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Research article

Analysis of the carbohydrate-binding-module from *Fragaria x ananassa* α-L-arabinofuranosidase 1

I.N. Sin [a, 1], M.A. Perini [a, 1], G.A. Martínez [b, c], P.M. Civello [a, c, *]

[a] INFIVE (CONICET-UNLP), Instituto de Fisiología Vegetal, Diag. 113 n°495 - C.c 327, 1900, La Plata, Argentina
[b] IIB-INTECH (CONICET-UNSAM), Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús, Avenida Intendente Marino km 8,2, B7130IWA, Chascomús, Pcia. Buenos Aires, Argentina
[c] Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115, 1900, La Plata, Argentina

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ABSTRACT

α-L-arabinofuranosidases (EC 3.2.1.55) are enzymes involved in the catabolism of several cell-wall polysaccharides such as pectins and hemicelluloses, catalyzing the hydrolysis of terminal non-reducing α-L-arabinofuranosil residues. Bioinformatic analysis of the aminoacidic sequences of *Fragaria x ananassa* α-L-arabinofuranosidases predict a putative carbohydrate-binding-module of the family CBM_4_9, associated to a wide range of carbohydrate affinities. In this study, we report the characterization of the binding affinity profile to different cell wall polysaccharides of the putative CBM of α–L-arabinofuranosidase 1 from *Fragaria x ananassa* (CBM-FaARA1). The sequence encoding for the putative CBM was cloned and expressed in *Escherichia coli*, and the resultant recombinant protein was purified from inclusion bodies by a Nickel affinity chromatography under denaturing conditions. The refolded recombinant protein was then subjected to binding assays and affinity gel electrophoresis, which indicated its ability to bind cellulose and also high affinity for homogalacturonans.

1. Introduction

α-L-arabinofuranosidases (α-L-AFases, EC 3.2.1.55) are enzymes involved in the plant cell wall catabolism, catalyzing the hydrolysis of terminal non-reducing α-L-arabinofuranosil residues present in pectic and hemicellulosic polysaccharides (Minic and Jouanin, 2006; Chavez Montes et al., 2008). In *Arabidopsis thaliana* these enzymes have been reported to be expressed in zones of cell proliferation, the vascular system, developing and regressing floral tissues and floral abscission zones (Fulton and Cobbett, 2003). They have also been involved in the hydrolysis of the carbohydrate moieties of arabinogalactan proteins in immature radish seeds (Kotake et al., 2006). They are thought to play an important role during fruit ripening and softening, with the modification of pectin composition in the cell wall one of the major phenomena observed during this process (Tateishi, 2008).

Three α-L-AFases (FaARA1, FaARA2, FaARA3) have been identified in *Fragaria x ananassa* (Rosli et al., 2009). These genes revealed a fruit-predominant expression profile and the overall α-L-AFase activity increased according to fruit ontogeny, being significantly higher in strawberry cultivars showing lower fruit firmness (Rosli et al., 2009). This observation suggests not only a direct relationship between these enzymes and fruit cell wall modification during development and ripening, but also that pectin degradation and loss of neutral sugars probably contribute to the fruit softening. The contribution of pectin disassembly to fruit softening has been particularly pointed out in the case of strawberry fruit (Jiménez-Bermúdez et al., 2002; Molina-Hidalgo et al., 2013; Rosli et al., 2004; Villarreal et al., 2008). Moreover, in strawberries a significant loss of arabinose during fruit ripening has been reported (Heng Koh and Melton, 2002; Redgwell et al., 1997).

Generally, glycosyl hydrolases (GH) involved in plant cell wall metabolism present a complex molecular architecture composed of discrete modules, one catalytic (characteristic of the enzyme activity) and one or more Carbohydrate Binding Modules (CBMs) linked by an unstructured amino acid chain (Shoseyov et al., 2006). A CBM is a contiguous amino acid sequence within a protein active on carbohydrates (30–200 residues long), which presents an independent folding with carbohydrate binding activity (Shoseyov

* Corresponding author. INFIVE (CONICET-UNLP), Instituto de Fisiología Vegetal, Diag. 113 n°495 - C.c 327, 1900, La Plata, Argentina
E-mail address: pmcivello@agro.unlp.edu.ar (P.M. Civello).
[1] Authors contributed equally to this work.

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et al., 2006; Carvalho et al., 2015). It could be located at the protein C- or N- terminus, and occasionally at the center of the protein. Its presence increases the effective concentration of the catalytic module on the substrate surface, thus increasing the overall enzyme activity. In some cases, it could also prompt the substrate relaxation, facilitating the enzyme access (Boraston et al., 2004; Shoseyov et al., 2006).

There are 1444 sequences listed in the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org, Lombard et al., 2014) that correspond to the GH51 family (74 from plants), from which only 76 are reported as characterized. From those 76, only five protein sequences are from plants (*Arabidopsis thaliana*, *Hordeum vulgare* and *Oryza sativa* Japonica Group), but none of them has been studied from the carbohydrate binding perspective. There are a few reports of carbohydrate binding characterization of α-L-AFases that correspond to families GH43 and GH54, mostly from Bacteria and Fungi (Ichinose et al., 2008; Miyanaga et al., 2006, 2004; Ribeiro et al., 2010), but none from plants or corresponding to the GH51 family.

The purpose of the present work was to characterize the carbohydrate binding properties of the putative CBM in the *Fragaria x ananassa* α-L-arabinofuranosidase 1 protein (FaARA1), making a contribution to the knowledge of the α-L-AFases members of the GH51 family in an aspect that has not been thoroughly studied before. We describe the cloning, heterologous expression and purification of the putative CBM, as well as the characterization of the recombinant-proteins affinity to different cell wall polysaccharides.

2. Methods

2.1. In silico analysis of strawberry arabinofuranosidases CBMs

A conserved domains search was performed on the three FaARA proteins reported for *Fragaria x ananassa* (FaARA1, FaARA2 and FaARA3, accession numbers: ABV08815.1, ABV08816.1 and EF635630.1, respectively), using the NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer et al., 2015). Putative domains were delimited and analyzed by a sequence alignment performed with Clustal Omega software (Sievers et al., 2011). Alignment was edited using JalView 2 software (Waterhouse et al., 2009).

2.2. Molecular cloning and heterologous expression of CBM-FaARA1

The construct designed for the heterologous expression of the carbohydrate-binding module of the *Fragaria x ananassa* α-L-arabinofuranosidase 1 gene (CBM-FaARA1) in *Escherichia coli* was engineered using the GateWay® recombination technology (Invitrogen™). An "*in silico*" analysis was performed to delimit the possible region where the putative CBM could be found in the FaARA1 gene, using the NCBI conserved domain database. Then, a fragment of the FaARA1 gene encoding the CBM was amplified by PCR using specific primers and a plasmid containing the full length FaARA1 gene as template (Rosli et al., 2009). The primers used were: Forward, **5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTA**ATCGAGGGCAGAGTTGAAGTCCA AACAGCACAG-3′; reverse, **5′-GGGGACCACTTTGTACAAGAAAGCTGGGTA**TTAAT GTCCCTTGTACGTGTCCAAAG-3′ (Fig. 1). The recombination sites attB1 and attB2 are indicated in bold. A stop codon was added in the reverse primer (underlined). An "*Entry Clone*" was generated by a standard BP recombination reaction between the PCR product and the pDONR221 plasmid (Invitrogen™). The "*Expression Clone*" was built by an LR recombination between the Entry Clone and the pDES17 plasmid (Invitrogen™), according to the manufacturer specifications. BL21(DE3) *E. coli* cells transformed with the expression clone were cultured in 100 mL LB medium supplemented with ampicillin (100 μg mL^{-1}), with shaking (200 rpm) at 37 °C until $OD_{600\ nm}$ = 0.5; then IPTG was added (1 mM final concentration) to induce protein expression in the same culture condition for 4 h. Cells were harvested by centrifugation at 8000x *g* for 10 min at 4 °C, and the pellet stored at −80 °C until use.

2.3. Protein purification and refolding

The induced cell pellet was resuspended in buffer A (8.0 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 286.0 mM NaCl, 2.6 mM KCl, pH 7.4) supplemented with 0.05% (w/v) lysozyme, 2 mM PMSF (Phenylmethylsulfonyl fluoride), 0.1 mM DTT (1,4-Dithiothreitol) and 5 mM EDTA (Ethylenediaminetetraacetic acid), 30 mL of buffer A per 1 g of wet pellet. The suspension was incubated on ice for 30 min and then subjected to 3 sonication cycles on ice (1 min sonication, 1 min rest), with a Sonics® Vibra-Cell VCX130 PB sonicator set at 30% amplitude. The lysate was centrifuged at 10,000x *g* and 4 °C for 20 min. The recovered protein aggregates were denatured with buffer B (buffer A with 1% w/v SDS [Dodecyl sulfate sodium salt]) and further purified by Nickel affinity chromatography (NiAC) under denaturing conditions, following the protocol described by Schlager et al. (2012), with minor modifications. The chromatography was adapted to a 1 mL HisTrap HP (GE Healthcare) column and an Äkta Prime Plus (GE Healthcare) chromatography system, using buffer C (buffer A supplemented with 0.1% w/v sarkosyl [N-Dodecanoyl-N-methylglycine sodium salt]) as washing and equilibration buffer and buffer D (buffer C supplemented with 500 mM imidazole [1,3-Diaza-2,4-cyclopentadiene]) as elution buffer. The concentration of the purified eluted protein was determined by the Bradford method using bovine serum albumin (BSA) as standard (Bradford, 1976); the protein was then diluted to final concentration of 50 μg mL^{-1} with 20 mM Tris-HCl, 8 M Urea, pH 8.5 buffer. The diluted protein was refolded by an overnight dialysis at 4 °C, against 20 mM Tris-HCl buffer, pH 8.0.

2.4. SDS-PAGE and Western blot

The recombinant protein purification procedure was followed by SDS-PAGE and Western blot analysis. SDS-PAGE was carried out in 12% (w/v) polyacrylamide gels according to Laemmli (1970) using a Mini-PROTEAN Tetra Cell equipment (Bio-Rad). Gels were stained with silver nitrate according to Blum et al. (1987). Western blot was performed using Penta·His™ Antibody (QIAGEN) according to the manufacturer specifications.

2.5. Binding assays

The affinity of the recombinant CBM-FaARA1 to different polysaccharides was assessed according to Nardi et al. (2013), with modifications. The following polysaccharides were used as substrates: microcrystalline cellulose, oat xylan, calcium polygalacturonate and starch (included as a possible "no affinity control"). Calcium polygalacturonate was prepared prior to performing the binding assay by adding 20 μl of a 500 mM $CaCl_2$ solution to 500 μl suspension of 5 mg mL^{-1} polygalacturonic acid (PGA) in 50 mM sodium acetate/acetic acid buffer, pH 4.5. Mixtures were incubated at room temperature for 30 min and then the insoluble gel was separated from the suspension by centrifugation (2 min at 15,000x g).

All binding substrates were prewashed and equilibrated with 20 mM Tris-HCl buffer pH 8.0 before use. In the case of calcium polygalacturonate, the insoluble gels were also subjected to disruption until a homogeneous suspension was obtained.

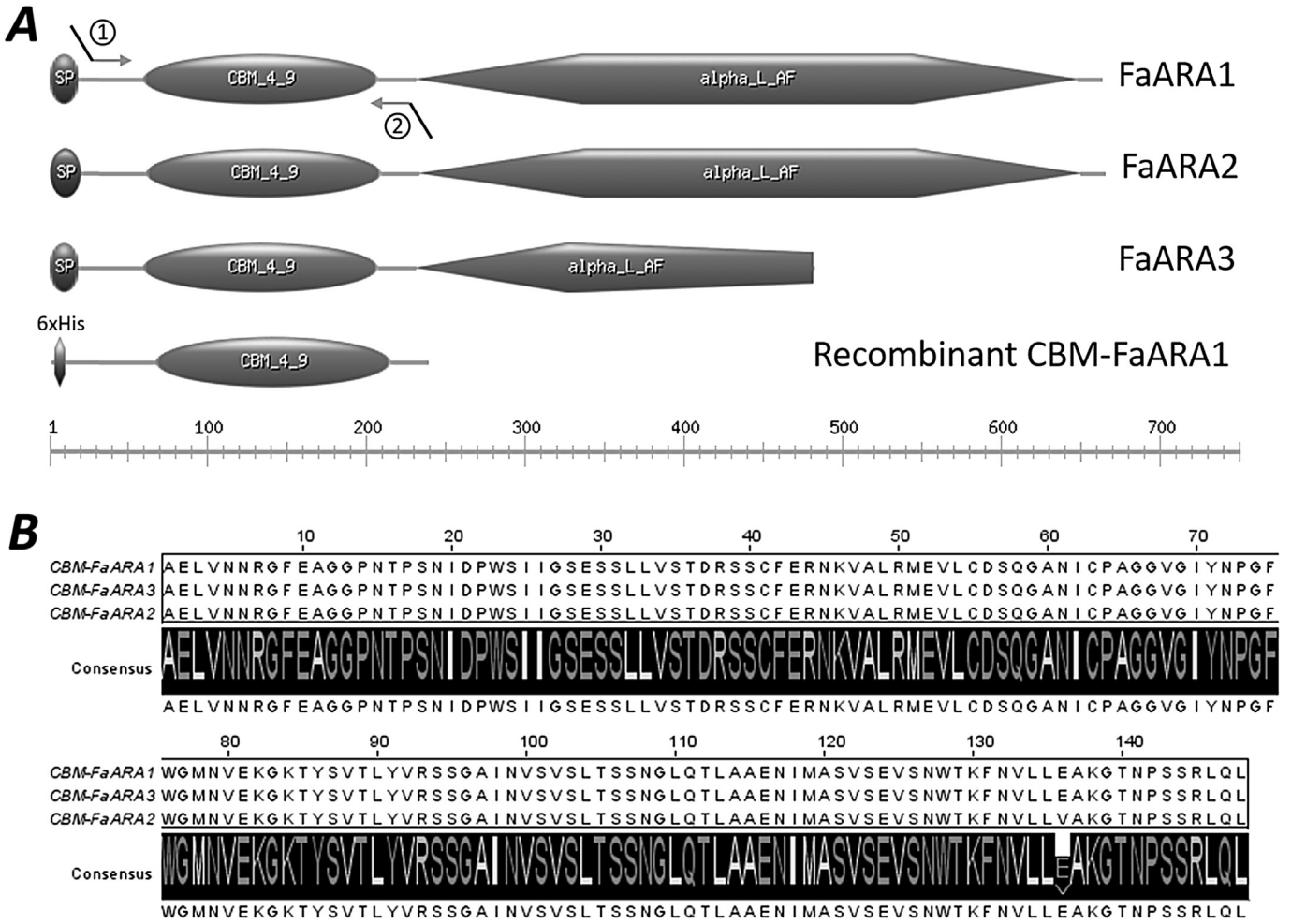

Fig. 1. A) Schematic representation of the three FaARAs and the recombinant CBM-FaARA1 protein. ***SP***: signal peptide; ***CBM_4_9***: carbohydrate binding module PF0218; ***Alpha-L-AF***: α-L-arabinofuranosidase domain; ***6xHis***: his tag for Ni-affinity purification; ***Scale***: protein chain length in amino acids. Arrows (1, 2) indicate annealing position of the primers used in the cloning of the putative CBM-FaARA1. *FaARA1*: SP, 1:18; CBM: 60–207; alpha-L-AF: 232–647; *FaARA2*: SP, 1:20; CBM, 62–209; alpha-L-AF, 234–649; *FaARA3*: SP, 1:18; CBM, 60–207; alpha-L-AF: 232–481. B) Sequence alignment of the three FaARAs putative CBM. Sequences encoding for the putative CBM were identified using the NBCI Conserved Domain Database. Alignment was performed with Clustal omega and edited with JalView 2.

Different amounts of the recombinant protein were added to 2.5 mg of substrate in a final volume of 0.5 mL (20 mM Tris-HCl buffer, pH 8.0). Reaction mixtures were incubated overnight at 4 °C with gentle shaking, and centrifuged at 15,000x *g* for 10 min. Free protein was determined on the supernatant and bound protein calculated subtracting it to the total protein added. To determine the latter, the experiment was repeated without carbohydrates. BSA was used as "non-carbohydrate-binding" control. Three replicates of each condition were performed.

Adsorption isotherms were constructed plotting bound protein (mg g^{-1}) against free protein (mg mL^{-1}), a linear regression from data was done, and the adsorption apparent constant (Kad) was calculated from the plot slope.

2.6. Affinity gel electrophoresis (AGE)

AGE was performed to make a qualitative assessment of CBM-FaARA1 binding affinities to carboxymethyl cellulose, citrus pectin, xylan and starch. The protocol used was adapted from the one described by Tomme et al. (2000). Native continuous PAGE was carried out at pH 8.7 in 6% (w/v) polyacrylamide gels polymerized in the absence (control) or presence of the carbohydrates at a final concentration of 0.1% (w/v). Electrophoresis was performed for 2 h at room temperature and a constant current of 15 mAmp per gel; three replicates for each condition. Carbonic anhydrase was used as "non-carbohydrate-binding" control. Gels were stained with silver nitrate to detect protein bands (Blum et al., 1987). The migrations of CBM-FaARA1 and Carbonic anhydrase were measured using *ImageJ* software (Schneider et al., 2012) and CBM-FaARA1 relative mobility to carbonic anhydrase calculated for each substrate.

2.7. Polygalacturonic acid hydrolysis and reducing sugar determination

PGA was subjected to a non-enzymatic hydrolysis according to Diaz et al. (2007), to obtain partially hydrolyzed fractions to be used in the protein stabilizations assays (see section 2.8). PGA solutions (1% w/v, pH 4.5) were heated at 95 °C for 1, 2, 3, 4 and 5 h, and then these hydrolyzed samples (hPGA 1 to hPGA 5) were ice cooled and stored at 4 °C until use.

Reducing sugar content was used as an estimate of PGA hydrolysis degree. It was determined according to the protocol described in Diaz et al. (2007); 100 μL of copper reagent (23.3% w/v NaCl, 5.4% sodium acetate, 0.5% $CuSO_4.5H_2O$, pH 4.8) were added to 100 μL of each hydrolysis sample. Mixes were incubated at 100 °C

for 15 min previous the addition of 0.8 mL of Folin-Ciocalteu reagent (diluted 1:40 with distilled water), and then the absorbance at 750 nm was measured. A calibration curve was made using galacturonic acid (GA) as standard, and the reducing sugar content of the samples was calculated as nmol of GA per μL.

2.8. Protein stabilization assay

A stock solution of recombinant CBM-FaARA1 protein purified under denaturing conditions (920 μg mL^{-1}) was subjected to 1:10 direct dilution in 20 mM Tris-HCl buffer pH 8.0 or 50 mM sodium acetate/acetic acid buffer pH 4.5 in the presence of PGA (0.5% w/v final concentration). The same dilution was carried out at pH 4.5 in the presence of the different PGA hydrolyzed fractions (hPGA 1 to hPGA 5, 5% w/v final concentration) or GA monomer (5% w/v final concentration). Mixes were incubated for 30 min at room temperature (time for protein aggregation to occur) and then centrifuged for 2 min at 15,000x *g*, to separate possible protein aggregates. Finally, protein concentration was determined in the supernatant by the Bradford method (Bradford, 1976). A direct dilution of the protein stock maintaining the chromatography denaturing conditions was used as "*no aggregation control*" (0% protein loss) and the percentage of protein loss after the renaturing process was calculated for each condition assayed. Direct dilutions with no stabilizing agent were carried out at pH 8.0 (mock pH 8.0) and pH 4.5 (mock pH 4.5) as "*no stabilization controls*". Three replicates of each condition were performed.

2.9. Protein quantification

All proteins concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

2.10. Data analysis

Data from binding assays were analyzed by ANOVA to evaluate the overall fit goodness (F test) of linear regressions, and t-tests were used to determine the statistical relevance of the calculated slopes ($p < 0.05$). In the case of AGE, data were subjected to an ANOVA analysis and means were compared by Tukey test ($p < 0.05$).

3. Results

3.1. In silico analysis of FaARA proteins

The NCBI Conserved Domain Database was used to predict putative domains in the three FaARAs protein sequences described for *Fragaria x ananassa* (Rosli et al., 2009). It was found that the three proteins present the characteristic architecture of the α-L-AFases of the GH51 family, consisting in a Signal Peptide at the protein N-terminus, followed by a putative CBM and an α-L-AF catalytic domain at the protein C-terminus (Fig. 1A). Interestingly, the FaARA3 gene presents an early stop codon, which would result in a truncated protein with a shortened α-L-AFase catalytic domain (Fig. 1A). A multiple alignment revealed a high similitude between the three proteins, being between 97% and 98% identity among the full length protein sequences (Supplementary Fig. 1).

When comparing the three putative CBMs found in the FaARA proteins there is above 99% identity among them. CBM-ARA1 is 100.0% identical to CBM-FaARA3, whereas FaARA2 turns out to be 99.3% identical to the other two, presenting a mutation in the position 140 where a valine is replaced by a glutamic acid residue (Fig. 1B).

3.2. Cloning, expression and purification of CBM-FaARA1

Because of the high similitude percentage between the amino acid sequences of the putative CBMs predicted on the three FaARA proteins, only the one found in the FaARA1 gene was selected to be cloned. Two constructs were generated as described in methods; an "*Entry clone*", pDONR221[CBM-FaARA1] and an "*Expression clone*", pDEST17[CBM-FaARA1], that encodes for a recombinant CBM-FaARA1 protein with a 6xHis tag at its N-Terminus for protein purification by Nickel affinity chromatography (Fig. 1A). The latter was used to transform *E. coli* BL21(DE3) competent cells and carry out the heterologous expression. Protein expressed in *E. coli* cells as protein aggregates in the form of inclusion bodies (Fig. 2). These were isolated, denatured and subjected to a Nickel affinity chromatography under denaturing conditions. Recombinant CBM-FaARA1 eluted as a single peak at 40% of the elution buffer (buffer D) gradient, which corresponds to a 200 mM Imidazole concentration (Fig. 3). The denatured purified protein proved to be prone to aggregation during refolding, making it difficult to obtain soluble protein after the process. Initial protein concentration as well as buffer pH turned out to be crucial, being particularly difficult to obtain soluble protein when the process was carried out at a protein concentration higher than 50 μg mL^{-1}, and pH lower than 8.0.

Different protein renaturing techniques such as direct dilution, dialysis and on column refolding, as well as different stabilizing agents such as glycerol, sucrose and urea, were assayed in order to reduce protein loss. The best results were obtained when the denatured purified protein was first diluted to 50 μg mL^{-1} with an 8.0 M urea containing buffer (pH 8.5) followed by dialysis to eliminate denaturing agents (pH 8.0). After this procedure, soluble recombinant CBM-FaARA1 protein could be obtained at a concentration of 50 μg mL^{-1}.

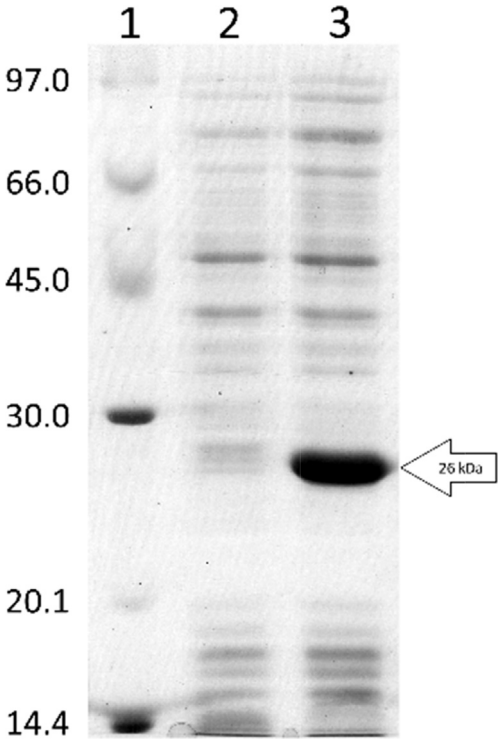

Fig. 2. Solubility assessment by SDS-PAGE. 1) MW Ladder; 2) soluble protein of induced *E. coli* cells harboring the expression clone; 3) total protein of induced *E. coli* cells harboring the expression clone. Recombinant CBM-FaARA1 band is indicated by the arrow.

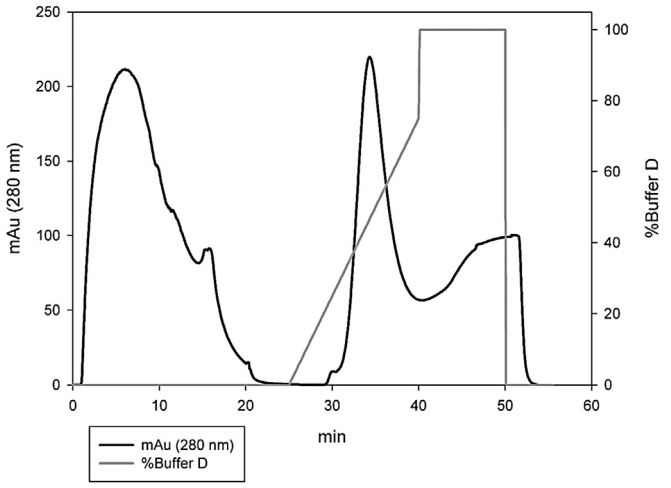

Fig. 3. Nickel affinity chromatography (*NiAC*). Black curve represents the absorbance at 280 nm. Grey curve represents chromatography elution gradient (% Buffer D). Flow through: 0–20 min; CBM-FaARA1 elution: 32–40 min. Washing and equilibration buffer (buffer C): 8.0 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 286.0 mM NaCl, 2.6 mM KCl, 0.1% w/v sarkosyl, pH 7.4. Elution Buffer: buffer C supplemented with 500 mM imidazole.

Protein expression and purification scheme were followed by SDS-PAGE and the recombinant protein identified by western blot (Fig. 4).

3.3. Binding assays

The binding assays reported here were used to assess the affinity of the recombinant CBM-FaARA1 protein to different insoluble carbohydrates and by doing so, validate the NCBI-CDD prediction of the presence of a putative CBM in the FaARA1 protein. Binding properties of the recombinant CBM-FaARA1 were tested against three cell wall polysaccharides (homogalacturonan [PGA], microcrystalline cellulose and oat xylan) and starch, which was included as a possible "no affinity control" substrate. Bovine serum albumin (BSA) was used as a "non-carbohydrate-binding" control for every substrate assayed. An absorption isotherm was constructed and linear regressions made for each protein/substrate combination (Fig. 5). ANOVA analysis was performed to evaluate the overall fit goodness (F test) and t-tests were used to determine the statistical relevance of the calculated slopes.

Absorption isotherms for CBM-FaARA1 protein with homogalacturonan or microcrystalline cellulose as substrates showed linear responses that could be fitted by statistically relevant simple linear regressions. The difference between the calculated slopes was also statistically relevant, showing that the recombinant CBM-FaARA1 protein has higher affinity to homogalacturan (K_{ad} = 795.41 mL g^{-1}) than to microcrystalline cellulose (K_{ad} = 17.93 mL g^{-1}). The scatter plots corresponding to the

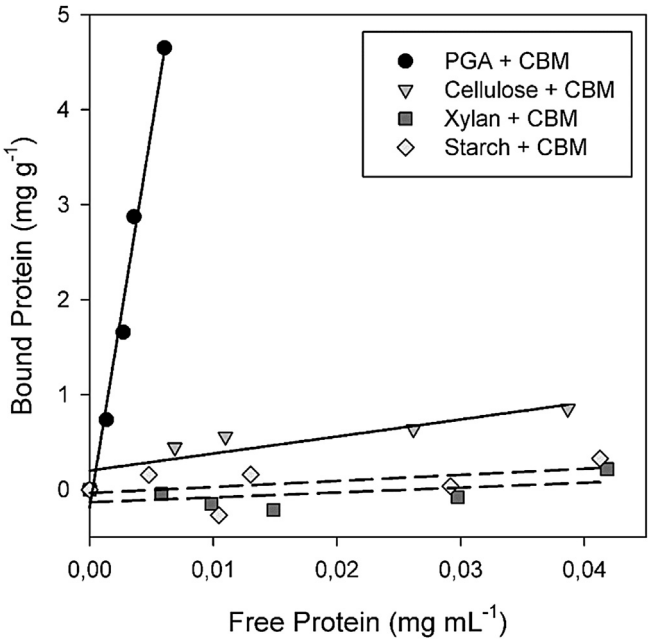

Fig. 5. Adsorption isotherms of purified CBM-FaARA1 to: starch, PGA, oat xylan and microcrystalline cellulose. Linear adsorption isotherms (full lines) indicate the apparent equilibrium distribution of CBM-FaARA1 between the solid phase (bound protein) and liquid phase (free protein) at various protein concentrations; each isotherm slope represents a Kad value. PGA + CBM: y = 795.41 x − 0.19, R^2 = 0,9854; Cellulose + CBM: y = 17.93 + 0.20, R^2 = 07,906.

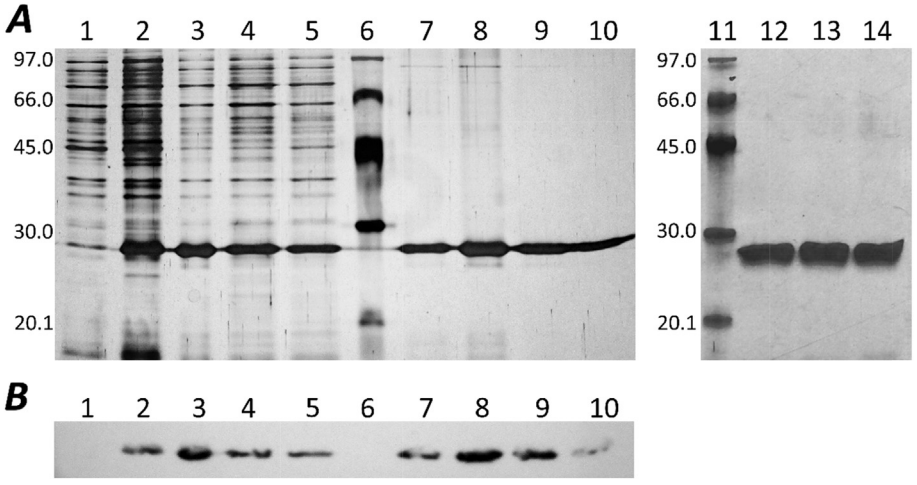

Fig. 4. (A) Recombinant protein expression and purification followed by SDS-PAGE (silver nitrate staining) and (B) Western blot using Penta·His™ Antibody, QIAGEN. 1) Non-induced *E. coli* cells; 2) Induced *E. coli* cells; 3) Denatured pre-isolated inclusion bodies; 4) NiAC Flow through 1; 5) NiAC Flow through 2; 6) and 11) MW ladder (20.1–97 kDa); 7) NiAC elution (fraction 1); 8) NiAC elution (fraction 2); 9) NiAC elution (fraction 3); 10) NiAC elution (fraction 4); 12–14) Independent replicates of refolded recombinant protein.

adsorption isotherms of the recombinant protein to oat xylan and starch as substrates presented and erratic behavior around 0 mg of bound protein per g of substrates, indicating that the protein binding to these substrates was too low be accurately measured. Moreover, no statistical relevance was obtained when trying to fit these plots by linear regressions.

For all BSA adsorption isotherms, data presented an erratic behavior around the zero value for bound protein (data not shown) and could not be fitted by linear regressions, also indicating a too low protein binding to be accurately measured by the procedures here described.

3.4. Affinity gel electrophoresis (AGE)

AGE is commonly used to evaluate protein affinity to a soluble substrate. In this case we used it to determine whether CBM-FaARA1 had the ability to bind to carboxymethyl cellulose, oat xylan, citrus pectins and soluble starch. Carbonic anhydrase was used as an internal reference, corresponding to a protein whose mobility is not affected by the carbohydrate presence. CBM-FaARA1 showed a significantly lower relative mobility when carboxymethyl cellulose or citrus pectin was immobilized in the gels, while the protein relative mobility was not affected by the presence of oat xylan or starch. No significant differences were found when comparing CBM-FaARA1 relative mobility on gels without carbohydrates (control) or containing oat xylan or starch (Fig. 6).

3.5. Protein stabilization

As mentioned above, recombinant CBM-FaARA1 proved to be prone to aggregation during the renaturing process, making it difficult to obtain soluble protein at higher concentration than 50 μg mL^{-1} at pH 8.0. The aggregation phenomenon was also observed when stable refolded protein was dialyzed to exchange the solution pH from 8.0 to 4.5.

Due to the high affinity to PGA showed by the recombinant CBM-FaARA1, this carbohydrate was assessed as a possible stabilizing agent for the protein in the refolding process. Direct dilution was the technique of choice for this assay for its simplicity and for being a fast protein renaturing procedure (Eiberle and Jungbauer, 2010; Gautam, 2012). No significant protein loss was observed at pH 8.0 or 4.5 when PGA 0.5% w/v was present in the refolding buffer, in comparison with a no-aggregation control (protein diluted under denaturing conditions; Fig. 7).

In order to assess if PGA size was involved in the protein-stabilizing phenomenon, the carbohydrate was subjected to chemical hydrolysis for 5 h prior its use as stabilizing agent, taking samples every hour (hPGA 1 to hPGA 5 fractions). The hydrolysis was followed measuring the reducing sugar content on every hydrolyzed sample and plotting the values expressed as nmol of GA per micro liter (nmol μL^{-1}) against the time of hydrolysis (Supplementary Fig. 2). The process showed a linear response, with almost one polymer bond breakage per hour (Supplementary Fig. 2).

The five different PGA hydrolyzed fractions were assayed as stabilizing agents at pH 4.5, as well as GA monomer representative of a complete PGA hydrolyzed sample. No significant soluble protein loss was observed when the first three hydrolysates (hPGA 1 to hPGA 3) were present in the refolding solution. Protein loss due to protein aggregation increased significantly when PGA was hydrolyzed for more than 3 h (hPGA 4, 33% protein loss; hPGA 5, 64% protein loss), being the protein loss observed with hPGA 5 comparable with the one observed with the pH 8.0 mock. Maximum protein loss was obtained when GA or pH 4.5 mock were added to the refolding buffer (Fig. 7).

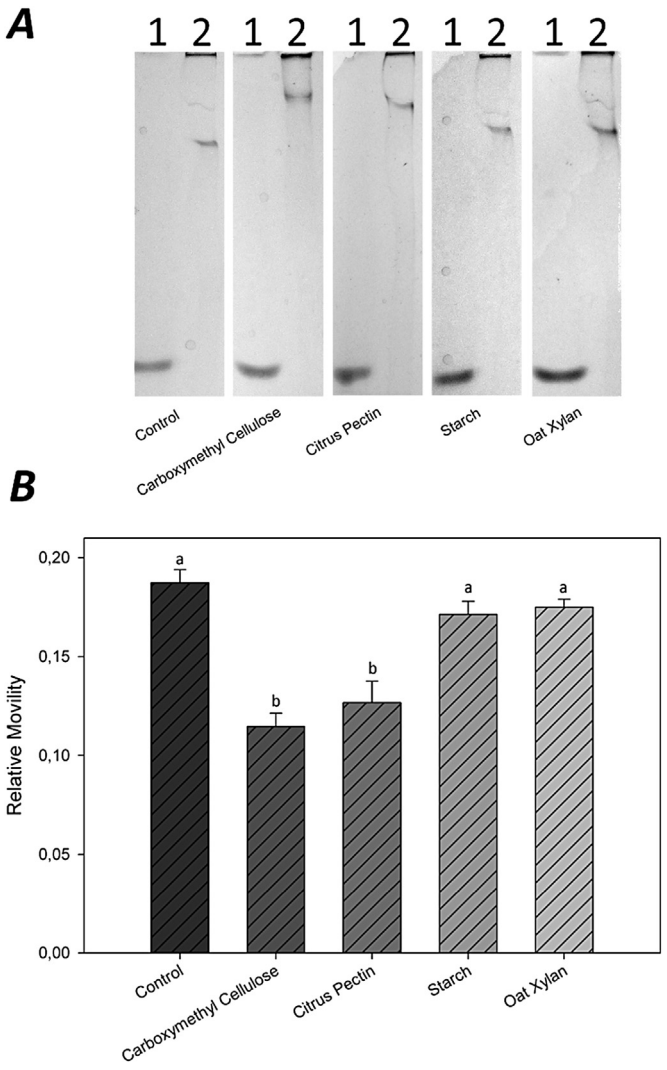

Fig. 6. A) Examples of affinity gel electrophoresis of CBM-FaARA1 against different polysaccharides. 1. Carbonic anhydrase; 2. CBM-FaARA1. B) Relative mobility of CBM-FaARA1 to Carbonic Anhydrase. Data were subjected to ANOVA analysis and means were compared by Tukey Test ($p < 0.05$). Different letters indicate significant difference.

4. Discussion

α-L-arabinofuranosidases (EC 3.2.1.55) are enzymes that have been reported to play important roles in plant development (Fulton and Cobbett, 2003) and in fruit ontogeny and softening (Tateishi, 2008; Sozzi et al., 2002). They are found in many GH families (2, 3, 43, 51, 54, 62) and have been associated to different CBM families (1, 2, 3, 6, 13, 22, 35, 42 and yet non classified {NC}) according to the CAZy database (Lombard et al., 2014). Within this diversity a wide range of carbohydrates affinities could be found (cellulose, xylan, arabinoxylan, arabinose, arabinans, arabinose) (Ichinose et al., 2008; Miyanaga et al., 2006, 2004; Ribeiro et al., 2010).

Three alpha-L-AFases from *Fragaria x ananassa* have been reported (Rosli et al., 2009) and enzyme activities and expression profiles during fruit ontogeny have been described. To our knowledge, this is the first report describing the properties of a CBM from a plant α-L-AFase, and also the first one describing a CBM belonging to the GH51 family. The recombinant protein was predicted to encode a putative CBM corresponding to the CBM_4_9 family (PF02018). To the date, there are 93 sequences uploaded in the

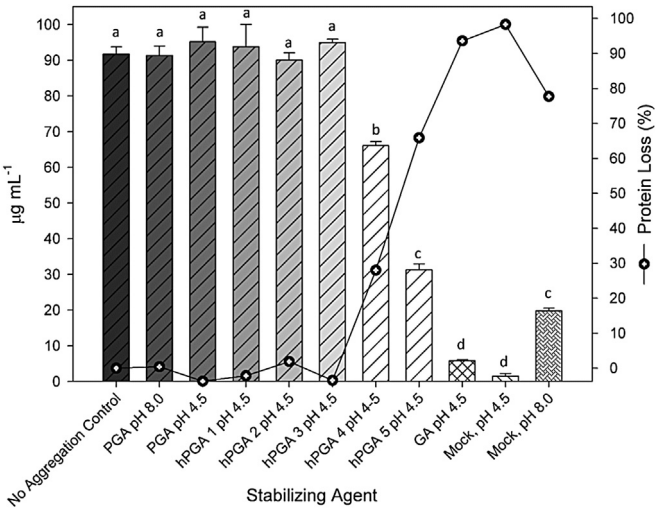

Fig. 7. Protein stabilization during refolding in the presence of different stabilizing agents. PGA and its hydrolyzed fractions (hPGA 1 to hPGA 5) were assayed as stabilizing agents, as well as GA monomer. Vertical bars correspond to soluble protein mean concentration (µg mL^{-1}) after refolding. Error bars indicate Standard error. Circular points represent protein loss (%) after refolding for each condition assayed. Data were subjected to an ANOVA analysis and means were compared with Tukey test. Different letters indicate significant differences ($p < 0.05$).

PF02018 family that correspond to GH51 α-L-AFases architecture, from which 5.4% (5 sequences) corresponds to Eukaryotes, whereas the other 94.6% (88 sequences) are from bacterial proteins. Regardless of their origin, most CBM identification in these proteins has been performed by sequence similarity rather than actual characterization.

We successfully cloned and expressed the recombinant CBM-FaARA1 in *E. coli* cells. As stated above, recombinant protein expressed in *E. coli* as insoluble protein aggregates in the form of inclusion bodies, thus necessitating a denaturing step prior to protein purification and refolding processes. We successfully adapted the protocol described by Schlager et al. (2012) to the purification of the recombinant protein, with the difference that an isolation of the protein aggregates was carried out prior the chromatography. This pre-isolation step proved to improve the protein purity after the affinity chromatography (data not shown). The purified denatured protein proved to be prone to aggregation, a frequent phenomenon observed when working with *E. coli* recombinant proteins expressed as inclusion bodies (Gautam, 2012; Yamaguchi et al., 2013). It was difficult to obtain soluble protein when the renaturing process was carried out at higher concentration than 50 µg mL^{-1} at pH 8.0. The same phenomenon was observed when stable refolded protein was dialyzed to exchange the solution pH from 8.0 to 4.5. Dialysis turned out to be the best choice as a renaturing technique, but prior protein conditioning was necessary for a successful refolding process. Protein denaturing condition had to be exchanged from the affinity chromatography elution buffer composition to 8 M Urea buffer in order to induce the dissociation of the detergent molecules from the recombinant protein and also achieve a protein concentration compatible with refolding (50 µg mL^{-1}). This unstable characteristic of the recombinant protein was the main reason why binding assays were carried out at pH 8.0 and 50 µg mL^{-1} as the maximum protein concentration.

FaARA1 protein has a pI of 5.0 and is predicted to be secreted to the apoplast where physiological pH is 4.5. In this condition, native FaARA1 protein as well as recombinant CBM-FaARA1 (pI 6.52) would be positively charged. The opposite situation occurs at pH 8.0, the conditions corresponding to the binding assay. There are some other reports of binding assays carried out at alkaline pH (Georgelis et al., 2011). Also, it has been suggested that a CBM's binding capacity could be attributed, at least in part, to several aromatic residues that constitute the hydrophobic module surface (Shoseyov et al., 2006), being then hydrophobic interaction one of the forces responsible for the association of a CBM to its substrate.

The results of binding assays show that the recombinant CBM-FaARA1 protein has high affinity to homogalacturonan; nonetheless, it was observed that the protein has also the capacity to bind microcrystalline cellulose with a significantly lower affinity (Fig. 5). This phenomenon suggests that the CBM present in FaARA1 would be to some extent promiscuous, being able to recognize more than one substrate. This substrate promiscuity phenomenon has been reported for other CBMs (Nardi et al., 2013; Obembe et al., 2007). There are terminal arabinofuranose residues on some of the side-groups of rhamnogalacturonan II and this carbohydrate has a homogalacturonan backbone. There are arguments suggesting that the pectin polysaccharides are all associated in a complex homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II pectin assemblage (Vincken et al., 2003). It is possible then that an α−L-arabinofuranosidase association with homogalacturonan might provide access to terminal arabinofuranose residues associated with various other wall polysaccharides or glycoproteins.

A typical adsorption isotherm shows a linear behavior at low protein concentrations and a "plateau" once the substrate becomes saturated. In our case, the maximum protein concentration used in binding assays was probably too low to achieve substrate saturation.

The analysis of relative mobility performed on gels containing different polysaccharides contributed to our characterization of the protein's carbohydrate affinities and these data are in agreement with conclusions based on the binding assays. These data also reveal that the recombinant CBM appears to be able to bind both soluble and insoluble forms of carbohydrates.

Due to the high affinity of the recombinant protein to homogalacturan we decided to test if this carbohydrate could act as a stabilizing agent in the protein refolding process. The addition to the refolding buffer of any substance that stabilizes the native form of protein, such as cofactors or even an appropriate substrate, is a very common approach to circumvent the difficulties of protein aggregation during a renaturing process (Eiberle and Jungbauer, 2010; Gautam, 2012). Homogalacturonan proved to be a suitable stabilization agent, allowing us to perform refolding by direct dilution even at pH 4.5 and at as high protein concentration as 100 µg mL^{-1} without significant loss. At this pH a 60-fold increase in soluble protein is observed when using homogalacturonan as stabilizing agent, compared to the same condition without the carbohydrate. Alkaline or acidic pHs seem not to interfere with the protein stabilization in the presence of homogalacturonan, evidence that the protein charge would not be relevant to the stabilization process. This could also indicate that the recombinant protein binding capacity to homogalacturonan is not subjected to the protein charge. The first three homogalacturonan hydrolyzed fractions (hPGA 1 to hPGA 3) also proved to be suitable stabilizing agents, but when homogalacturonan hydrolysis went beyond 3 h (hPGA 4 and hPGA 5) the stabilizing effect was gradually lost until no stabilization was observed when using monomer GA as an example of a PGA complete hydrolysis. The fact that the phenomenon is lost when homogalacturonan is hydrolyzed suggests that it is size dependent and that a minimum homogalacturonan size is needed for stabilization. The latter could also indicate that there is a minimum structure required for the CBM to recognize and bind its substrate. Taking into account the homogalacturonan capacity to bind and stabilize the recombinant CMB-FaARA1 "*in vitro*", it would

be interesting to analyze a possible "*in vivo*" role of these polysaccharides in the stabilization of α-L-AFases in the cell wall.

5. Conclusion

A region of the FaARA1 gene was successfully expressed in *E. coli* BL21(DE3) cells, and purified to apparent SDS-PAGE homogeneity by Ni-affinity chromatography under denaturing conditions.

The cloned fragment was predicted to encode a CBM corresponding to the CBM_4_9 superfamily (PF02018). Our results show that the recombinant protein has a low affinity to cellulose and a strong affinity to homogalacturonans, being the latter a suitable agent for the protein stabilization.

Contributions

Ignacio Sin and Mauro Perini performed all the experimental work, and participated actively of the data analysis, discussion and the manuscript. Both authors contributed equally to this work.

Dr. Gustavo Martínez and Dr. Marcos Civello direct the research project, designed the experimental approach, and participated actively of data analysis, discussion and the manuscript writing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.05.028.

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