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Cloning of an α-L-Arabinofuranosidase and Characterization of Its Action on Mono- and Di-Substituted Xylopyranosyl Units

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Abstract

An α-L-arabinofuranosidase (ARF) gene of 1503 bp was synthesized, subcloned into pET26b vector, and expressed in *Escherichia coli*. The enzyme was purified in active form, and consisted of 500 amino acid residues, corresponding to 55 kD based on SDS-PAGE. The affinity-purified protein was characterized using arabinofuranosyl xylooligosaccharides (AXOS) as substrates. The pH effect was investigated showing an optimum at pH 5.5. XaARF catalyzed the cleavage of arabinose at C3 of the xylopyranosyl unit efficiently if the arabinofuranosyl substitution was at the terminal compared to internal xylose units. The enzyme was able to act on di-substituted xylopyranosyl units with the first cleavage at C3 followed by C2 linkages.

Keywords

α-L-Arabinofuranosidase, Mono-Substituted Xylopyranosyl Unit, Di-Substituted Xylopyranosyl Unit, Arabinofuranosyl Xylooligosaccharides (AXOS)

1. Introduction

The structural complexity of cellulosic biomass is due to the large hemicellulose fraction consisting of mostly xylan. The characteristic feature of xylan in its native state is heteropolysaccharide (heteroxylan) with a β-(1,4)-xylopyranosyl (Xyl*p*) backbone carrying arabinofuranosyl, acetyl, and glucuronyl side groups, and oligosaccharide chains containing galactose, xylose, and arabinose [1] [2]. The fine architecture and the side group branching vary from plant to plant and even in different tissues of the same plant [3]. Arabinoxylans from cereal grains are highly branched. Those from wheat and barley endosperm contain consi-

derable levels of main chain Xyl*p* residues singly or doubly substituted with arabinofuranosyl units (Ara*f*) attached to C-2/C3 positions [4] [5]. Adding to the complexity, arabinofuranosyl side units may be ester-linked at C5 with ferulic acid moieties, which can further form diferulate crosslinks. It has been shown that diferulate dimerization contributes to linkages between xylan and lignin and also between other polysaccharides, such as pectin and lignin.

The cooperative interactions between main chain enzymes and enzymes liberating side chain substituents (collectively known as accessory enzymes) are involved as key processes in depolymerization of xylan. Notably, endo-xylanase has been known to act synergistically with increased efficiency when arabinofuranosidase (ARF, EC 3.2.1.55) is used to remove the Ara*f* substituents [6]. In arabinoxylans (as in the case of wheat xylan) where Ara*f* substituents may be ester-linked with ferulic acids, the feruloyl-arabinose (Ara*f*-FA) ester bonds can first be cleaved by feruloyl esterase (FAE, EC 3.1.1.73). This treatment would undecorate arabinofuranosyl side groups available for ARF action. A further issue in the degradation of cereal arabinoxylan concerns the complexity due to the main chain Xyl*p* residues that may be doubly substituted with Ara*f* residues.

ARFs are divided into two groups: The major group contains enzymes active on Xyl*p* mono-substituted with arabinose, either at position 2 or 3, and thus are referred to as ARFm2,3. Less common is another group containing enzymes active specific for doubly arabinofuranosylated Xyl*p*, releasing either 1,2- or 1.3-linked Ara*f*. These enzymes are referred to as ARFd2 or ARFd3 [7]. The objective of this paper is to report the cloning and purification of *Xanthomonas* ARF, and comparative studies of the enzyme action with similar known ARFs using arabinofuranosyl xylooligosaccharides (AXOS) as substrates. The XaARF in this study may be useful for enhancing the efficiency of enzymatic degradation in biomass conversion.

2. Materials and Methods

The following were obtained from Megazyme (Wicklow, Ireland): wheat arabinoxylan, *α*-L-arabinofuranosidase from *Aspergillus niger* (GH51, AnARF), and from *Bifidobacterium adolescentis* (GH43, BaARF), and arabinofuranosyl xylooligosaccharides (AXOS) including A^2XX, $A^{2+3}XX$, A^3XX and XA^3XX. The pET vector and *E. coli* BL21(DE3) were from Novegen (Madison, WI). Precast protein gels were purchased from Novex (San Diego, CA). The ni-Sepharose fast flow was obtained from GE Healthcare (Piscataway, NJ). Restriction and DNA modifying enzymes were from New England Biolabs (Beverly, MA). Microbiological culture medium components and agar were from Difco Laboratories (Detroit, MI). Thin-layer plates and substrates were purchased from Analtech (Newark, De). All chemicals and reagents were of analytical grade.

2.1. Gene Synthesis and Plasmid Construction

The gene encoding XaARF was synthesized by GenScript (Piscataway, NJ) as a 1572 bp open reading frame ligated at the *Hin*dIII site of a modified pUC57

plasmid. The gene was sub-cloned into pET26b expression vector after *Eco*RI and *Hin*dIII digestion. The resulting gene vector construct was transformed into BL21 competent cells.

2.2. Expression and Purification

A single transformant was inoculated in 2 ml LB-Kan medium and cultured at 225 rpm and 30˚C overnight. The culture was diluted 1:50 in 30 ml LB-Kan and grown under the same conditions overnight. Ten ml of the ON culture was added to 400 ml LB-Kan in baffled flasks, and incubated for 5.5 hr (37˚C, 200 rpm) to an OD of 0.7 at 600 nm. Expression was initiated by 0.1 mM IPTG and incubation continued for 15 hr (18˚C, 140 rpm). The cells were pelleted and lysed using Cellytic B cell lysis reagent following supplier's instruction (Sigma-Aldrich, St. Louos. MO). The lysate was run through a Ni-Sepharose affinity column, using a binding (starting) buffer of 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole at pH 7.4 with gradient elution to 300 mM imidazole. The peak fractions (OD280) were combined and buffer exchanged into 20 mM sodium phsophate, pH 7.5 with 10% glycerol, and concentrated using centrifugal filters (Millipore, Burington, MA).

2.3. Bioinformatics

The gene/protein sequence was retrieved from GenBank AAM36157.1.

Geneious (Biomatters Ltd., Auckland, New Zealand) was used for sequence analysis and vector construction. Multiple sequence alignment was performed using Clustal Omega. For graphics and statistics, KaleidaGraph software (Snergy, Reading, PA) was used for calculating standard errors and for plotting.

2.4. Electrophoresis

The purified enzyme was run on a Bis-Tris NuPAGE gradient gel (4% - 12%) using 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS) buffer using denaturing and reducing conditions (constant 100 V for 2 h). The developed gel was stained with SimplyBlue Safe stain (Invitrogen, Carlsbad, CA, USA). The protein marker was Precision plus Kaleidoscope standards (BioRad, Hercules, CA). Gel bands were analyzed by image analysis software (Alpha Innotech, AlphaImager, San Jose, CA, USA).

2.5. Analysis of Hydrolytic Release of Arabinose from AXOS Substrates

The ability of the enzyme to hydrolyze AXOS was analyzed by monitoring the release of arabinose. The analysis was performed on a Shimadzu LC-10AD delivery system using a RCM monosaccharide Ca^{2+} column (200 × 10.0 mm, Agilent) with H_2O as the eluant, at a flow rate of 0.2 ml/min at 85˚C. The eluant was monitored with a refractive index detector. Arabinose peaks were identified by retention time and spiking with standards.

Hydrolysis of AXOS was also estimated by monitoring the formation of oligomeric products using thin-layer chromatography. TLC separation was performed on silica gel plates, and developed twice with a mobile phase of n-BuOH/HCOOH/H_2O (2:3:1) [8]. The sugars were detected by spraying the developed plate with 10% H_2SO_4 in methanol with 1 mg/ml orcinol.

3. Results and Discussion

3.1. Cloning and Bioinformatics of the XaARF Gene

The 1503 nt sequence of the XaARF gene was synthized (GenScript, Piscataway, NJ) and sub-cloned into pET26b expression vector. A BLASTP search reveals the sequence related to the primary structures of *Xanthomonas campestris* α-L- arabinofuranosidase (Q8PBD1) and *Rhodanobactor fulvus* α-L-arabinofuranosidase (14W0Z9) with 82.6% and 73.2% identitiy. Santos *et al.* [9] has identified this enzyme XacARF51 having a trimeric structure that is active in cleaving arabinofuranosyl linkages of internal di-substituted xylopyranosyl units.

3.2. Enzyme Protein Purification

The XaARF expressed in *E. coli* clone was purified by Ni-Sepharose affinity column. A single band was obtained on 10% Tris-Glycine gel and SDS-PAGE 4% - 12% gradient gel. **Figure 1** shows the affinity chromatogram with the insert of the 10% Tris-Glycine gel (run under denaturing and reducing conditions following Invitrogen's protocol). The enzyme protein shows a single band at 0.15 mg and 0.3 mg sample loading.

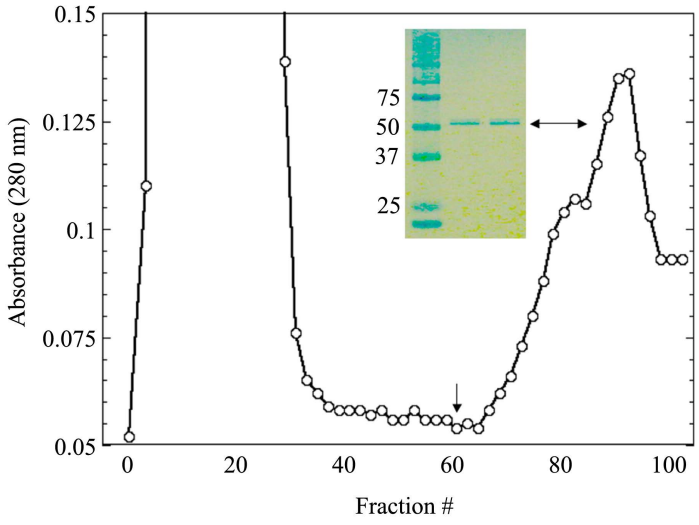

Figure 1. Affinity purification of XaARF. Conditions: Ni-Sepharose affinity column, using a binding (starting) buffer of 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole at pH 7.4 with gradient elution to 300 mM imidazole. For the SDS-PAGE insert, the purified enzyme was run on a precast 10% Tris glycine gel using 50 mM 3- morpholinopropane-1-sulfonic acid (MOPS) buffer solution using denaturing and reducing conditions (constant 100 V for 2 h). The protein marker was Precision plus Kaleidoscope standards (BioRad, Hercules, CA).

3.3. Effect of pH on XaARF on Arabino-Xylooligosaccharides (AXOS)

The pH effect on the enzyme action was tested using A^2XX AXOS substrate with the arabinose product monitored by HPLC. The substrate (see Figure 2 for chemical structures) was constituted in universal buffer. The reaction mixture contained 0.6 mM buffer, 1.7 nmole XaARF, 10 mM A^2XX in a final volume of 60 ml. The digestion was observed to decrease with basic pH, from 143.40 ± 3.03 at pH 4.0, and 136.25 ± 4.03 at pH 5.0 to 25.38 ± 6.29 at pH 8.0 (Figure 3).

The pH effect was further studied using 1,5-*α*-L-arabinoheptaose (Ara7) as substrate. The increase in arabinose and the decrease in the substrate during digestion were monitored by HPLC. The results confirm that the enzyme action was favored at a low pH range (Figure 4). A pH 5.5 sodium acetate buffer was hence used for enzyme action analyses.

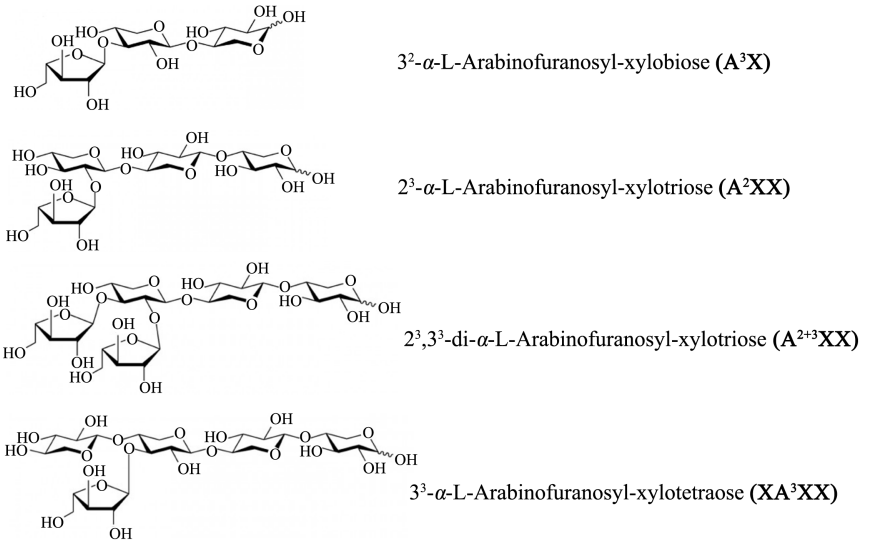

Figure 2. Chemical structures of AXOS substrates from Megazyme used in the analysis of enzyme actions.

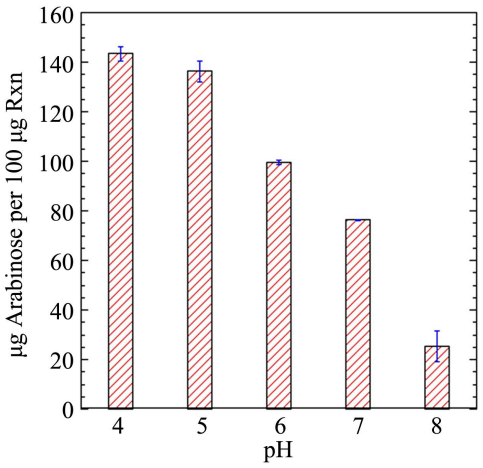

Figure 3. pH effect on XaARF action with A^2XX as substrate.

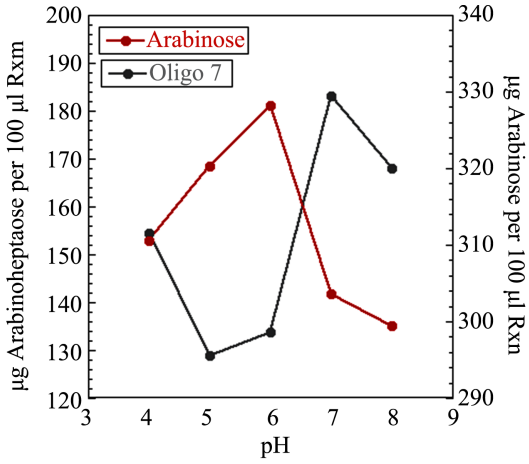

Figure 4. pH effect on XaARF action using 1,5-*α*-L-Aarabinoheptaose as substrate.

3.4. Comparison of Enzyme Specificity on Four Arabino-Xylooligosaccharides (AXOS)

The enzyme action on 4 arabino-xylooligosaccharides of varying structural architecture was investigated. The order of reactivity showed the following: A^3X > XA^3XX > $X^{2+3}XX$ > A^2XX. The graph in **Figure 5** suggests that XaARF catalyzed efficient cleavage of the arabinose substituted at C3 of the xylopyranosyl unit. The action was relatively faster if the xylopyranosyl unit for the singly arabinofuranosyl substitution was at the terminal (non-reducing) end (reaching a maximum yield of 180 mg arabinose per 100 ml reaction of A^3X in 1 hr). This was compared to the slower process observed for internal units which attained a maximum yield of 182 mg arabinoase per 100 ml reaction of XA^3XX in 2 hr. In contrast, arabinofuranoyl linked to C2 of the xylopyranosyl unit, even at the terminal end (for example, A^2XX), exhibited the lowest yield of arabinose for the first three time points. In conclusion, XacARF showed a preference for C3-linked over C2-linked arabinose from singly substituted xylopyranosyl terminal ends over internally located units.

The enzyme action on $A^{2+3}XX$ warrants further investigation. The yield amount of arabinose was significantly higher than those observed for A^3X and XA^3XX, producing 231 mg arabinose per 100 ml reaction at 2 hr incubation (**Figure 6**). This result clearly suggests that the enzyme was able to act on di-substituted xylopyranosyl units. It is likely that the C3-linked arabinose was cleaved in the first stage of the reaction, followed by the cleavage of the C2-linked arabinose.

The XaARF action was compared with two commercially available ARFs: BaARF and AnARF (both from Megazyme Ltd.). BaARF belongs to family GH43 and has been known to specifically cleave terminal arabinofuranosyl residues linked to C3 of double-substituted xylopyranosyl units [10] [11]. AnARF is a family 51 enzyme, which is known to hydrolyze terminal arabinofuranosyl linkages [12]. The graph in **Figure 6** supports such activity description. Furthermore, the enzyme XaARF produces twice or more the yield of arabinose under the same reaction conditions because it catalyzes the release of both C2- and C3-linked

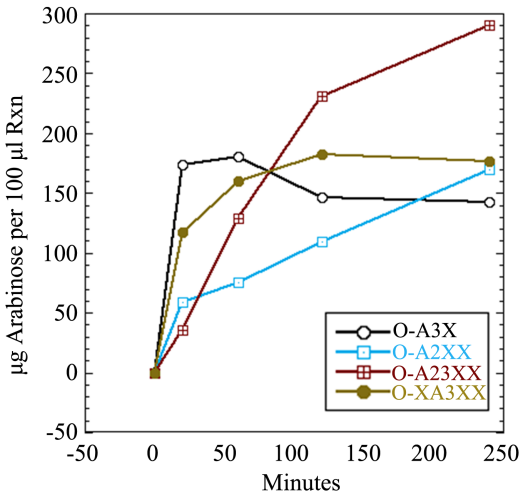

Figure 5. XaARF action on $A^{3}X$, $A^{2}XX$, $A^{2+3}XX$, and $XA^{3}XX$. Conditions: 0.02 nmole enzyme, 10 mM AXOS, 50 mM sodium acetate, pH 5.5, 40˚C incubation.

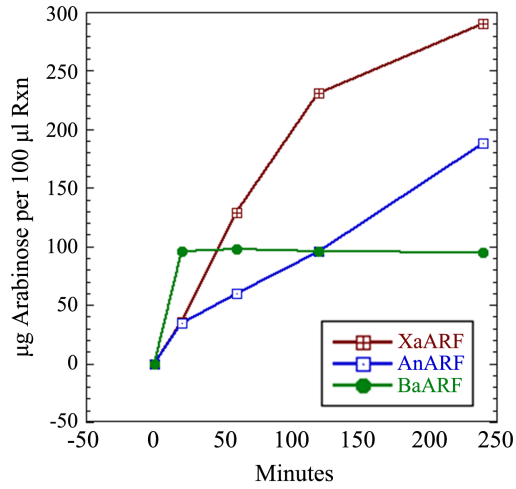

Figure 6. Comparison of XaARF, BaARF, and AnARF activities on hydrolysis of $A^{2+3}XX$. Conditions: 0.02 nmole enzyme, 10 mM $A^{2+3}XX$, 50 mM sodium acetate, pH 5.5, 40˚C incubation.

arabinofuranosyl residues from double-substituted xylose units (as also confirmed by the results presented in **Figure 5**).

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

References

[1] Biely, P., Vršanskáa, M., Tenkanen, M. and Kluepfel, D. (1997) Endo-β-1,4-Xylanase Families: Differences in Catalytic Properties. *Journal of Biotechnology*, **57**, 151-166. https://doi.org/10.1016/S0168-1656(97)00096-5

[2] Biely, P., Singh, S. and Puchart, V. (2016) Towards Enzymatic Breakdown of Complex Plant Xylan Structures: State of the Art. *Biotechnology Advances*, **34**, 1260-1274. https://doi.org/10.1016/j.biotechadv.2016.09.001

[3] Biely, P. (2003) Xylanolytic Enzymes. In: Whitaker, J.R., Voragen, F. and Wong, D., Eds., *Handbook of Food Enzymology*, Marcel Dekker, New York.

[4] Izydorczyk, M.S. (2021) Arabinoxylan. In: Phillips, G. and Williams, P., Eds., *Handbook of Hydrocolloids*, 3rd Edition, Elsevier, Ltd., Amsterdam, 399-441. https://doi.org/10.1016/B978-0-12-820104-6.00016-4

[5] Zannini, E., Nunez, A.B., Sahin, A.W. and Arendt, E.K. (2022) Arabinoxylans as Functional Food Ingredients: A Review. *Foods*, **11**, Article 1026. https://doi.org/10.3390/foods11071026

[6] Sørensen, H.R., Pedersem. S., Jørgensen, C.T. and Meyer, A.S. (2007) Enzymatic Hydrolysis of Wheat Arabinoxylan by a Recombinant "Minimal" Enzyme Cocktail Containing β-Xylosidase and Novel Endo-1,4-β-Xylanase an α-L-Arabinofuranosidase Activities. *Biotechnology Progress*, **23**, 100-107. https://doi.org/10.1021/bp0601701

[7] Wilkens, C., Andersen, S., Dumon, C., Berrin, J.-G. and Svensson, B. (2017) GH62 Arabinofuranosidase: Structure, Function and Application. *Biotechnology Advances*, **35**, 792-804. https://doi.org/10.1016/j.biotechadv.2017.06.005

[8] Koller, A. and Neukom, H. (1964) Detection of Oligogalacturonic Acids by Thin-Lauer Chromatography. *Biochimica et Biophysica Acta* (*BBA*)—*Specialized Section on Mucoproteins and Mucopolysaccharides*, **83**, 366-567. https://doi.org/10.1016/0926-6526(64)90020-5

[9] Santos, C.R.D., De Giuseppe, P.O., De Souza, F.H.M., Zanphorlin, L.M., Domingous, M.N., Pirolla, R.A.S., Honorato, R.V., Tonoli, C.C.C., De Morais, M.A.B., De Matos Martin, V.P., Fonsecam, L.M., Büchli, F., De Oliveira, P.S.L., Gozzo, F.C. and Murakami, M.T. (2008) The Mechanism by Which a Distinguishing Arabinofuranosidase Can Cope with Internal Di-Substitutions in Arabinoxylans. *Biotechnology for Biofuels*, **11**, Article No. 223. https://doi.org/10.1186/s13068-018-1212-y

[10] Van Laere, K.M.J. and Voragen, A.G.J. (1997) A New Arabinofuranohydrolase from *Bifidobacterium adolescentis* Ablt to Remove Arabinosyl Residues from Double-Substituted Xylose Units in Arabinoxylan. *Applied Microbiology and Biotechnology volume*, **47**, 231-235. https://doi.org/10.1007/s002530050918

[11] Van Den Broek, L.A.M., Lloyd, R.M., Beldman, G., Verdoes, J.C., McCleary, B.V. and Voragen, A.G.J. (2005) Cloning and Characterization of Arabinoxylan Arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* DSM20083. *Applied Microbiology and Biotechnology*, **67**, 641-647. https://doi.org/10.1007/s00253-004-1850-9

[12] Kaneko, S., Ishii, T., Kobayashi, H. and Kusakabe, I. (1998) Substrate Specificities of α-L-Arabinofuranosidases Produced by Two Species of *Aspergillus niger*. *Bioscience, Biotechnology, and Biochemistry*, **62**, 695-699. https://doi.org/10.1271/bbb.62.695