SEC COUPLED WITH UV AND FLUORESCENCE DETECTION, AN EFFICIENT METHOD FOR B-GLUCOSYL-YARIV ARABINOGLACTAN PROTEIN (AGP) MONITORING

1SOUPI MARIETTA, 2BOURVEN ISABELLE, 2SIMON STEPHANE, 1LHERNOULD SABINE, 1OMOKOLO DENIS, 2GUIBAUD GILLES, 2COSTA GUY

1Laboratoire de Chimie des Substances Naturelles (EA 1069), Faculté des Sciences et Techniques, 123 Avenue Albert Thomas, F-87060 Limoges Cedex, France. Tel : 33 (0) 555 457 393, Fax : 33 (0) 555 457 386 http://www.unilim.fr/lcsn
2Groupe de Recherche Eau Sol et Environnement, Faculté des Sciences et Techniques, 123 Avenue Albert Thomas, F-87060 Limoges Cedex, France. Tel : 33 (0) 555 457 3XX, Fax : 33 (0) 555 457 3XX http://www.unilim.fr/grese

ABSTRACT

The arabinogalactan proteins (AGPs) are O-glycosylated proteins consisting of less than 10 % proteins involved in many aspects of plant growth and development. Our work focuses on the development of a sensitive and easy method for monitoring partially purified AGP. Fluorescence excitation-emission matrix (EEM) spectrophotometry was used to select excitation-emission wavelengths of arabic gum and plant β-glucosyl Yariv AGP for the SEC fluorescence system. Commercial as plant AGP were then separated by high performance size exclusion chromatography (SEC) on two columns in series (Agilent bio SEC 100 Å and 300 Å) coupled with 215 nm UV and 221/350 nm Ex/Em fluorescence detection. Separate molecules were then collected and analyzed by biochemical and immunological techniques, as well as by mass spectrometry. Here we demonstrated that AGP could be well separated and monitored by SEC UV/fluorescence proceeding. Biochemical, immunological and mass spectrometry confirmed that the analyzed peaks correspond to AGP molecules. If this method was not able to separate all the AGP expressed in plant, it was suitable to monitor β-glucosyl Yariv AGP evolution during plant development, such as in cocoa embryogenesis.

Keywords: Arabinogalactan proteins, size exclusion chromatography, fluorescence and UV detection, AGP fingerprint

1. INTRODUCTION

Arabinogalactan proteins (AGP) are glycosylated members of the Pro/Hyp-rich glycoprotein super family (HRGP), (Nothnagel, 1997; Showalter et al., 2010). The complexity of glycan structures and the polymorphism of the aglycon moiety are responsible for the very large molecular diversity of these proteoglycans (Tan et al., 2004; Estévez et al., 2006). The protein content of AGPs reaches 1 to 10 % and peptides sequences consisted of a central domain rich in Pro, Ala, Ser, Thr (Schultz et al., 2000). Mostly, AGPs are O-glycosylated at one or more hydroxyprolin (Hyp) residues by arabinogalactan (AG) type arabino-3,6-galactan groups (Seifert and Roberts, 2007). Structural diversity of the aglycon moiety, suggested by some preliminary studies Chen et al., 1994; Du et al., 1994), was confirmed by the sequencing of whole genome of Arabidopsis thaliana, which proved that AGPs belong to a complex multigenic family. An algorithm, the BIO OHIO software program (Showalter et al., 2010), based on properties of polypeptide core, was developed to identify and classify hydroxyproline-rich glycoproteins including classical AGPs, AG peptides, fasciclin-like AGPs, lysine-rich AGPs, plastocyanin AGPs, and other chimeric AGPs. All HRGPs are not AGPs or AGP-like proteins. This software was also used to analyze the diversity of the AGP-like family. Two approaches based on the properties of proteins or glycans have been developed. For example, to characterize the glycan fraction of AGPs, a NMR-based sequencing of AG chains was realized.
(Göllner et al., 2011) and these data gave support to the Hyp glycosylation and the carbohydrate composition.

If the physico-chemical properties of AGPs are very similar, a micro structural heterogeneity is probably responsible of their multiple biological functions. In fact, AGPs or AGP-like proteins are involved in plant growth and development and play an important role in cell-cell signaling, programmed cell death, cell differentiation, cell wall plasticity and zygotic and somatic embryogenesis (Seifert and Roberts, 2007; Gao and Showalter, 2000; Motose et al., 2001; Hu et al., 2006; Chapman et al., 2000).

To investigate the function of AGPs or AGP-like molecules, their extraction, purification and isolation are an essential prerequisite. But purification and separation of native AGP or AGP-like proteins remain difficult. The purification of AGP has been facilitated by the ability of most, but not all AGPs, to bind the β-glucosyl Yariv reagent (Yariv et al., 1967). This reagent, used for AGPs quantification (Van Holst and Clarke, 1995), is also a tool for their detection and localization, as well as AGP-directed monoclonal antibodies. The Yariv reagent has been largely used to isolate AGPs from number of species: Arabidopsis thaliana (Schultz et al., 2000); Ipomoea batatas (Ozakiet et al., 2010) and Triticum aestivum (Göllner et al., 2010). The molecular weight (MW) of AGPs from Ipomoea batatas and Triticum aestivum was determined through a size exclusion chromatography (SEC) coupled to online Multi-Angle Laser Light Scattering (MALLS). It corresponds, respectively, to 126.8 and 125 kDa. The native AGPs of arabic gum, also known as acacia gum, has been separated on Hydrophobic Interaction Chromatography (HIC) in three mains fractions: an AG fraction, an AG fraction and a glycoprotein fraction (Osman et al., 1995; Göllner et al., 2010). The determination of the absolute MW of these fractions was obtained using SEC-MALLS (Renard et al., 2006; 2012). To analyze peak homogeneity of gum AG fractions after reversed-phase chromatography RP-HPLC followed by UV detection monitored at 220 nm, (Goodrum et al., 2000) have sequenced the protein backbone after AGPs deglycosylation. For Arabidopsis thaliana the precipitation of AGP by β-glucosyl Yariv was followed by separation on RP–HPLC and UV detection at 215 nm (Schultz et al., 2000). Protein backbone analysis of each peak, after deglycosylation by anhydrous hydrogen fluoride, exhibited a mixture of AGPs and AG peptides.

In this paper, we investigate a rapid and easy method with enough resolution to separate native β-D-glucosyl Yariv AGP from plants with a new methods based on SEC chromatography [1250-0.5 kDa] coupled with UV and fluorescence detection. With this technic, commercial arabic gum, as native AGP from cocoa embryo and poplar leaves have been well separated and their molar mass estimated

2. MATERIAL AND METHODS

2.1. Biological materials

Arabinogalactan proteins have been extracted from 1 years old hybrid poplar leaves clone 717 B4 (INRA Orléans) and from mature cacao embryos clone SNK630. Cocoa pods and buds were harvested from IRAD at Nkolbissou, Yaoundé, Cameroon. Somatic embryos were produced from staminodes and/or petals flowers, according to (Maximova et al., 2002). Commercial Arabic gum (Sigma), has been used as reference molecule, as suggested by (López-Franco et al. 2012).

2.2. AGPs Purification

AGPs were extracted from 5 g of fresh material, ten leaves of poplar or a hundred somatic embryos, with 5 mL of buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 % (v/v) β-mercaptoethanol, and 1 % (w/v) Triton X-100. After 3 h incubation at 4°C, samples were centrifuged for 10 min at 14000 g. The pellet was resuspended by vortex-mixing in 5 mL of 50 mM Tris-HCl, pH 8.0. The insoluble material was removed by two successive centrifugations. Both supernatants were pooled before being freeze-dried. The freeze-dried extract was dissolved in 250 µL of 1 % (w/v) NaCl. AGPs were precipitated with the β-D-glucosyl Yariv reagent (1, 3, 5-tris[4-β-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) previously synthesized according to the method of (Van Holst et al. 1995). AGP precipitation was performed over-night at 4°C by mixing an equal volume of sample and β-D-glucosyl Yariv at a final concentration 2 mg/mL in 1 % (w/v) NaCl, as described by (Schultz et al. 2000). The insoluble β-D-glucosyl Yariv AGP complex was collected by centrifugation (1 h at 14000 g). The β-D-glucosyl Yariv was removed from AGP by washing the pellet three times in 1 % (w/v) NaCl, and centrifuged after each washing at 14000 g for 10 min. Next, AGP precipitated were washed twice fold more in methanol. The pellet was dissolved in dimethyl sulfoxide with 5 mg of
sodium dithionite. Ultra pure water was added by vortex-mixing until the mixture became clear yellow-stained. This resulting solution was then desalted on a Sephadex G25 column (10 mL) and equilibrated with ultra pure water. Fractions containing AGPs were identified by the β-D-Glucosyl Yariv test before being pooled and freeze-dried.

2.3. Excitation–emission matrix (EEM) fluorescence spectroscopy

Freeze-dried AGP were resuspended in 50 mM phosphate buffer at pH 7.0 ± 0.1, filtered using a 0.45 μm pore size nitrate cellulose filter (Nalge, Nunc International). 3-Dimensional -Fluorescence spectra (EEM) were performed on AGPs at 0.05 mg/mL using a Shimadzu RF-5301 PC spectrofluorophotometer. Spectra were collected with subsequent scanning emission spectra, from 250 to 550 nm, by varying the excitation wavelength from 220 to 500 nm, with 5 nm increments. The software Panorama Fluorescence was employed for handling EEM data.

2.4. Size exclusion chromatography (SEC)

For SEC analysis, 100 μL of filtered sample (0.45 μm) at 1-2 mg/mL were injected in a Merck Hitachi LAC chromatograph composed of a L7200 autosampler, a L7100 quaternary pump, a L7000 interface and a spectrofluorophotometer detector (L7485). The SEC method was carried out with two columns (100 Å and 300 Å Agilent bio SEC) connected in series. The theoretical resolving range reported by the manufacturer is 0.1 to 100 kDa and 5 to 1250 kDa respectively. The mobile phase was composed of 50 mM phosphate buffer, at pH 7.0 ± 0.1. All measurements were made using a mobile phase flow of 0.7 mL min⁻¹. Before use, the mobile phase was filtered through a 0.22 μm filter and vacuum degassed for 20 min. Molar mass calibration were performed using a set of proteins with molecular weight ranging from 670 to 0.1 kDa [tryoglobulin 670 kDa (Sigma), ferritine 440 kDa (Sigma), human immunoglobulin 155 kDa (Sigma), serum albumin albumin bovine 69.3 kDa (Sigma), ovalbumin 45 kDa, ribonuclease 13.7 kDa (Sigma), tyrotrpin releasing hormone 0.3 kDa (Sigma.) and Tyrosin 0.1 (Fluka)]. For molar mass calibration curve, the logarithm of the molecular mass was plotted as a function of the elution volume (Log MW = -0.3164 Ve + 9.4676, R²= 0.982). The permeation volume, determined with NaN₃, was 22.5 mL.

2.5. Biochemical and immunological analysis

SEC fractions were collected for biochemical, immunological.

To identify neutral sugar-containing glycoconjugate, 10 μg of proteins were spotted on thin layer chromatography (TLC), sprayed with 1 % orcinol/ferric chloride reagent before being heated 20 min at 120°C.

To identify some of the possible AGP epitopes present in our extract, 30 μg of proteins were loaded onto 0.45 μm nitrocellulose membrane (BioTrace NT, PALL), coated with TBST 5 % of non-fat-milk for 1 h at room temperature under shaking. Primary monoclonal antibodies against AGP epitopes, LM2 (β-linked glucuronic acid) from Plant Probes (Smallwood et al., 1996) and JIM4 (β-GlcA-(1,3)-α-GalA-(1,2)-Rha) from Carbosources Knox et al., (1989) were diluted in TBS and incubated for 2 h, at room temperature under shaking. After three washings in TBST 1 % of non-fat milk, the membrane was subsequently incubated for 1 h with secondary antibody coupled to alkaline phosphatase (Sigma), 1/10000 diluted. Three washings more in TBST 1 % of non-fat milk, the alkaline phosphatase activity was revealed with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Thermo-scientific). Until desired color develops, the reaction was stopped with ultra pure water.

3. RESULTS AND DISCUSSION

3.1. Commercial arabic gum separation

The commercial arabic gum was analyzed by 3-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM) and by SEC coupled with UV and fluorescence detection (Fig. 1). The 3D-EEM revealed for arabic gum AGP a maxima fluorescence area corresponding to Excitation/Emission (Ex/Em) wavelengths of 200-250 nm and 280-380 nm respectively (Figure 1A). These particular spectra correspond to Ex/Em of aromatic amino acids such SEC as tyrosine and tryptophane, where tryptophan generally dominates the absorption fluorescence spectra (Beaven et al., 1950; Wetlaufer, 1962). This couple of Ex/Em has been then chosen for AGP separation by SEC chromatography (Fig. 1C). Arabic gum chromatograms (Fig. 1B and C) exhibited a complex profile suggesting that the commercial solution is made of a mixture of macromolecules. SEC arabic gum chromatogram was first recorded at 215 nm (Fig. 1B) wavelength that has been
previously used to detect AGPs (Schultz et al., 2000). Six main peaks were detected and considered as six different fractions (Fig. 1B). In order to compare the molecular weight of these fractions with those of the literature, we used proteins standards for molar mass calibration. The apparent molecular weights, MW\textsuperscript{app}, obtained were similar to absolute molecular weights given by multangle laser light scattering (Randall et al., 1989; Idris et al., 1998; Picton et al., 2000; Al-Assaf et al., 2005; Beltrán et al., 2005; Renard et al., 2012) (Table 1). The MW\textsuperscript{app} is the resultant of the hydrodynamic volume of the molecule, determine by its conformation. AGP folding from arabin gum seems to appear having a similar globular conformation than proteins use for molar mass calibration. Ionic and/or hydrophobic interactions between AGP and column matrix probably explain such globular folding. However, only the first three fractions were described in the literature as a glycoprotein (GP), arabinogalactan protein (AGPs) and arabinogalactan peptide (AG) fractions having respectively 47.8, 11.8 and less than 0.4 % of proteins (Randall et al., 1989; Idris et al., 1998; Picton et al., 2000; Al-Assaf et al., 2005; Beltrán et al., 2005; Renard et al., 1006; 2012). To have a better understanding on the molecular composition of each of these fraction, UV at 280 nm as fluorescence spectra were purchased (Fig. 1B, C). The chromatograms obtained at 280 and 215 nm are quite similar (Fig. 1B) except slightly differences in the intensity ratio for some peaks. Gao and Showalter (2000) have demonstrated that amino acid sequence of GP is quit different from those of AG and AGP. AG(P) have in common HPro and Ser as the most abundant amino acid while Asp was the most one for GP. AGP from arabic gum have little aromatic amino acid which represent less than 5 % of its content. Then, the two first peaks were probably a mixture of GP and AGP. According to UV 280 nm chromatogram, peak 2 seems to contain a higher rate of AGP than GP if expected that no feruloylated polysaccharides were linked to AGP. The third peak (Fig. 1B) is barely detected at 280 nm that could be linked to low protein content. From this hypothesis, the third fraction could correspond to the AG fraction of arabic gum. Because arabic gum glycoprotein is described as a twisted hairy rope (Qi et al., 1001), peaks 4, 5, 6 (Fig. 1B) having MW\textsuperscript{app} lower than 7 kDa probably correspond to a mixture of partial deglycosylate glycopeptides. The protein fluorescence detection (Ex/Em: 221/350 nm) displays one more peak (Fig. 1C, arrow) not resolved 215/280 nm corresponding to a not absorb and/or an undetectable compound due to low UV detector sensitivity. If the 3 first peaks are quit similar, the chromatogram profile for low molecular weight (peaks 4, 5, 6) is strongly different allowing us to separate multiple compounds and demonstrating that these peaks are consisting of peptides and/or glycopeptides. Each six chromatographed peaks have been analyzed regarding their reactivity to β-D-glucosyl Yariv, their immunogenicity against AGP antibodies (LM2, JIM4) and by their carbohydrate content (Fig. 2). Peak 2 exhibited a positive labeling for all the 3 main biochemical markers suggesting that protein from it seems to be the AGP fraction of arabic gum. Our data are in accordance with those found in the literature (Renard et al., 2006). But our data also demonstrated that peak 1 and 3 contained probably AGP-like molecules (Fig. 2).

3.2. Poplar leaves and cacao somatic embryos AGP separation

The AGP from poplar leaves and cacao somatic embryos have been first isolated according to Schultz et al. (2000). β-D-glucosyl Yariv AGP were then analyzed by 3-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM) and by SEC coupled with UV and fluorescence detection (Fig. 3). As demonstrated for commercial arabic gum, the β-glucosyl Yariv AGP from plants show one peak at Ex/Em of 221/350 nm (Fig. 3A). The β-glucosyl Yariv AGPs have been then chromatographed by a double BIOSEC 100 Å/300 Å serial column coupled with a double UV and fluorescence detectors. The purified β-glucosyl Yariv AGPs exhibited similar UV/fluorescence profile especially for high MW (Fig. 3B). More, the sensitivity and the number of detectable peaks are much higher with fluorescence than UV detectors. For poplar leaves (Figure 3B1) and cacao embryos (Fig. 3B2), 2 main UV peaks could be well separated at respectively 903/1280 kDa (peak 1) and 405/583 kDa (peak 2) whereas 4 to 5 peaks at 221/350 nm Ex/Em. The peak 1 and 2 detected at Ex/Em 221/350 nm are slightly offset from the peak 1 and 2 detected by UV suggesting a molecular heterogeneity of the UV/fluorescence detected fractions. The question now is about the macromolecular composition of all these peaks. The β-glucosyl Yariv reactivity, AGP antibodies (LM2, JIM4) immunogenicity and carbohydrate content of all the main detected peaks are presented in table 2. Data suggested that AGP and/or AGP-like are found in peak 1, 2 and 3. Glycopeptides collected from peak 1 and 2 of cacao embryos (Fig. 3B2) have been then analyzed by capillary
purification is an easy, and fast method suitable for the development of plants and more particularly AGP and follow their differential expression during the development of plants and more particularly during the maturation of the somatic embryos. This technique associated with plant β-glucosyl Yariv AGP purification is an easy, and fast method suitable for monitored plant AGP.

REFERENCES:


Figure 1: 3D Fluorescence spectra and SEC chromatograms of commercial arabic gum (sigma) previously solubilized in phosphate buffer pH 7. (A), 3D spectra; (B), SEC chromatogram [215 nm (—); 280 nm (---)]; (C), SEC chromatogram [215 nm (—); 221/350 nm Ex/Em (---)].
Figure 2: Biochemical analysis of the six fractions of arabic gum separated by SEC. (A) β-Glc Yariv precipitation (np, no precipitation; p, precipitation); (B), dot blot against JIM4 AGP mAb (+, positive reaction; - negative reaction); (C), dot blot of carbohydrate staining (+, positive reaction; - negative reaction).

Figure 3: 3D Fluorescence spectra and SEC chromatograms of purified β-Glc Yariv AGP from (1) poplar leaves clone 717 1B4 and (2) cacao somatic embryo genotype SNK 630 previously solubilized in phosphate buffer pH 7. (A), 3D spectra; (B), SEC chromatograms [215 nm (---); 221/350 nm Ex/Em (---)].
Table 1. Comparison of molecular mass calibration of arabic gum fractions from our studies (row 1) and from data available in literature (row 2, 3); Column number 1, 2, 3, 4, 5, 6 considered picks on figure 1A for our study.

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Table 2. Biochemical investigation of AGPs from poplar leaves and cocoa somatic’s embryos fractions collected after SEC separation (+, precipitation of AGPs with β-Glc Yariv; -, no precipitation; + presence; - absence)

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