# Three novel rhamnogalacturonan I- pectins degrading enzymes from Aspergillus aculeatinus: Biochemical characterization and application potential

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# Three novel rhamnogalacturonan I- pectins degrading enzymes from *Aspergillus aculeatinus*: biochemical characterization and application potential

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**ABSTRACT (149 words)**

Rhamnogalaturonans I (RGI) pectins, which are a major component of the plant primary cell wall, can be recalcitrant to digestion by commercial enzymatic cocktails, in particular during fruit juice clarification process. To overcome these problems and get better insights into RGI degradation, three RGI degrading enzymes (RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase) from *Aspergillus aculeatinus* were expressed in *Pichia pastoris*, purified and fully biochemically characterized. All three enzymes showed acidic pH optimum, and temperature optima between 40 to 50 °C. The *Km* values were 0.5 $mg.ml^{-1}$, 1.64 $mg.ml^{-1}$ and 3.72 $mg.ml^{-1}$ for RHG, ABF, GAN, respectively. NMR analysis confirmed an endo-acting mode of action for RHG and GAN, and exo-acting mode for ABF. The application potential of these enzymes was assessed by measuring changes in viscosity of RGI-rich camelina mucilage, showing that RHG-GAN enzymes induced a decrease in viscosity by altering the structures of the RGI backbone and sidechains.

**KEY WORDS**

RGIases, *Aspergillus aculeatinus*, camelina mucilage, RGI-type pectins

## INTRODUCTION (7701words)

In plants, pectin is a heteropolysaccharide mainly present in the middle lamella and primary cell wall, and is deposited during early stages of growth and cell expansion (Voragen, Coenen, Verhoef & Schols, 2009). Four pectic domains can be described: Homogalacturonan (HG), Rhamnogalacturonan type I (RGI), Rhamnogalacturonan type II (RGII) and Xylogalacturonan (XGA) (Mohnen, 2008). RGI, which is the second most abundant and complex polysaccharide in the primary cell wall (i.e. 20-35% of pectins) consists of a backbone composed of the repeating diglycosyl units (→2)-α-L-Rhap-(1→4)-α-D-GalpA-(1→), partly substituted, at *O*-4 and/or *O*-3 positions of α-L-Rhap residues, with side chains of (1→5)-α-L-arabinans, (1→4)-β-D-galactans, arabinogalactans I (AGI), arabinogalactans-II (AG II), and possibly galactoarabinans (GAs) (Silva, Jers, Meyer & Mikkelsen 2016; Yapo, 2011).

To effectively degrade this complex network, one of the most important group of enzymes used in fruit and vegetable processing industry is pectinases, and in particular microbial-derived RGI-degrading enzymes (RGIases), which lowers viscosity, allows efficient mash pressing during juice clarification process and remove the mucilage coat, for instance in coffee beans. (Grassin & Fauquembergue, 1996; Tapre & Jain, 2014, Kashyap, Vohra, Chopra & Tewari, 2001). A number of RGIases have been described from *Aspergillus aculeatus*, due to its fermentation capabilities and high level of protein secretion (De Vries, Benen, de Graaff & Visser 2002; Rytioja et al., 2014). Genome sequencing among species in the *Aspergillus* genus combined to a phylogenetic and phenotypic approaches, showed that there are closely related novel species including *A. aculeatinus*, which was used in this study. It has an extensive and highly conserved set of genes encoding RGIases (Vesth et al., 2018), and therefore, considerable potential to secrete cell wall degrading enzymes.

The degradation of RGI backbone is realized by RGIases specific for cleaving bonds in the repetitive (→2)-α-L-Rhap-(1→4)-α-D-GalpA-(1→) disaccharidic units (Silva et al., 2016). Endo-rhamnogalacturonase, RHG (GH28 - EC 3.2.1.171), catalyze the hydrolysis of the α-D-GalpA-(1→2)-α-L-Rhap glycosidic linkages, releasing the non-reducing end of Rha*p* (Suykerbuyk et al., 1997). The first discovered endo-rhamnogalacturonase enzyme was the RhgA from *A. aculeatus*, and it is one of the more extensively characterized RGI modifying enzymes (Kofod et al., 1994). Besides, accessory RGIases, which catalyze the hydrolysis of internal or

terminal linkages of the arabinan and galactan sidechains (Silva et al., 2016) can also be found. For example, α-Arabinofuranosidase, ABF (GH54 - EC 3.2.1.55) can remove terminal non-reducing arabinose residues on arabinans and on the short side chains of arabinogalactans (Chacón-Martínez et al., 2004; de Wet, Matthew, Storbeck, van Zyl & Prior 2008; Miyanaga et al., 2006). Several α-Arabinofuranosidases have been purified from *A. niger*, and only one from *A. aculeatus*, reporting an activity on α-(1→2), α-(1→3) and α-(1→5) linked arabinose residues (Beldman, Searle-van Leeuwen, De Ruiter, Siliha, & Voragen 1993; de Vries & Visser, 2001; Saha, 2000). Among accessory RGIases, arabinogalactanases, GAN (GH53 - EC 3.2.1.89), catalyze the degradation of the galactan side chains at β-(1→4) linkages and galactanases, GAL (GH35 - EC 3.2.1.-) at β-(1→3) and β-(1→6) linkages (Le Nours et al., 2003; Silva et al., 2016). So far, the majority of arabinogalactanases from *A. aculeatus* are endo-β-1,4-galactanase (Christgau, Sandal, Kofod, & Dalbøge,1995; Le Nours et al., 2003; Ryttersgaard, Lo Leggio, Coutinho, Henrissat, Larsen, 2002; Torpenholt, Poulsen, Muderspach, De Maria & Lo Leggio, 2019). To assess the application potential of these enzymes, one can follow the changes in viscosity of RGI-rich pectic matrix upon their application. If pectins from fruits can be, to some extent, rich in RGI, it presents as well some HG domains which can interfere in the viscosity assay (Wefers, Flörchinger, & Bunzel, 2018; Arnous & Meyer, 2009). In contrast, seed coat mucilage appears as a substrate of interest to test the effects of the enzymes, considering its polysaccharide composition, and its use as hydrocolloid in the pharmaceutical and food industry (Ubeyitogullari and Ciftci, 2020). The seed coat mucilage composition appears highly variable, depending upon species. In Arabidopsis it is mainly composed of unbranched RG-I, while in *Plantago ovata* the predominant polysaccharide is arabinoxylan (Macquet, Ralet, Kronenberger, Marion-Poll, & North, 2007; Guo, Cui, Wang, & Young, 2008). Camelina sativa, a Brassicaceae, has received considerable interest for its potential use (Malik, Tang, Sharma, Burkitt, Ji, Mykytyshyn et al., 2018) in a large panel of applications. Camelina mucilage is rich in RG-I, even though its fine chemical composition remains unresolved. The rheological properties of the polysaccharide could for instance change by the presence or absence of neutral side chains.

The aims of this study are to 1) to produce three *A. aculeatinus* RG-I-active enzyme by recombinant expression in *Pichia pastoris* and determine their

biochemical properties, 2) to isolate Camelina seed coat mucilage and characterize its RG-I content and 3) to apply the enzymes (singularly and in combination) to establish their ability to modify (decrease) viscosity of Camelina RG-I mucilage. Our hypothesis is that the combined action by the recombinant *A. aculeat*inus RG-I enzymes can significantly reduce viscosity of Camelina mucilage.

## MATERIALS & METHODS

### Commercial RGI substrates

Pectic galactan - P-PGAPT, Arabino galactan - P-ARGAL, Arabinan - P-ARAB, debranched Arabinan - P-DBAR, azurine-cross-linked Rhamnogalacturonan I - I-AZRHI and soybean Rhamnogalacturonan - P-RHAGN substrates were purchased from Megazyme® (Bray,Co. Wicklow, Ireland).

### *Aspergillus aculeatinus* culture and cDNA obtention

*Aspergillus aculeatinus* SCCO822 strain was kindly provided by Soufflet Research & Innovation Centre (CRIS, Nogent-sur-Seine, France). *Aspergillus aculeatinus* strain was cultivated in M3 medium (Mitchell, Vogel, Weimann, Pasamontes & van Loon, 1997) with different carbon sources (rich and poor RGI medium) in order to stimulate RGase expression (**Table S1)**. After 14 days of culture at 27 °C, mycelium was harvested and ground in liquid nitrogen with a mortar and pestle. Total RNAs isolation was performed with RNA easy plant mini kit (Qiagen,Hilden, Germany). Freshly extracted RNAs (14 ng.µl$^{-1}$) were treated with TURBO DNA-free Kit (Invitrogen, Carlsbad, California, United States) and cDNA was synthesized with Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) using anchored-oligo(dT)18 primer.

### Cloning and heterologous expression of RGase

*A. aculeatinus (*Genome reference: NW_020291493.1*)* endo-rhamnogalacturonase, RHG (GH28 - EC 3.2.1.171) (GenBank Gene ID: 37148665), endo-β-1,4-galactanase, GAN ( GH53 - EC 3.2.1.89) (GenBank Gene ID: 37150470) and α-Arabinofuranosidase, ABF (GH54 - EC 3.2.1.55) (GenBank Gene ID: 37152657) were amplified from *A. aculeatinus* cDNAs. Cloning primers used are listed in **Table 1**. ORFs from RHG and ABF were cloned into pPICZαB, including

their signal peptide, with the use of BstBI (to remove α-factor from vector) and NotI restriction enzymes (New England Biolabs, Hitchin, UK). As GAN ORF contain a BstBI restriction site, it was not possible to clone it using the same procedure. GAN ORF was cloned downstream of pPICZαB α-factor with EcoRI and NotI restriction enzymes (New England Biolabs, Hitchin, UK). The three sequences were cloned in frame with polyhistidine tag. *Pichia pastoris* (X-33) cells were used as host cells for heterologous expression and were transformed as detailed in the EasySelect Pichia Expression Kit manual (Invitrogen, Carlsbad, California, United States).

**Table 1.** Primers used for cloning *Aspergillus aculeatinus* RGIases into pPICzαB expression vectors. RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

| Enzyme | Gene ID | Forward | Reverse |
|---|---|---|---|
| RHG | 37148665 | AATTTCGAAACGATGATGCGTGCTCTTTTCCTTCTTG | TGCACGCGGCCGCGCCTGCCAAGGCACTGTAC |
| GAN | 37150470 | TCTAAGAATTCACGCGCTCACCTATCGCGG | TGCACGCGGCCGCGAGCTCCCCGAGAGTCTCGA |
| ABF | 37152657 | AATTTCGAAACGATGATGCCTTCACGACGAACCCTC | TGCACGCGGCCGCCGCGGACGCAAAGCCC |

## Recombinant protein expression and purification

Recombinant RGIases were produced and purified by affinity chromatography as described in Voxeur et al., 2019. Briefly, transformed *P. pastoris* cells were grown on buffered glycerol-complex medium at 30°C in baffled flasks before the induction step, which begins with the transfer of the cells into a medium without glycerol but with 0.5% methanol. During 72h, the medium is kept at this concentration by daily addition of methanol. Supernatants are recovered after centrifugation (1 500×g, 8 min, 4 °C) and filtered with GD/X 0.45 µm PES filter Media (Whatman, Maidstone, United Kingdom). Purification is performed by affinity chromatography (IMAC) with a 1 ml HisTrap excel column (GE Healthcare, Chicago, Illinois, United States). Every buffer and supernatant were kept on ice during purification. 100 ml supernatant was loaded onto the column at a 1 $ml.min^{-1}$ flow rate. HisTrap column was washed with 10 column volumes of wash buffer (25 mM imidazole, 250 mM NaCl, 50 mM NaP pH 7,5). Target protein was recovered with 15 ml of elution buffer (250 mM imidazole, 250 mM NaCl, 50 mM NaP pH 7.5). RGIases were concentrated with Amicon Ultra Centrifugal filter with a 10 kDa cut-off (Merck Millipore, Burlington, Massachusetts, United States). Buffer exchange of concentrated and purified RGIases was performed using PD Spintrap G-25 column (GE Healthcare).

## Protein Electrophoresis

Protein concentrations were determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with Bovine Serum Albumin (A7906, Sigma) as standard. Molecular weight and homogeneity

were estimated by polyacrylamide gel electrophoresis under denaturing conditions using mini-PROTEAN 3 system (BioRad, Hercules, California, United States). Proteins were revealed using PageBlue Protein Staining Solution (Thermo Fisher Scientic) according to the manufacturer's protocol. Deglycosylation of RGIases were performed using Peptide-N-Glycosidase F (PNGase F) at 37 °C for one hour according to the supplier's protocol (New England Biolabs, Hitchin, UK).

**Microplate assay**

The activities of the endo-β-1,4-galactanase (GAN), α-arabinofuranosidase (ABF) and endo-rhamnogalacturonase (RHG) enzymes were determined using the DNS method (Miller, 1959). The reaction consisted of 0.5 % (w/v) pectic galactan or arabinogalactan diluted in 50 mM sodium acetate (pH 4.5) for GAN; 0.5 % (w/v) arabinan or debranched arabinan diluted in 50 mM McIlvaine buffer (McIlvaine, 1921) (pH 4.0) for ABF and 0.5 % (w/v) rhamnogalacturonan diluted in sodium acetate buffer (pH 4.5) for RHG. Three µg of enzyme in 25 µL final volume was incubated at 40 °C for 15 min for all reactions. The reactions were stopped with 150 µL of DNS reagent, boiled at 95 °C for 8 min and cooled down for color stabilization. The absorbance was measured at 540 nm using Powerwave XS2 (BioTek Instruments, Inc, VT, USA) equipment. L-(+)-Arabinose and D-(+)-Galactose were used as standards for ABF and GAN, activity tests, respectively. The recombinant RHG activity was also assayed with 0.5 % (w/v) azurine-cross-linked Rhamnogalacturonan-I (AZCL-RGI) at 40 °C for 15 min in 100 µl of 50 mM sodium acetate buffer (pH 4.5). The reaction was terminated by adding 100 µl of 10 % Tris pH 10 and measured at 595 nm. A calibration curve of AZCL-RGI was made by 3 h-incubation with concentrated RHG at optimal activity. All experiments were conducted in triplicates with a blank sample and the increase in the absorbance was proportional to the amount of substrate released in all assays.

**Effects of pH and temperature**

The optimum pH was determined between pH 3 and 8 using 50 mM citrate acid buffer (pH 3 to 3.5), sodium acetate buffer (pH 3.5 to 5) and McIlvaine buffer (pH 5 to 8) and pectic galactan for GAN, AZCL-RGI for RHG and arabinan for ABF, at 40°C. The pH stability was assessed by incubating the enzymes at pHs values from 3 to 8, at 40 °C for 90 min, using 50 mM of the above-mentioned buffer solutions. To

determine the optimum temperature, enzymatic reactions were performed between 30°C and 70°C in 50 mM sodium acetate buffer pH 4.5 with pectic galactan for GAN, AZCL-RGI for RHG, and 50 mM McIlvaine buffer pH 4.0 with arabinan for ABF. Temperature stability was determined by measuring the residual activity after 30 min of preincubation of the enzyme at temperatures between 30 °C and 70 °C in 50 mM sodium acetate buffer, pH 4.5 and McIlvaine buffer pH 4.0. For the pH stability the residual enzyme activity was assayed using 0.5 % (w/v) pectic galactan for GAN, AZCL-RGI for RHG and arabinan for ABF as substrate in the optimal condition for each enzyme after 90 min preincubation at given pH between 3 and 8 at 30°C.

**Determination of Km, Vmax, and specific activity**

The kinetic parameters Vmax and Km were calculated by GraFit7 software (Michaelis-Menten/Hill; Erithacus Software, Horley, Surrey, UK), using pectic galactan for GAN, AZCL-RGI for RHG and arabinan for ABF. The reactions were performed at the optimum pH and temperature, using 0.05 to 4 $g.mL^{-1}$ substrate concentrations.

**Mucilage extraction and hydrolysis**

Seeds from *Camelina sativa* for mucilage extraction were kindly provided by Biogis Center (SAS PIVERT, Compiègne, France). Mucilage from *Camelina sativa* was extracted following an adapted protocol (Sarv et al., 2017). It consists of 1h water imbibition of seeds at room temperature under slow stirring followed by filtration, ethanol precipitation and freeze-drying. For monosaccharides composition determination, 1 mg of freeze-dried mucilage was treated during 2 h at 120 °C with 1 $ml.mg^{-1}$ of 4 M trifluoroacetic acid to maximize monosaccharide recovery. Monosaccharides were dried under a nitrogen stream and resuspended in 1 ml of $H_2O$. Samples were filtered through a 13 mm 0.2 µm nylon econofilter (Agilent technologies) before HPAEC analysis.

**Monosaccharide composition determination**

The cell wall monosaccharide composition was determined by high-performance anion-exchange chromatography (HPAEC) with a pulsed amperometric detector (Dionex ICS 3000 system). After hydrolysis of mucilage, aliquots of the extract were injected through a 4x50 mm CarboPac PA1 pre-column (Dionex) before

separation of anionic compounds on a 4x250 mm CarboPac PA1 column (Dionex) at 30 °C. For neutral and acid monosaccharides, a multi-step gradient analyse was used as in Pillon et al., 2010. Peak analysis was performed using Chromeleon software, version 7.0. Standard of fucose, arabinose, rhamnose, glucose, galactose, xylose, mannose, galacturonic acid and glucuronic acid from.5.0 x $10^{-4}$ to 2.5 x $10^{-1}$ mg.ml$^{-1}$ concentration were used for quantification (Bertin et al., 2013).

**NMR Spectra analysis**

Before NMR analysis, samples were rinsed twice with 99.9 % $D_2O$ (Euriso-top), dried under vacuum, and 1 mg dissolved in 99.96% $D_2O$ (0.5 ml$^{-1}$). $^1H$ NMR spectra were recorded, at room temperature on a Bruker Avance 500 spectrometer equipped with a 5 mm BBI probe and Topspin 3.1 software. $^1H$ NMR spectra were accumulated using a 30° pulse angle, a recycling time of 1 s and an acquisition time of 2 s for a spectral width of 3 000 Hz for 32 K data points with or without a presaturation of the HOD signal using a presaturation sequence provided by Bruker. The 2D $^1H/^1H$ COSY, $^1H/^1H$ TOCSY, $^1H/^1H$ NOESY, $^1H/^{13}C$ HSQC and $^1H/^{13}C$ HMBC spectra were acquired with a standard pulse sequence delivered by Bruker (Bertin et al., 2015).

**Viscosity analysis**

The influence of RGIases on camelina mucilage viscosity was measured using a sino-wave Vibro viscometer (A&D Company). Two µg of the enzyme were incubated in a 13 ml water solution of camelina mucilage 4 g.l$^{-1}$ at 30°C pH 5. Measurements were taken at 0 min, 1 h, 2 h, and 4 h. A quarter of two µg of each enzyme (0.5 µg) was added in combination assays to allow the enzyme-effect to be discriminated and avoid dependent-dose effect. Measures were taken at 0 min, 30 min, 1 h, 2 h, and 4 h.

**Size-Exclusion Chromatograph analysis**

SEC analysis was conducted using a GPCmax/Viscotek TDA305 (Malvern) equipped with quadruple detection. Columns used for the SEC separation were a Malvern A5000 and a A6000M (Malvern) connected in series, calibrated on PEO 24K and verified with Dextran T73K.

All the analyses were carried out using $NaNO_3$ 50mM + 0.02% sodium azide as eluent. Detectors were temperature regulated at 30 °C. After viscosity assays, 500 µL of all samples were diluted to 1/2 in water and inactivated by a bath at 95 °C for 10 minutes. After filtration on 0.45 µl syringe filter, 100 µl was injected with a flow-rate of 0.7 mL min−1.

**Statistical analysis**

Analyses were performed with R software (http://www.R-project.org/). Statistical comparisons among groups were carried out with one-way ANOVA and Tukey HSD multiple comparisons, $p< 0.05$ was considered statistically significant. Data were expressed as means ± SD.

## RESULTS AND DISCUSSION

### *Cloning and expression of recombinant RGIases*

The full-length genomic DNA sequences of endo-rhamnogalacturonase (RHG) (GenBank Gene ID: 37148665), α-Arabinofuranosidases (ABF) (GenBank Gene ID: 37152657) and endo-β-1,4-galactanase (GAN) (GenBank Gene ID: 37150470) consisted of 1517 (3 introns), 1754 (no introns) and 1597 (1 intron) base pairs, respectively. RHG and GAN were amplified from cDNA from *A. aculeatinus* culture grown on soy bean RG as the expression of the genes was induced on this substrate (**Table S1**). As ABF gene does not have intron, its amplification was directly performed on genomic DNA. At the amino-acid level, the sequences were aligned as shown in **Fig. 1**. The deduced amino acid sequence showed high similarities with enzymes previously annotated from *A. aculeatus*, 99.32 % for RHG (**Fig. 1A**), 98.80 % for ABF (**Fig. 1B**) and 100 % for GAN (**Fig. 1C**). Thus, most of changed residues are conservative mutations. In addition, the structure-based sequence model revealed conserved RGIases active sites between *A. aculeatus* and *A. aculeatinus* (data not shown).

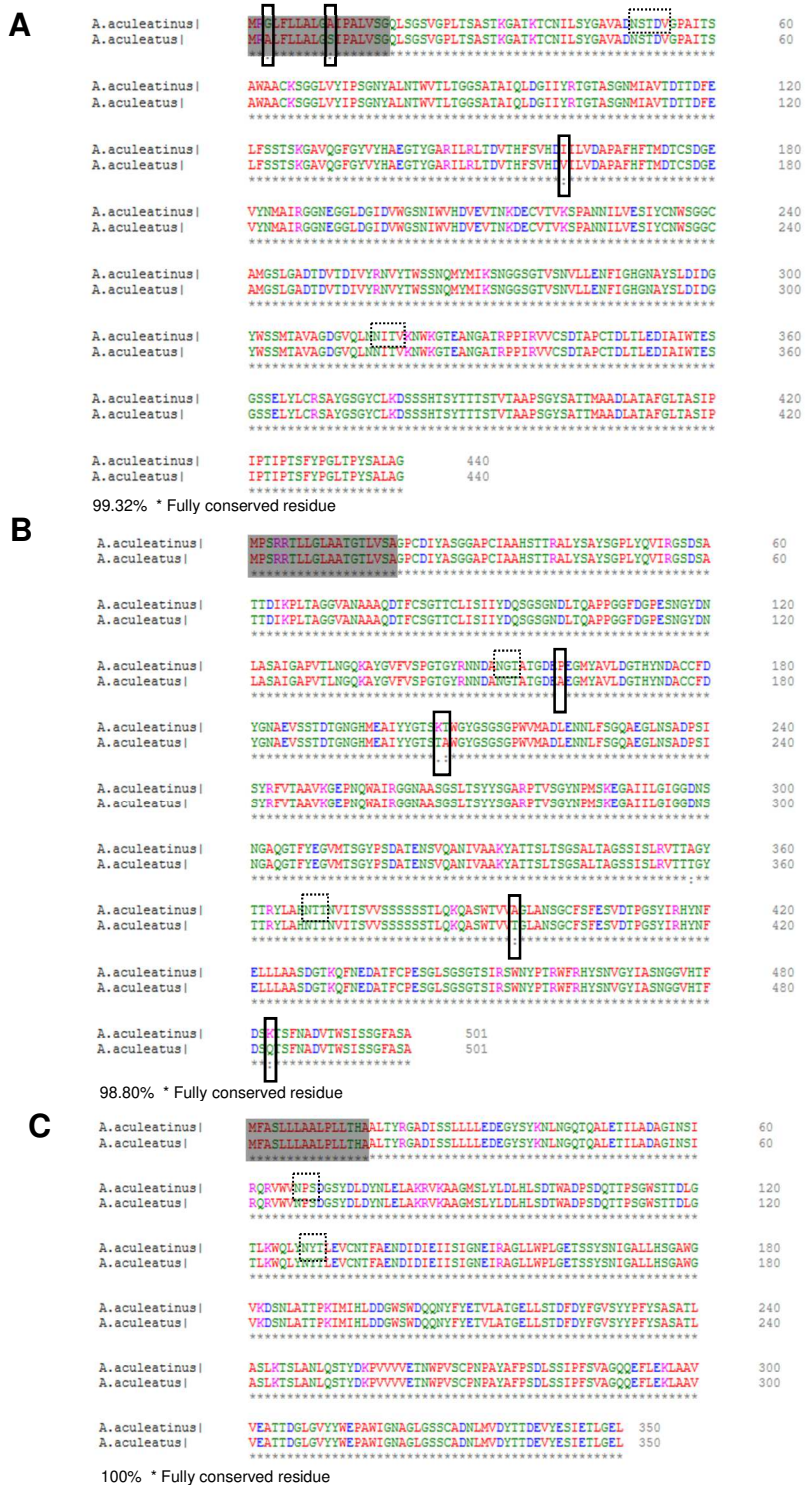


**Fig. 1.** Multiple alignment of amino acid sequences from *A. aculeatinus* and *A. aculeatus* for the three RGIases. The alignments were performed using ClustalW. Sequences used were as follow: (A) RHG (*A. aculeatinus,* XM_025645590.1, NCBI REFSEQ*; A. aculeatus,* Q00001.1, Uniprot); (B) ABF (*A. aculeatinus,* XP_025505686.1, NCBI REFSEQ*; A. aculeatus,* XP_020052596, NCBI REFSEQ); (C) GAN (*A. aculeatinus,* XP_025504038.1, NCBI REFSEQ*; A. aculeatus,* 1FHL_A, PDB). Potential N-glycosylation sites are dotted-box and potential residues changes are black-boxed. Signal peptide are

highlighted in grey. RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

### *Purification of recombinant RGlases*

The three enzymes were successfully produced and purified with high purity (**Fig. 2A-B**). The purified recombinant enzymes have a slightly higher apparent molecular weight compared to the predicted protein from *A. aculeatinus* (**Fig. 2. and Table 2**). RHG and ABF were secreted at ~66 kDa and GAN was secreted at ~50 kDa, while theoretical sizes were ~48 kDa for RHG, ~53 kDa for ABF and ~40 kDa for GAN, suggesting the presence of N- or O-Glycosylation sites. Two putative N-glycosylation sites were indeed identified for RHG (32 Asn, Ser, Thr, Asp and 299 Asn, Ile, Thr, Val), ABF (134 Asn, Gly, Thr, Ala and 349 Asn, Thr, Thr, Asn) and GAN (55 Asn, Pro, Ser, Asp and 116 Asn, Thr, Thr, Leu). However, the first potential site of N-glycosylation on GAN, could be hindered by the presence of Pro just after the Asn. None *O*-glycosylation sites were identified for the three enzymes by NetO-Glyc Server analysis. After treatment by a peptide -N-Glycosidase enzyme (PNGase F), a slight decrease in molecular mass of the proteins was observed, confirming the presence of N-glycosylation on the three enzymes (**Fig. S1**). It has been previously reported that heterologous proteins that are expressed by *P. pastoris* yeast can be glycosylated on the Asn, Ser or Thr hydroxy groups with N-linked and *O*-linked saccharides (usually 8–14 mannose residues) (Bretthauer & Castellino, 1999; Habrylo, Evangelista, Castilho, Pelloux & Henrique-Silva, 2018). Two potential *N*-glycosylation sites and discrepancies in molecular weight have been reported for RHG and GAN from *A. aculeatus* (Christgau et al., 1995; Kofod et al., 1994). In contrast, no carbohydrate was shown to be bound at the potential *N*-glycosylation sites in the crystallographic structure of *A. aculeatus* GAN (Ryttersgaard et al., 2002). The molecular weight obtained for the recombinant RHG is comparable to rRGase A from *A. aculeatinus* (~62Kda) with a significant amount of glycan structures attached to the enzyme reported by Kofod et al., 1994. While no reports have been found in literature for ABF from *A. aculeatus*, ABF from *A. niger* has a 64/67 kDa molecular weight and the presence of two *N*-linked glycosylation sites (Crous, Pretorius & van Zyl, 1996; Flipphi, van Heuvel, van der Veen, Visser, & de Graaff, 1993). In addition, literature shows that native and recombinant enzymes expressed in *P. pastoris* have similar biochemical properties, and when the N-linked glycans are not close to the

active site tunnel loops (as for the three characterized RGIases, **Fig. S2**), there is no impact on thermal stability and enzymatic activities (Koseki et al., 2006., Adney et al., 2009).

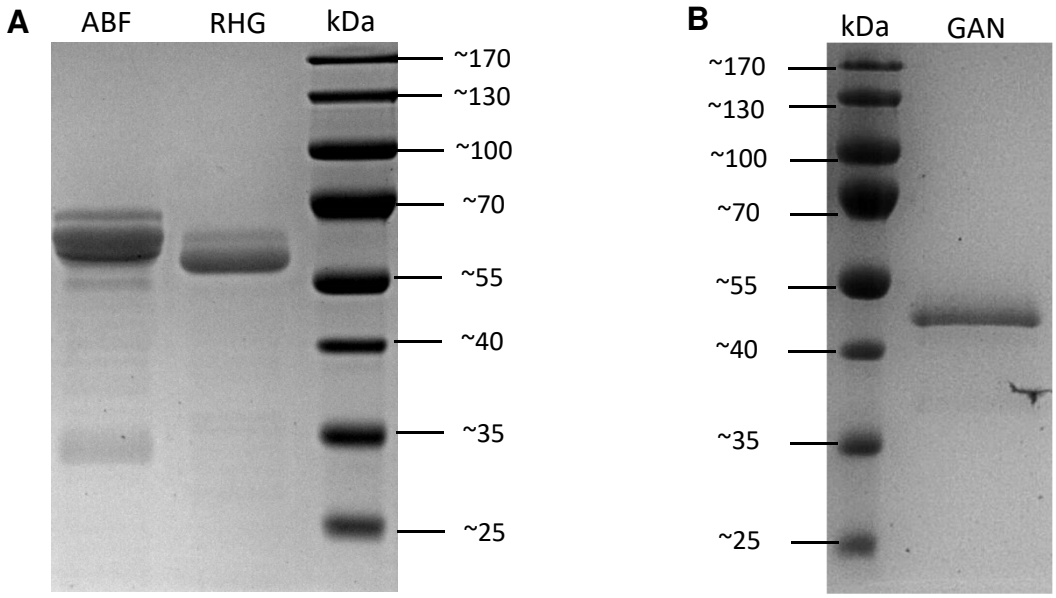


**Fig. 2.** SDS-PAGE of purified recombinant *A. aculeatinus* RGIases. (A) *Line 1* purified α-Arabinofuranosidases (ABF); *Line 2* purified endo-rhamnogalacturonase (RHG). *Lane 3* molecular weight marker. (B) *Lane 1* molecular weight marker; *Line 2* purified endo-β-1,4-galactanase. (GAN). Each line of purified protein contains ~ 3 µg. Purified proteins were resolved on a 12% acrylamide gel and stained with Coomassie brilliant blue.

**Table 2.** Biochemical characteristics of the purified recombinant *A. aculeatinus* RGIases. RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

| Characteristics | RHG | ABF | GAN |
|---|---|---|---|
| Classification | GH28 - EC 3.2.1.171 | GH54 - EC 3.2.1.55 | GH53 - EC 3.2.1.89 |
| MW * (kDa) | 47.3 | 53.0 | 40.2 |
| pI * | 5.05 | 5.18 | 4.54 |
| pH optimum | 4.5 | 3.5 - 4.5 | 3.5 - 4.0 |
| pH stability | 5.5 - 8 | 3 - 8 | 3.5 - 6 |
| Temperature optimum (°C) | 50 | 40 | 50 |
| Temperature stability (°C) | < 60 | < 50 | < 40 |
| Km (mg/ml) | 0.50 ± 0.19 | 1.64 ± 0.17 | 3.72 ± 0.59 |
| Vmax (mg/min) | 1.59 ± 0.37 | 1.14 ± 0.05 | 4.02 ± 0.39 |
| Vmax/Km | 3.18 ± 0.56 | 0.71 ± 0.21 | 1.08 ± 0.10 |

* Theoretical value

### *Biochemical characterization of recombinant RGIases*

The optimal activity conditions and the specificity for commercial substrate were determined for each RGIases (**Fig. 3, Fig. 4 and Table 2**). The optimum temperature for RHG was 50 °C (**Fig. 3A and Table 2**), remaining stable up to 40 °C and losing 30 % of its activity at 50 °C. The effects of pH on enzyme activities reported that RHG has maximal activity at pH 4.5 (**Fig. 3B and Table 2**). Although, RHG was less stable at lower acidic pHs, losing 50 % of its activity for pH 3 to 5. However, its activity remained stable (80 % activity) from pH 5.5 to 7. The biochemical properties are conserved within their GH 28 family. Hence, our results were strikingly different from those previously reported, (Kofod et al., 1994). This could be related to different substrates used for the assays as the use of AZCL-RGI to test Rhamnogalacturonan hydrolase and lyase activities was only reported from the 2000s onwards (De Fine Licht, Schiøtt, Mueller, & Boomsma, 2010). RHG showed the highest activity for AZCL-RGI substrate (**Fig. 4A**). In contrast, when using rhamnogalacturonan (RG) and camelina mucilage, RHG activity was dramatically reduced, and close to null. The RHG activity was also evaluated by $^{1}H$

NMR (**Fig. 4B-C**). Due to the non-solubility of AZCL-RGI in an aqueous solvent, no signal was observed on the control spectrum (**Fig. 4B**). In contrast, the characteristic signals of RGI appeared during the hydrolysis of the chromophore part showing the activity of the RHG. The activity of RHG on RG was also assessed by $^{1}$H NMR (**Fig. 4C**): Signals observed in the spectrum at 5.24 ppm (H-1 →2)-α-Rha-(1→), 5.02 ppm (H-1 →4)-α-GalA-(1→) and 4.63 ppm (H-1 →4)-β-Gal-(1→) (Mikshina et al., 2015b; Zheng & Mort, 2008), were characteristic of the hydrolysis of the backbone of RG by an endo-RHG as no signals from rhamnose or galacturonic acid monosaccharides were detected.

For ABF if the optimum temperature was 40 °C (**Fig. 3C and Table 2**), the activity remained stable up to 60 °C and was inactivated at 70 °C. ABF had an optimum activity between pH 3.5 to 4.5 (**Fig. 3D and Table 2**). The ABF enzyme showed a stable activity from pH 3 to pH 7, for which more than 80 % of the activity was maintained. The ABF results are in concordance to those previously reported for ABF-GH54 family enzymes: a pH optimum between 3.5 and 5, and a temperature optimum between 50 and 60 °C (de Wet et al., 2008). ABF showed 100 % of activity for arabinan (ARAB), 20 % for debranched arabinan (D-ARAB), and very low activity was detected for camelina mucilage (MUC) (**Fig. 4D**). The activity of ABF was also studied by $^{1}$H NMR. (**Fig. 4E-F**). In the spectra related to the activity of ABF on arabinan (**Fig. 4E**), the hydrolysis of the sidechains was observed by the losses of 5.13 ppm (H-1 →3,5)-α-Ara-(1→) and 5.16 ppm (H-1 →3)-α-Ara-(1→) signals, as well as the appearance of the two signals of arabinose at 5.24 ppm (α-Ara) and 4.51 ppm (β-Ara) (Ding, 2015; Shinozaki, Kawakami, Hosokawa & Sakamoto, 2014). These results indicated that the ABF is an exo-enzyme. ABF was also active on debranched arabinan (**Fig. 4F**) albeit to a lower extent compared to arabinan. ABF acted preferentially on →3)-α-Ara-(1→ compared to →5)-α-Ara-(1→.

GAN activity showed an optimum temperature of 50 °C (**Fig. 3E and Table 2**), although its activity decreased by 80% after a 30min incubation at 50°C and was completely inactivated above 60°C. GAN enzyme had an optimum activity for pH 3.5 to 4.5 (**Fig. 3F and Table 2**). Despite its stability in the pH 4.5 to pH 5.5 range, it lost 20% of its activity at pH 4 and 60 % at pH 6. GH53 enzymes – GAN have a pH optimum between 4 and 4.5 and temperature optima between 45 and 65 °C (Christgau et al., 1995; Le Nours et al., 2003). For the substrate specificity, GAN showed significant activity on galactan, while no activity was detected on

arabinogalactan and MUC substrates (**Fig. 4G**). NMR spectra for GAN on galactan showed the presence of signals at 4.61 ppm (H-1 β-Gal) and 5.28 ppm (H-1 α-Gal) characteristics of galactoses linked in position 4 (**Fig. 4H-I**). As no signal for galactose monosaccharide was detected, we can conclude that GAN is an endo-enzyme (Cruz et al., 2010; González-Ayón et al., 2019). Moreover, based on the $^{1}$H NMR spectra, and consistent with the colorimetric assay, no GAN activity was detected on arabinogalactan. As this substrate is in part constituted by →3)-β-Gal-(1→, we can conclude that GAN is specific for →4)-β-Gal-(1→.

Most of these results are in accordance with the reports for RHG and GAN proteins from *A. aculeatus* (Christgau et al., 1995; and Kofod et al., 1994), showing an endo-acting activity on RGI backbone and galactan, respectively. Also, the present study for *A. aculeatinus* ABF is in agreement with the ABF from *A. niger* which had action on arabinan (Flipphi et al., 1993). The kinetics parameters reported for the three RGIases characterized in our study (**Table 2**) are not easily comparable between species as substrates, pH and temperature used in the assays varied. For example, *Km* values reported for GAN from *A. aculeatus,* tested with potato arabinogalactan were 10-fold lower than what we report in the present study (Van de Vis, Searle-van Leeuwen, Siliha, Kormelink, & Voragen., 1991). To our knowledge, no values have been established for RHG and ABF from *A. aculeatus*, making comparisons difficult with our present study.

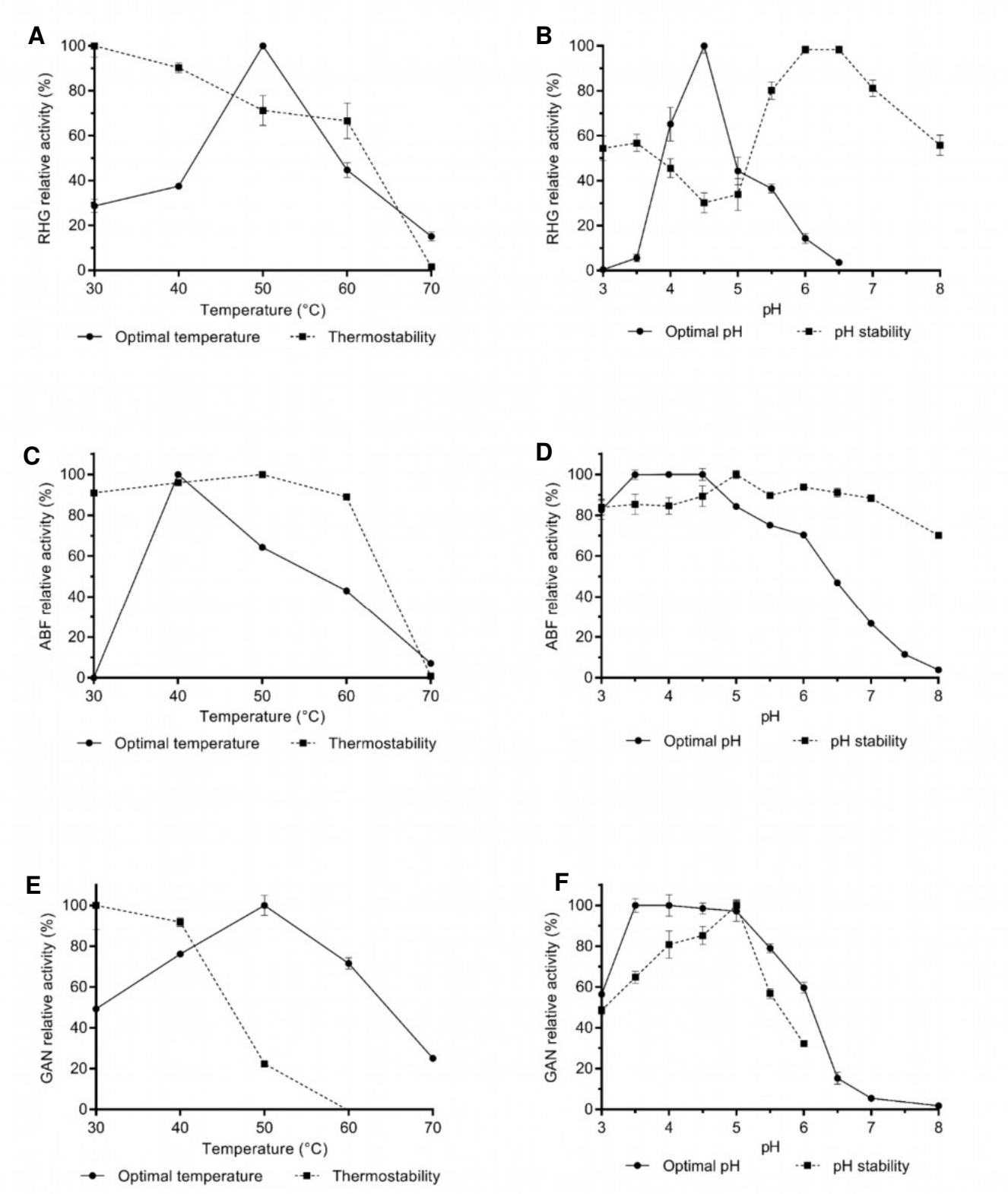


**Fig. 3.** pH and temperature optimum for *A. aculeatinus* recombinant RGIases. (A) Influence of temperature on RHG activity; (B) Influence of pH on RHG activity; (C) Influence of temperature on ABF activity; (D) Influence of pH on ABF activity; (E) Influence of temperature on GAN activity; (F) Influence of pH on GAN activity. The values are mean ± SD for three replicates. RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

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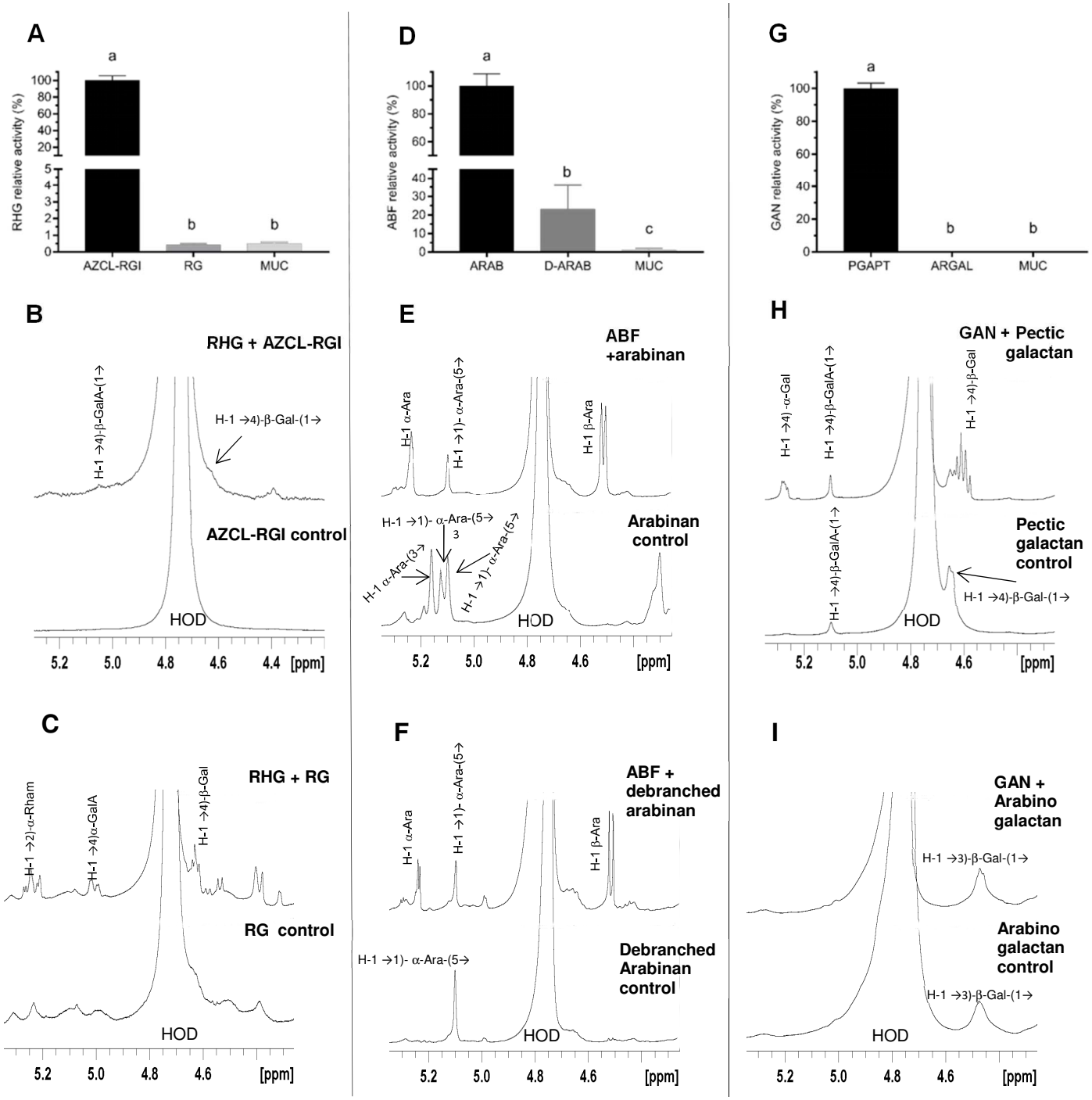



437

438 **Fig. 4.** Substrate specificity of *A. aculeatinus* recombinant RGIases. (A) RHG relative activity on AZCL-
439 RGI: Azurine-cross-linked rhamnogalacturonan I, RG: Rhamnogalacturonan and MUC: Camelina
440 mucilage. (B) $^{1}$H proton NMR spectra of RHG activity on AZCL-RGI and (C) RG. (D) ABF relative activity
441 on ARAB: Arabinan, D-ARAB: Debranched arabinan and MUC. (E) $^{1}$H proton NMR spectra of ABF
442 activity on Arabinan and on (F) Debranched arabinan. (G) GAN relative activity on PGAPT: Pectic
443 galactan, ARGAL: Arabino galactan and MUC. (H) $^{1}$H proton NMR spectra of GAN activity on Pectic
444 galactan and (I) Arabino galactan. The values of the relative activity are mean ± SD for three replicates.
445 RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

### *Polysaccharide content of Camelina sativa mucilage*

To further evaluate the RGI enrichment in *Camelina sativa* mucilage and describe the activity of RGIases recombinant enzymes, the monosaccharides content of MUC was determined (**Table 3**). A 1 h stirring of the seeds in water allowed obtention of 1.15 g of freeze-dried mucilage per 100 g of camelina seeds. To determine sugar composition, freeze-dried mucilage was treated during 2 h at 120 °C with 1 $ml.mg^{-1}$ TFA to maximize monosaccharide recovery. The HPAEC analysis showed that *Camelina sativa* mucilage was rich in galacturonic acid, galactose and rhamnose (~30 mol %) while minor amounts of arabinose, glucuronic acid (~5 mol %), glucose (~3 mol %), xylose and fucose (>~1 mol %) were detected, (**Table 3**).

**Table 3.** Monosaccharide composition of Camelina mucilage. The values are mean ± SD for three replicates.

| Monosaccharide | Relative content (mol %) |
|---|---|
| L-Fucose | 0.16 ± 0.01 |
| L-Rhamnose | 27.15 ± 1.04 |
| L-Arabinose | 5.54 ± 0.06 |
| D-Galactose | 29.55 ± 1.89 |
| D-Glucose | 2.77 ± 0.33 |
| D-Glucuronic acid | 4.11 ± 0.05 |
| D-Galacturonic acid | 30.42 ± 2.87 |
| D-Xylose | 0.30 ± 0.02 |

These values are similar to that reported by North, Berger, Saez-Aguayo, & Ralet, 2014. The higher galacturonic acid, rhamnose and galactose contents indicate a substrate rich in RGI, which may be highly substituted by short side-chains of galactose and, to a lesser extent, by arabinose side-chains at Rhap O (2) and O (3) atoms. In contrast in most cell walls, RGI are present as long side-chains of galactans and arabinans attached to Rhap at the O(4) position (Mikshina, Petrova & Gorshkova., 2015a). The NMR signals from MUC were identified by 2D NMR and chemical shifts were reported in **Table 4**. The glycosidics linkages were further

468 determined by 2D NMR and selected region of the 2D NMR COSY, HSQC and
469 NOESY spectra are reported in **Fig. S3**. The cross-peaks observed on NOESY
470 spectrum: H-1 of α-Rha*p* residues at 5.23 ppm with H-4 of α-GalA*p* at 4.42 ppm and
471 H-1 of α-GalA*p* at 5.02 ppm with H-2 of α-Rha*p* at 4.13 ppm indicate the presence of
472 RG-I backbone. Cross peaks were also observed between H-1 of β-Gal*p* at 4.62 ppm
473 with H-4 of α-Rha*p* at 3.68 ppm indicating the presence of side chain mainly
474 composed of galactose. Very small intensity cross-peaks were also observed
475 between H-1 of Ara*f* (5.11 ppm) and H-3 of β-Gal*p* (3.73 ppm) corroborating a partial
476 substitution of galactose side chains by arabinose. The sugar composition allowed
477 estimating that one side chain of galactose out of five was substituted by arabinose.
478 The signal intensity of methyl group protons at 1.23 ppm and 1.30 ppm allowed
479 estimating the proportion of non-branched rhamnose (53 %) and branched rhamnose
480 (47 %) residues, respectively (Mikshina et al., 2015).
481 This result was compared with the average length of the galactose chains that were
482 approximately 2 monomers (evaluated by the ratios of galactose and rhamnose in the
483 polymer). These results confirmed that RGI backbone is highly substituted by short
484 side chains of galactose which led us to propose structure of camelina mucilage:
485 Backbone :
486 →2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→
487 Main side chain:
488 β–D-Gal*p*-(1→4)-β-D-Gal*p*-(1→ substituted partially by α-L-Ara*f*-(1→3)

**Table 4.** Chemical shifts of $^{13}C$ and $^{1}H$ (*italics*) in ppm of the main carbohydrate sugars residues from camelina mucilage (in $D_2O$ at room temperature). Not-determined (nd)

| Residue | C-1<br>*H-1* | C-2<br>*H-2* | C-3<br>*H-3* | C-4<br>*H-4* | C-5<br>*H-5* | C-6<br>*H-6* |
|---|---|---|---|---|---|---|
| →4)-α-GalA-(1→ | 99.5<br>*5.02* | 69.8<br>*3.90* | 70.6<br>*4.08* | 78.9<br>*4.42* | 72.5<br>*4.90* | 175.0 |
| →2)-α-Rha-(1→ | 100.2<br>*5.23* | 78.1<br>*4.10* | 70.2<br>*3.88* | 74.0<br>*3.39* | 70.8<br>*3.77* | 18.5<br>*1.23* |
| →2,4)-α-Rha-(1→ | 100.2<br>*5.25* | 78.1<br>*4.13* | 71.4<br>*4.06* | 82.3<br>*3.68* | 70.8<br>*3.79* | 18.5<br>*1.30* |
| →4)-β-Gal-(1→ | 105.6<br>*4.62* | 73.5<br>*3.69* | 75.0<br>*3.73* | 79.2<br>*4.21* | 76.3<br>*3.68* | 63.0<br>*3.84; 3.79* |
| β-Gal-(1→ | 105.6<br>*4.59* | 73.4<br>*3.53* | 74.2<br>*3.67* | 70.5<br>*3.90* | 76.8<br>*3.71* | 62.6<br>*3.83;3.78* |
| α-Ara-(1→ | 109.2<br>*5.11* | 82.0<br>4.09 | nd | nd | nd | nd |

Considering this, it is noteworthy that camelina mucilage was different from flax seed (Elboutachfaiti, et al 2017) or Arabidopsis thaliana seed mucilage (Yapo, 2011).

***Effects of RGlases on mucilage: from polysaccharide chemistry to viscosity***

In order to analyze the effects of RGlase on mucilage structure, and relate to the above-mentioned results (**Fig. 4.A-D-G)** NMR analyses were carried out for all recombinant enzymes independently or in combination (**Fig. 5**). As mentioned, the relative activity on MUC was less than <10% for ABF, <1% for RHG, and null for GAN (**Fig. 4A-D-G**). Due to the complexity and the viscosity of MUC, the three enzymes were applied on this substrate in order to simplify its structure and to determine the $^{1}H$ NMR spectrum (**Fig. 5**). The digestion of MUC by GAN showed signals at 4.61 ppm (H-1 β-Gal) and 5.28 ppm (H-1 α-Gal) characteristic of galactose linked in position 4. Thus, the 30% of galactose in MUC were mainly →4)-β-Gal-(1→, and were the main side chains of MUC. The two signals at 5.24 ppm (α-Ara) and 4.51 ppm (β-Ara) in $^{1}H$ NMR spectrum (**Fig. 5**) inferred from ABF activity on MUC were characteristic of arabinose monosaccharide. This arabinose represented 5 % of the monosaccharide and was probably linked on galactose side chains. Spectrum of the RHG activity on MUC (**Fig. 5**) showed signals deriving from a depolymerization of the RGI backbone: →2)-α-Rha-(1→4)-α-GalA-(1→. The synergistic action of

enzymes on MUC was also tested. The main activity was observed for RHG with the depolymerization of the backbone and minor action of the two other enzymes was detected on sidechains. Despite their short size, the presence of side-chains of arabino galactan and galactan could impact RGI polymer rheology. It was indeed previously reported that the reduction of neutral sugar concentration can induce a significant decrease in gelling temperature and viscosity (Sousa, Nielsen, Armagran, Larsen & Sørensen, 2015).

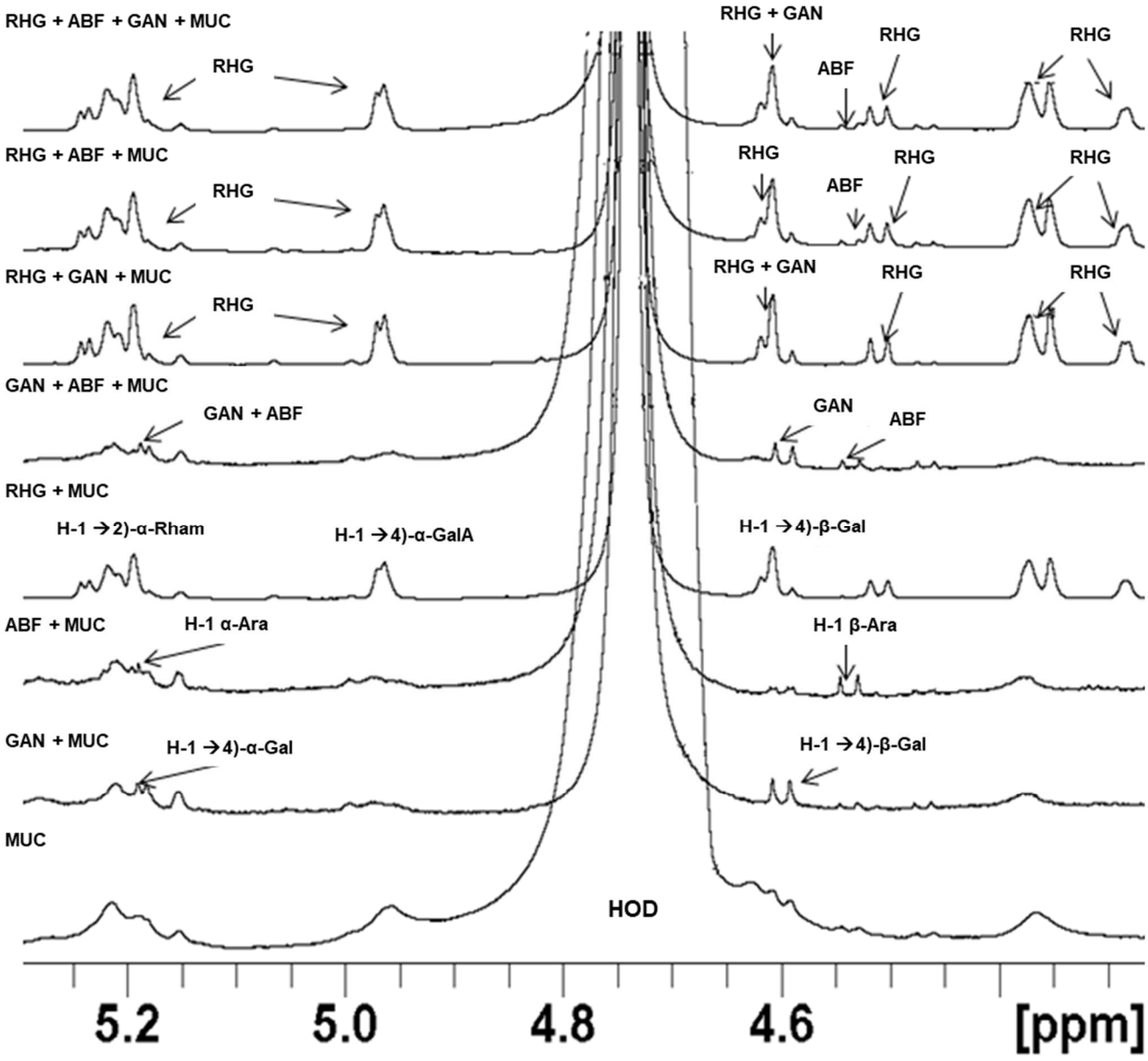
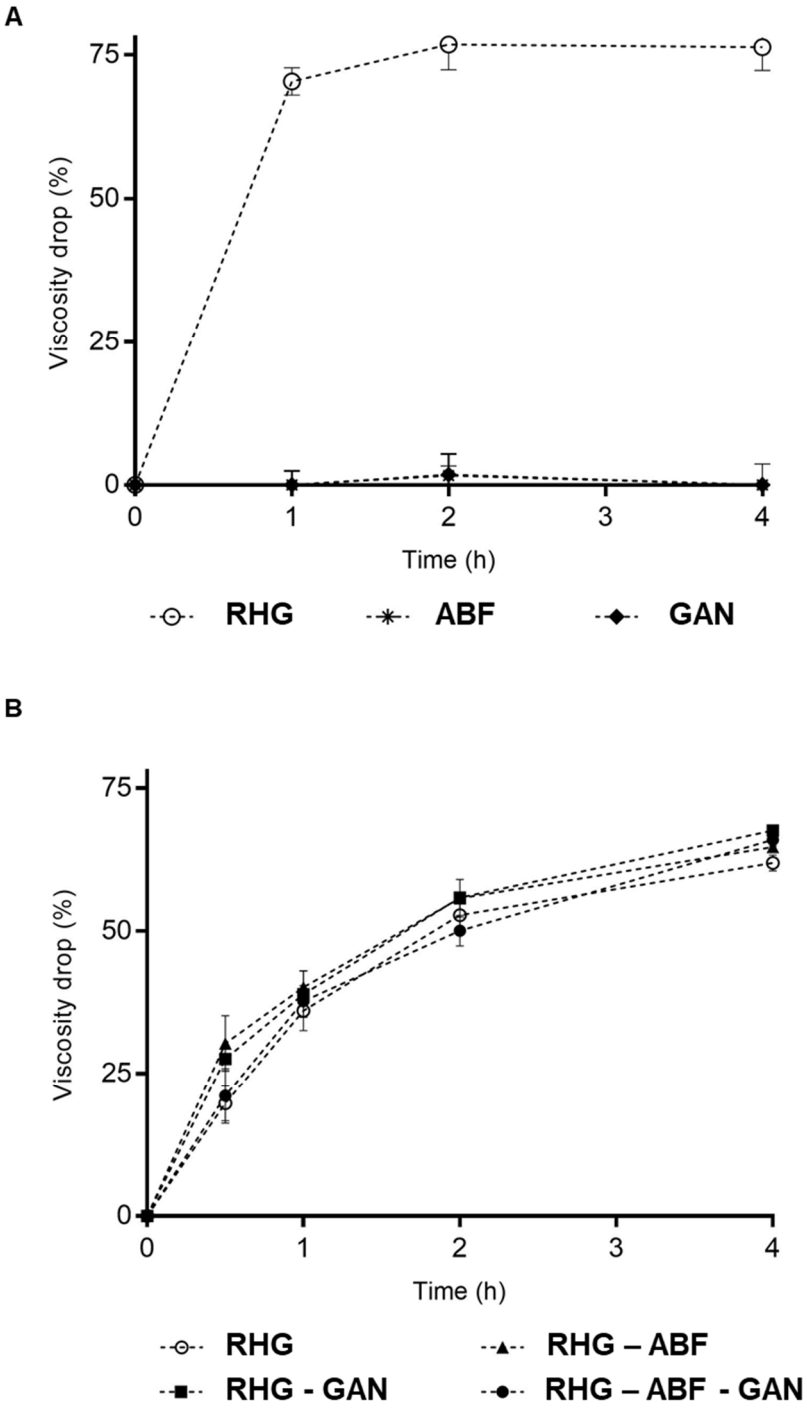



**Fig. 5.** $^{1}$H proton NMR spectra of Camelina mucilage following treatment with RHG, ABF and GAN recombinant enzymes. RHG: Endo-rhamnogalacturonase; ABF: α-Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

To determine the applicative potential of characterized RGIases, the changes in MUC viscosity were measured through a viscosity drop curve assay following application of the enzymes for 1 h to 4 h (**Fig. 6A-B**). The synergistic action of enzymes on MUC was studied by treating the substrate with each of the enzymes (RHG, ABF, and GAN) or binary-ternary mixtures (RHG-ABF, RHG-GAN, ABF-GAN, and RHG-ABF-GAN). *A. aculeatinus* RHG showed the highest efficiency in decreasing the viscosity on MUC compared to ABF and GAN, at a similar enzyme dose of 2 µg (**Fig. 6A - Table S2**). The results revealed a significant drop (70 %) in viscosity as early as 1 h. Thus, to better discriminate the potential additive effects of the enzymes in mixture, 0.5 µg of each enzyme was added and an early point of 30 min was monitored. Binary and ternary mixtures lead to a further reduction in MUC viscosity as compared to RHG enzyme alone. Indeed, at 30 minutes RHG lowered the viscosity by 19 %, while RHG-GAN and RHG-ABF binary combinations led to a significant ~30 % drop in viscosity **(Fig. 6B - Table S2**). In contrast, the effects of the RHG-ABF-GAN ternary combination was only a 21 % reduction in viscosity.
At 4h, the highest viscosity drop was observed when using RHG-GAN and RHG-ABF-GAN combinations. A significant viscosity drop of 67.5 % was observed when using RHG-GAN mixture, compared to a 61.9 % viscosity drop generated by the single RHG enzyme (**Fig. 6B - Table S2**). As previously observed, the effect of the ternary combination on mucilage viscosity was less significant than that of binary mixtures. Therefore, it is likely that viscosity drop produced by RHG-GAN mixture was due to an additive effect of GAN, which potentially revealed cleavage sites for the RHG. This is consistent with the NMR analysis (**Fig. 5**), where RHG was shown to act on the RGI backbone (GalA α-(1→2) Rha) while ABF-GAN were acting for the sidechains of arabinose (Ara α-(1→3) Ara and Ara α-(1→2) Ara) and galactose (Gal β-(1→4) Gal).

Despite the statistical differences measured (**Table S2**), the viscosity was not drastically modified after 4 hours of treatment, probably related to the low contribution of GAN and ABF, considering the presence of short side-chains present in mucilage. Consistent with this results, HPSEC analyses (**Fig. S4**) revealed a shift in MW of the polymer after incubation with RHG enzyme, and a much lower effect mediated by ABF and GAN. Previous studies on apple pectins showed that the presence of RG-I side-chains allow pectin molecules to interact and entangle more, eventually creating a tighter conformation and decreasing molecular mobility (Hwang

565 & Kokini, 1992). However, the intrinsic viscosity is highly related to the number of
566 side branches (Yapo, 2011). In our study, the RGI from *Camelina sativa* have a
567 higher number of substitutions by galactose. Even so, the length of the branches
568 appears to be insufficient to translate into long-term changes in viscosity following
569 action of the accessory enzymes. It should also be noted that our results, by the
570 chemical analysis of mucilage, provide a basis for identifying novel RGIases activities
571 that can target short RGI side-chains. Probably, by increasing the dose of GAN
572 enzyme or by having a two-step application (first GAN, second RHG), we could
573 potentiate the changes in the viscosity of the polymer. Enzymatic treatment
574 (pectinases) has been mostly used for plant seed mucilage to reduce viscosity before
575 further processing (Wu, Li, Wang, Zhou & Mao., 2010). Hence, considering various
576 doses and/or combinations of enzymes, a cocktail containing *A. aculeatinus* RGIases
577 enzymes degrading sidechains and backbone, could be useful to improve the
578 degradation process of RGI seed coat, allowing its use as structuring and texturizing
579 agents for food or nutraceutical applications.




580

581 **Fig. 6.** Effects of RHG, ABF and GAN on mucilage viscosity over 4h incubation. (A) Single effect of
582 RHG, ABF, and GAN on the viscosity properties of camelina mucilage. (B) Synergism of RHG, ABF,
583 and GAN on the viscosity properties of camelina mucilage. RHG: Endo-rhamnogalacturonase; ABF: α-
584 Arabinofuranosidases; GAN: Endo-β-1,4-galactanase.

585

## CONCLUSION

This study is the first full biochemical characterization of three RGI-degrading enzymes, a rhamnogalacturonase, a galactanase and an arabinofuranosidase, from *A. aculeatinus*. We were able to show that the three enzymes had an acidic pH optimum and that their optimal temperature for activity was 40-50°C. We assessed the substrate specificity of these three enzymes first on commercial substrates, showing that RHG acts on RGI as an endo enzyme, while ABF had a strong activity on arabinan with an exo mode of action. GAN was specific for →4)-β-Gal-(1→ linkages on galactan, with an endo mode of action. Using combined technical approaches, including NMR and viscosity assays, we were further able to relate the effects of the enzymes on a complex substrate, *Camelina sativa*'s mucilage, to potential industrial application. We first determined mucilage structure using 2D-NMR showing that it harbors short side-chains of galactose. We next showed that the use of binary combination of enzymes, led to a transient higher effect on mucilage viscosity, compared to the use of a single enzyme. This suggest that the short side galactose chains could explain the differential effects on mucilage's RGI-chemistry and viscosity. We believe that our study shed new light on the use, and the potential formulation, of these enzymes in industrial processes for which RGI hydrolysis is required. For instance, these novel enzymes could be used in fruit juice clarification, where RGI is known to be recalcitrant to digestion with commonly-used enzymatic cocktails.

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