficulties in isolating a single type of AGP and the microheterogenity of the constituent glycan chains (Ellis et al. 2010). Double AGP precipitation by βGlcY led to a purified rye AGP with only one big signal in size-exclusion chromatography of about 110 kDa. Compared with other AGPs of cereal origin, the AGP of rye is in the same range of molecular mass as AGPs of oat grain (83 kDa) and wheat grain (125 kDa) and also shows to have similar structural features (Göllner et al. 2010, 2011a). The precise molecular mass of wheat flour AGP is still unclear with a broad range of 22 kDa to 70 kDa (Tryfona et al. 2010).

From cereals especially the AGP of wheat flour is in the focus of interest and has already been characterized thoroughly. Early reports characterized cereal AGPs to have a β-1→6-linked Gal*p* backbone substituted in position 3 with galactose or/and arabinose residues (Loosveld et al. 1998, van den Bulck et al. 2005). For other members of the Poaceae like *Lolium multiflorum* or *Phleum pratense* a different AGP model was established by Anderson et al. (1977) and Sims et al. (2000) following the classical type II AG with a β-1→3 Gal*p* backbone with short β-1→6-linked Gal*p* side chains which are substituted with α-1→3-linked Ara*f* arabinose residues.

The most recent structural characterization of AGPs from wheat flour was done by Tryfona et al. (2010) by digesting the AG part with recombinant enzymes. After chromatographic separation, the liberated oligosaccharides were identified by MALDI-TOF and a very detailed model of the AG part of wheat flour AGP was presented. Interestingly the basic model was identical with the previous findings of Anderson et al. (1977) and Sims et al. (2000), except for detecting longer β-1→6-galactose side chains with DP 5 to DP 20. Furthermore, β-linked pyranosidic arabinose residues have been found, also known from arabinogalactan from larch (Göllner et al. 2011b).

These differences between the model of the wheat flour AGP and the proposed structural model of AGP of rye grain (Fig. 2) could be due to the different isolation processes of the glycoproteins. Besides the different starting material (flour vs. whole grain), the wheat flour AGP was isolated by different ethanol precipitation steps and digestion of interfering polysaccharides with enzymes. The AGP investigated in this work was isolated very specifically by repeated precipitation with βGlcY. As not each AGP is precipitable with βGlcY, the wheat flour AGP preparation is probably consisting of various types of AGPs, already indicated by the higher yield of AGP using the stepwise precipitation with ethanol (0.14%, van den Bulck et al. 2005).

Arabinogalactans have been shown to have immunomodulating (Alban et al. 2002) as well as prebiotic effects (Robinson et al. 2001). Isolation and characterization of the AGP from whole

Table 2: Linkage types of purified rye AGP and its product after partial acid hydrolysis

		Relative amount (mol%)	
Monosaccharide	Deduced linkage	Purified rye AGP	Purified rye AGP after partial acid hydrolysis
Gal	t*p*	3.3	16.2
	3*p*	14.3	10.1
	6*p*	6.4	44.9
	3,6*p*	36.2	23.0
Ara	t*p*	32.1	5.8
	5*p*	7.7	n.d.*

* not detectable

grain of rye was the first step to clarify the nutritional and health benefits of this cereal cell wall constituent.

3. Experimental

3.1. Isolation

Grains of rye were obtained from the Institute of Phytopathology, Christian-Albrechts-University, Kiel, Germany. The AGP isolation was carried out according to Göllner et al. (2010).

3.2. Gel diffusion assay

Four cavities were stamped in an autoclaved agarose gel (10 mM Tris-HCl, 1 mM $CaCl_2$, 0,9% NaCl, 1% agarose). The middle cavity was filled with βGlcY solution (1 mg/mL), the three surrounding cavities with solutions of isolated AGPs (1 mg/mL). The characteristic positive reaction for the presence of AGPs was a sharp precipitation line between the Yariv-reagent cavity and the AGP cavity.

3.3. Size-exclusion-chromatography (SEC)

Molecular mass of the rye AGP was determined by using multi angle laser light scattering detection (mini DAWN, Wyatt Technology, Santa Barbara, CA, USA) in combination with RI-detection (Polymer Laboratories GmbH, Darmstadt, Germany) after size exclusion chromatography on three PL aquagel-OH MIXED 8 μm columns connected in series (Polymer Laboratories GmbH). The eluent was 0.1 M $NaNO_3$ at a flow rate of 1.0 mL/min. The refractive index increment (dn/dc) was 0.141 mL/g (Göllner et al. 2011a).

3.4. Monosaccharide composition

The neutral sugar analysis was carried out following Göllner et al. (2011a). For detection of uronic acids the corresponding trimethyl silyl ethers (TMS-derivates) were analyzed by GLC according to Inngjerdingen et al. (2005).

3.5. Methylation analysis

Linkage analysis of the carbohydrate moiety of the AGPs was performed following the method of Harris et al. (1984) by GLC-MS on a Permabond® OV-1701 (25 mL, 0.25 mm ID) column (Macherey & Nagel, Düren, Germany).

3.6. Hydroxyproline determination

Hydroxyproline determination was done according to Stegemann and Stalder (1967) by oxidation of the imino acid by chloramine-T and coupling of the chromogen formed with Ehrlich's aldehyde in strong perchloric acid. The colored product was measured photometrically at 558 nm.

3.7. Determination of amino acids

The amino acid composition of the AGP samples was determined by HPLC after column derivatization of the amino acids with ninhydrin as described by Göllner et al. (2011a).

3.8. Partial acid hydrolysis

AGP was dissolved in 2 mL of 12.5 mM oxalic acid and heated at 100 °C for 5 h (Gleeson and Clarke 1979; Göllner et al. 2010). The hydrolysate was cooled to room temperature, precipitated by the addition of ethanol (final concentration = 80% v/v), left overnight at 4 °C, and centrifuged (20000 g, 10 min). After two washing steps with 80% ethanol, the precipitated residual polysaccharide was freeze-dried.

3.9. Alkaline hydrolysis

Freeze-dried AGP was hydrolyzed in 0.44 M NaOH (105 °C, 18 h) (Volk et al. 2007), neutralized with HCl, dialyzed extensively against demineralized water (MWCO = 6000 - 8000, Spectra / Por, Houston, TX, USA) and finally freeze-dried.

Acknowledgement: We thank Prof. Berit Smestad Paulsen, School of Pharmacy University of Oslo, for silylation data.

References

Aizat WM, Preuss JM, Johnson AAT, Tester MA, Schultz CJ (2011) Investigation of a His-rich arabinogalactan-protein for micronutrient biofortification of cereal grain. Physiol Plant 143: 271–286.

Alban S, Classen B, Brunner G, Blaschek W (2002) Haemolytic complement assay for the differentiation between the complement modulating effects of an arabinogalactan-protein from *Echinacea purpurea* and heparin. Planta Med 68: 1118–1124.

Anderson RL, Clarke AE, Jermyn MA, Knox RB, Stone BA (1977) A carbohydrate-binding arabinogalactan-protein from liquid suspension cultures of endosperm from *Lolium multiflorum*. Aust J Plant Physiol 4: 143–158.

Clarke AE, Anderson RL, Stone BA (1979) Form and function of arabinogalactans and arabinogalactan-proteins. Phytochem 18: 521–540.

Classen B, Witthohn K, Blaschek W (2000) Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the β-glucosyl Yariv reagent. Carbohydrate Research 327: 497–504.

Ellis M, Egelund J, Schultz CJ, Bacic A (2010) Arabinogalactan-proteins: Key regulators at the cell surface? Plant Physiol 153: 403–419.

Gleeson PA, Clarke AE (1979) Structural studies on the major component of Gladiolus style mucilage, an arabinogalactan-protein. Biochem J 181: 607–621.

Gleeson PA, McNamara M, Wettenhall REH, Stone BA, Fincher GB (1989) A hydroxyproline-rich protein core of an arabinogalactan-protein secreted from suspension cultured *Lolium multiflorum* (Italian ryegrass) endosperm cells. Biochem J 264: 857–862.

Göllner EM, Blaschek W, Classen B (2010) Structural investigations on arabinogalactan-protein from wheat, isolated with Yariv reagent. J Agric Food Chem 58: 3621–3626.

Göllner EM, Ichinose H, Kaneko S, Blaschek W, Classen B (2011a) An arabinogalactan-protein from whole grain of *Avena sativa* L. belongs to the wattle-blossom type of arabinogalactan-proteins. J Cereal Sci 53: 244–249.

Göllner EM, Utermöhlen J, Kramer R, Classen B (2011b) Structure of arabinogalactan from *Larix laricina* and its reactivity with antibodies directed against type-II-arabinogalactans. Carbohyd Polym 86: 1739–1744.

Harris PJ, Henry RJ, Blakeney AB, Stone BA (1984) An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. Carbohydr Res 127: 59–73.

Inngjerdingen KT, Debes SC, Inngjerdingen M, Hokputsa S, Harding SE, Rolstad B, Michaelsen TE, Diallo D, Paulsen BS (2005) Bioactive pectic polysaccharides from *Glinus oppositifolius* (L.) Aug. DC., a Malian medicinal plant, isolation and partial characterization. J Ethnopharmacol 101: 204–214.

Jermyn MA, Yeow YM, Woods EF (1975) Class of lectins present in the tissues of grain plants. Aust J Plant Physiol 2: 501–531.

Lee KJD, Sakata Y, Mau SL, Pettolino F, Bacic A, Quatrano RS, Knight CD, Knox JP (2005) Arabinogalactan proteins are required for apical cell extension in the moss *Physcomitrella patens*. Plant Cell 17: 3051–3065.

Loosveld AMA, Grobet PJ, Delcour JA (1997) Contents and structural features of water-extractable arabinogalactan in wheat flour fractions. J Agric Food Chem 45: 1998–2002.

Qi W, Fong C, Lamport DTA (1991) Gum Arabic is a twisted hairy rope. A new model based on O-Galactosylhydroxyproline as the polysaccharide attachment site. Plant Physiol 96: 848–855.

Robinson RR, Feirtag J, Slavin JL (2001) Effects of dietary arabinogalactan on gastrointestinal and blood parameters in healthy human subjects. J Am Coll Nutr 20: 279–285.

Seifert GJ, Roberts K (2007) The biology of arabinogalactan proteins. Annu Rev Plant Biol 58: 137–161.

Showalter AM (2001) Arabinogalactan-proteins: structure, expression and function. Cell Mol Life Sci 58:1399–1417.

Sims IM, Middelton K, Lane AG, Cairns AJ, Bacic A (2000) Characterisation of extracellular polysaccharides from suspension cultures of members of the Poaceae. Planta 210: 261–268.

Stegemann H, Stalder KH (1967) Determination of hydroxyproline. Clinica Chimica Acta 18: 267–273.

Steinhorn G, Sims IM, Carnachan SM, Alistair JC, Schlothauer R (2011) Isolation and characterization of arabinogalactan-proteins from New Zealand kanuka honey. Food Chem 128: 949–956.

Tryfona T, Liang H-C, Kotake T, Kaneko S, Marsh J, Ichinose H, Lovegrove A, Tsumuraya Y, Shewry PR, Stephens E, Dupree P (2010) Carbohydrate structural analysis of wheat flour arabinogalactan protein. Carbohydr Res 345: 2648–2656.

Tsumuraya Y, Hashimoto Y, Yamamoto S (1987) An L-Arabino-D-galactan and an L-Arabino-D-galactan-containing proteoglycan from radish (*Raphanus sativus*) seeds. Carbohydr Res 161: 113-126.

Van den Bulck K, Swennen K, Loosveld AMA, Courtin CM, Brijs K, Proost P, Van Damme J, Van Campenhout S, Mort A, Delcour JA (2005) Isolation of cereal arabinogalactan-peptides and structural comparison of their carbohydrate and peptide moieties. J Cereal Sci 41: 59–67.

Volk RB, Blaschek W, Classen B (2007) Characterization of an arabinogalactan-protein from pressed juice of *Echinacea purpurea* - investigations on linkage-type between protein and polysaccharide moiety. J Nat Med 61(4): 397–401.

Wack M, Classen B, Blaschek W (2005) An acidic arabinogalactan-protein from the roots of *Baptisia tinctoria* (L.) *R. Brown*. Planta Med 71: 814–818.

Yariv J, Rapport MM, Graf L (1962) The interaction of glycosides and saccharides with antibody to the corresponding phenylazoglycosides. Biochem J 85: 383–388.