Carbohydrate Polymers 98 (2013) 522–531

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Arabinogalactan protein cluster from *Jatropha curcas* seed embryo contains fasciclin, xylogen and LysM proteins

Maria Sehlbach[a], Simone König[b], Michael Mormann[c], Jandirk Sendker[a], Andreas Hensel[a,*]

[a] University of Münster, Institute for Pharmaceutical Biology and Phytochemistry (IPBP), Hittorfstraße 56, D-48149 Münster, Germany
[b] University of Münster, Interdisciplinary Centre for Clinical Research, Röntgenstr. 21, D-48149 Münster, Germany
[c] University of Münster, Institute for Hygiene, Robert-Koch-Strasse 41, D-48149 Münster, Germany

ARTICLE INFO

Article history:
Received 11 March 2013
Received in revised form 3 June 2013
Accepted 4 June 2013
Available online 24 June 2013

Keywords:
Jatropha curcas L.
Arabinogalactan proteins
Fasciclin
Xylogen
LysM
N-/*O*-glycosylation

ABSTRACT

An non-GPI-anchored AGP cluster (Y2) was isolated from the seeds of *Jatropha curcas* L. (Euphorbiaceae) composed of 4.8% polypeptides (mainly Ala, Ser, Gly, Hyp, Glu) and a carbohydrate moiety composed of Gal, Ara, GlcA, Rha, Man and GlcN. Besides the typical structural features of arabinogalactan proteins, typical *N*-glycan linker of the complex type ($GlcNAc_4Man_3Gal_2Fuc_1Xyl_1$) were identified. *O*-glycosylation occurred mainly *via* Hyp and to a lesser extent *via* Thr and Ser. N-glycans from the complex type, carrying at the innermost GlcNAc at position O-3 one α-Fuc-residue, were also present.

MS analysis of the tryptic digest assigned peptides of three major protein groups: fasciclin-like arabinogalactan proteins, xylogen-like proteins and LysM domain-containing proteins. They could not be separated further and it is indicated that various homologous protein forms co-exist. Histological investigation of *J. curcas* seeds revealed the presence of AGPs in the vessels of cotyledons and in the procambium ring of the embryo.

1. Introduction

Jatropha curcas L. (Euphorbiaceae) is a deciduous and drought resistant tree, widely distributed in tropical and subtropical areas of Central and South America, Africa, India and Southeast Asia. Plants have been used for ecosystem restoration of disturbed areas and biodiesel production (Fairless, 2007). The seeds contain high amounts of fatty oil, a lectin (Lin, Zhou, Wang, Jiang, & Tang, 2010) and diterpens from the tigliane-type in form of phorbol esters with tumour-promoting and inflammative activity (Goel, Makkar, Francis, & Becker, 2007). The seeds of *J. curcas* are used traditionally for wound-healing, fractures and burns. Recently a crude arabinogalactan protein (AGP) fraction from *J. curcas* seeds has been described to influence human skin cell physiology with the stimulation of mitochondrial activity of keratinocytes and dermal fibroblasts and significant increase of the ATP status of primary keratinocytes (Zippel, Wells, & Hensel, 2010). Additionally, the fraction induced keratinocyte differentiation by stimulation of growth factors GM-CSF, HGF, KGF and TGFβ. This *in vitro* activity profile pointed to a potent induction of cellular differentiation *via* stimulation of growth hormones and TGFβ-induced cell signalling, rationalizing the traditional medicinal use of aqueous plant extracts from *J. curcas* for improved wound-healing. Therefore, the present study aimed at the isolation, purification and advanced structural analysis of the AGP by detailed analysis.

2. Experimental

2.1. General

If not stated otherwise all chemicals were purchased from Sigma (Deisenhofen, Germany) and VWR (Darmstadt, Germany). *J. curcas* L. seeds were obtained from KPR – Gardeners Club, Slovakia. Identification was performed by senior author A. Hensel. A voucher species is deposited under code number 283 in the archives of the Institute of Pharmaceutical Biology and Phytochemistry (IPBP), University of Münster, Germany.

Abbreviations: AEC, anion exchange chromatography; AGP, arabinogalactan protein; DP, degree of polymerization; FLA, fasciclin-like arabinogalactans; GPC, gel permeation chromatography; HPAEC-PAD, high pressure anion exchange chromatography with pulsed-amperometric detection; LC, liquid chromatography; LYSM, LysM domain-containing proteins; MS, mass spectrometry; MALLS, multi-angel laser light scattering; MW, molecular weight; SPB, sodium phosphate buffer; TFMS, trifluoromethanesulphonic acid; XLP, xylogen-like proteins.

* Corresponding author. Tel.: +49 251 8333380; fax: +49 251 8338341.
E-mail address: ahensel@uni-muenster.de (A. Hensel).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.06.003

2.2. Isolation of Y2

2.2.1. Isolation procedure

The testa of *J. curcas* seeds (3 kg) were removed. The resulting material (1.474 g) was ground in a mortar and defatted with petroleum benzine for 24 h in a Soxhlet apparatus. 630 g of the defatted endosperm were extracted with the 14-fold amount of water for 24 h at 4 °C. After centrifugation (14,000 × *g*, 13 min) high molecular weight material of the supernatant was precipitated by drop-wise addition of the 4-fold amount of ice-cold 96% ethanol under stirring. The precipitation was completed overnight at 4 °C. After centrifugation (2300 × *g*, 10 min) the remaining residue was dissolved in water, dialyzed (molecular weight cut-off 3.5 kDa) and centrifuged at 5300 × *g* for 13 min to remove any insoluble residue. This process yielded 1.1% (related to the starting seed material) of soluble fraction JC1. Non-glycoproteins were depleted by a 5 min treatment of JC1 solution at 100 °C, followed by immediate ice-cooling. The precipitated protein was removed by centrifugation (10 min at 3100 × *g*). The remaining supernatant was lyophilized to obtain JC2.

2.2.2. Chromatographic purification of JC1

Anion exchange chromatography (AEC) of JC2 (4.5 g in 60 mL water) was performed on DEAE-Sephacel® (GE Healthcare, Freiburg, Germany): 5.2 cm × 16 cm i.d., phosphate form, flow 3 mL/min, fraction size 10 mL, 85 fractions per gradient step, stepwise gradient with deionized water and $NaPO_4$ buffers (pH 5, ion strength 0.1, 0.25, 0.5, 1 mol/L). Carbohydrate-containing fractions were pooled, concentrated *in vacuo*, dialyzed and lyophilized. The main fraction named R2 was isolated from the 0.1 mol/L eluate (yield 0.08% related to the starting seed material).

2.2.3. Precipitation of AGPs from R2 by Yariv reagent

AGPs were isolated from R2 by precipitation with β-D-glucosyl-Yariv reagent, which was synthesized from *p*-nitrophenyl-β-D-glucopyranoside according to Yariv, Rapport, and Graf (1962). Identity and purity of the product was confirmed by UV–vis, HR-MS, ^1H and ^{13}C NMR (COSY, HSQC, HMBC) in DMSO-d_6.

100 mg of β-D-glucosyl Yariv reagent were dissolved in 100 mL of NaCl solution (0.15 M) and 400 mg R2 were added. The solution was mixed, AGPs were allowed to precipitate for 18 h at 4 °C, followed by centrifugation (1700 × *g*, 5 min). The resulting sediment was washed 4 times with 50 mL NaCl (0.15 M) with centrifugation after each washing step. The residue was suspended in 30 mL of water. Solid sodium dithionite was added to a final concentration of 10% (w/v) and the mixture was heated under stirring to 50 °C until the red colour disappeared. The solution was extensively dialyzed (MWCO 12–14 kDa) against water, centrifuged (3100 × *g*, 5 min) and lyophilized to yield Y2 (yield 0.01% related to the starting seed material).

2.3. Carbohydrate analysis

Standard methods of carbohydrate analysis (carbohydrate quantification, uronic acids, polysaccharide and protein hydrolysis, HPAEC-PAD for carbohydrates and amino acids, GC-FID analysis of alditol acetates, linkage analysis by GC–MS identification of partially methylated alditol acetates, D/L-configuration, low pressure GPC on Superose®) were performed according to methods previously described by Hermann et al. (2012) and Zippel, Deters, Pappai, and Hensel (2009). Quantitative analysis of monosaccharide composition of Y2 after TFA hydrolysis was performed by HPAEC-PAD using external standard calibration with the respective reference carbohydrates and, for verification, by GC-FID of the respective alditol acetates. The total amount of uronic acids was determined by colourimetric assay according to Blumenkrantz and Asboe-Hansen (1973). Individual uronic acids were determined after hydrolysis of the polymers by HPAEC-PAD. D-/L-configuration was determined *via* capillary electrophoresis after hydrolysis and derivatisation with S-(−)-1-phenylethylamine (Noe & Freissmuth, 1995). Linkage analysis was performed *via* methylation analysis after reductive deuteration of acidic polymers and evaluation of partially methylated alditol acetates by GC/MS.

2.3.1. Determination of molecular weight

HP-SEC was performed on a SECurity GPC system (PSS Polymer Standard Service, Mainz, Germany) consisting of three Suprema® columns from PSS (i.d. 8 mm, particle size; 10 μm 100 Å, 300 mm; 3000 Å, 300 mm; guard column, 50 mm) coupled online to a refractive index detector (Agilent series 1200 RID, Agilent Technologies, Santa Clara, USA), a UV detector (Agilent 1200 Series Variable Wavelength Detector, Agilent Technologies), a viscosimetric detector (PSS SECurity ETA2010) and MALLS detection (PSS SECurity SLD7000 MALLS) equipped with a 5 mW HeNe laser, operating at λ = 632.8 nm. Phosphate buffer (50 mM, pH 7) served as eluent at 0.7 mL/min; calibration was performed with pullulans (PSS Calibration Kit (1.32, 5.9, 10, 22.8, 47.3, 112, 212, 404, 710 kDa) dn/dc = 0.149 (pullulan)) and data analysis by PSS WinGPC Unity V.7.3.0.

2.3.2. NMR spectroscopy

For NMR, 16 mg of Y2 were dissolved in 0.8 mL D_2O (Uvasol®, Merck, Darmstadt, Germany) and filtered through prewashed cotton. ^1H and ^{13}C NMR measurements were obtained at 500 MHz and 125 MHz, respectively (Agilent VNMRS 500). The NMR spectra were referenced to the C6 signal of rhamnose (17.20 ppm in ^{13}C NMR, 1.25 ppm in ^1H NMR, 26 °C). The signals of the anomeric centre were compared to reference standards. To that end, 40 mg of the soluble gum arabic fraction (Caelo, Hilden, Germany) (Defaye & Wong, 1986) and 40 mg of sugar beet arabinan (Westphal, Kuehnel, Schols, Voragen, & Gruppen, 2010) (Südzucker, Obrigheim, Germany) were measured in D_2O containing 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium (TMSP). Signals were referenced to the C-6 of rhamnose at 1.25 ppm in ^1H and 17.20 ppm in ^{13}C NMR spectrum.

Data were processed by MestReNova® 8.0.1, Mestrelab Research S.L., Santiago de Compostela, Spain.

2.3.3. Mass spectrometry

Mass spectrometric analysis of oligosaccharides was performed as described recently for the characterization of *N*-glycopeptides derived from plant lectins by use of a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) equipped with a nanoelectrospray ionisation (nanoESI) ion source (Kumar, Shivappa, Pohlentz, Mormann, & Kumar, 2013). Neutral sugars were dissolved in methanol/water/formic acid (49/49/2; v/v/v) and analysed in the positive ion mode using a capillary voltage of 1100 V and a cone voltage of 30 V to minimize in-source fragmentation. For the characterization of acidic oligosaccharides analytes were dissolved in methanol/water (50/50; v/v) and submitted to ionisation in the negative ion mode employing a capillary voltage of −1100 V and a cone voltage of 30 V. The source temperature was set to 80 °C and a desolvation gas (N_2) flow rate of 75 l/h was used. Homemade nanospray capillaries were used. For low-energy CID experiments, the glycan-derived precursor ions were selected in the quadrupole analyzer and fragmented in the collision cell using a collision gas (Ar) pressure of 3.0×10^{-5} mbar and collision energies of 15–40 eV (E_{lab}).

2.3.4. X-ray fluorescence analysis

Total reflection X-ray fluorescence (TXRF) was carried out at the Institute of Inorganic and Analytical Chemistry, University of Münster, Germany. 1.6 mg of Y2 in nitric acid 0.2% were analysed using