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Detailed Mode of Action of Arabinan-Debranching α-L-Arabinofuranosidase GH51 from *Bacillus velezensis*

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Running title: Arabinan-debranching AFase from *Bacillus velezensis*

Abstract

The gene encoding an α-L-arabinofuranosidase (BvAF) GH51 from *Bacillus velezensis* FZB42 was cloned and expressed in *Escherichia coli*. The corresponding open reading frame consists of 1,491 nucleotides which encodes 496 amino acids with the molecular mass of 56.9 kDa. BvAF showed the highest activity against sugar beet (branched) arabinan in 50 mM sodium acetate buffer (pH 6.0) at 45ºC. However, it could hardly hydrolyze debranched arabinan and arabinoxylans. The time-course hydrolyses of branched arabinan and arabinooligosaccharides (AOS) revealed that BvAF is a unique *exo*-hydrolase producing exclusively L-arabinose. BvAF could cleave α-(1,2)- and/or α-(1,3)-L-arabinofuranosidic linkages of the branched substrates to produce the debranched forms of arabinan and AOS. Although the excessive amount of BvAF could liberate L-arabinose from linear AOS, it was extremely lower than that on branched AOS. In conclusion, BvAF is the arabinan-specific *exo*-acting α-L-arabinofuranosidase possessing the high debranching activity towards α-(1,2)- and/or α-(1,3)-linked branches of arabinan, which can facilitate the successive degradation of arabinan by *endo*-α-1,5-L-arabinanase.

Keywords: *Bacillus velezensis*, α-L-arabinofuranosidase, arabinan-debranching activity, mode of action

Introduction

L-Arabinose is one of the representative pentose sugars in nature, which is abundant as the component of hemicellulosic biomass in plant cell wall. Especially, the food scientists are taking an increasing interest in the health-beneficial functionality of L-arabinose as a low-calorie alternative sweetener [1]. The inhibitory action of L-arabinose against the intestinal sucrase has known to reduce the digestion and absorption of sucrose [2]. Recently, the prebiotic effects of arabinoxylan oligosaccharides, linear arabinan, and arabino-oligosaccharides (AOS) were also reported on the growth-promotion of probitic microorganisms such as *Bifidobacterium* and *Lactobacillus* species [3-5]. Sugar beet (branched) arabinan is an α-1,5-linked L-arabinofuranosyl polymer with α-(1,2)- and/or α-(1,3)-linked arabinofuranosyl branches. The cost-effective production of L-arabinose has been accomplished via the enzymatic hydrolysis of sugar beet arabinan by the concerted actions of *endo*-α-(1,5)-L-arabinanase (ABNase; EC 3.2.1.99) and *exo*-acting α-L-arabinofuranosidase (AFase; EC 3.2.1.55) [6, 7].

The AFase can specifically cleave the terminal non-reducing L-arabinofuranosidic linkages in various arabinose-containing polymers such as arabinans and arabinoxylans [8, 9]. For the efficient degradation of heteropolysaccharides such arabinoxylans, AFase should be the essential accessory enzyme which works in concert with other main hemicellulases including β-xylanase and β-xylosidase. A variety of bacteria, fungi, and plants has been known as the main producer of AFase which mainly belongs to the Glycoside Hydrolase (GH) family 2, 3, 43, 51, 54, and 62 [10]. Among these six GH families, the AFases GH43 and GH51 include the majority of hydrolases being specific for the degradation of arabinans. The three-dimensional structure of AFase GH51 from *Geobacillus stearothermophilus* revealed that it possesses the catalytic $(\beta/\alpha)_8$-barrel domain and the C-terminal jelly-roll architecture

[11]. Meanwhile, *Streptomyces avermitilis* sp. was reported to produce the novel *exo*-(1,5)-α-L-arabinofuranosidase GH43 consisting of the catalytic five-bladed β-propeller fold with a C-terminal carbohydrate-binding module [12].

The gene clusters involving in the enzymatic degradation and utilization of arabinans and L-arabinose were found from *Bacillus subtilis* [13, 14], *Geobacillus stearothermophilus* [15], and *Corynebacterium glutamicum* [16]. From the genome of *B. subtilis* 168T^{+}, two *exo*-AFases GH51 (AbfA and Abf2) [14] and two *endo*-ABNases GH43 (AbnA and Abn2) [17, 18] were comparatively studied. An intracellular AFase of AbfA is active towards both sugar beet arabinan and debranched arabinan, while the other intracellular enzyme of Abf2 can hydrolyze only sugar beet arabinan. However, the detailed mode of action of Abf2 has not been reported yet.

In the present study, the putative gene encoding an AFase (hereafter abbreviated as BvAF) was found and cloned from the genome of *Bacillus velezensis* FZB42. The enzymatic properties and the detailed mode of actions towards sugar beet arabinan and branched AOS were investigated by using BvAF as a model enzyme to understand the debranching AFases.

Materials and Methods

Enzymes and reagents

Restriction endonucleases and *Pyrobest* DNA polymerase were purchased from Takara Bio (Japan). *AccuPrep* plasmid extraction kit, PCR purification kit, and oligonucleotide primers were provided by Bioneer (Korea). DNeasy Blood & Tissue kit (Qiagen, Germany) was used for the genomic DNA preparation. Sugar beet arabinan, debranched arabinan, arabinoxylans, and AOS were procured from Megazyme (Ireland).

Gene amplification and cloning

Genomic DNA was prepared from *B. velezensis* FZB42 (DSM 23117) grown in nutrient broth (0.5% peptone, 0.3% meat extract, and 1.0% $MnSO_4$) at 30ºC. A set of PCR primers, BvAF-N (5'-TTTTGAGCTCATGTCTCACAATACGGC-3') and BvAF-C (5'-TTTTCTCGA GAGATGGATCCGCAGG-3'), were designed to amplify the gene encoding BvAF (GenBank ID: WP_012118129.1). PCR was performed using C-1000 thermal cycler (Bio-Rad, UK) as follows: an initial denaturation step at 98ºC for 30 sec, followed by 30 cycles consisting of denaturation at 98ºC for 10 sec, annealing at 54ºC for 30 sec, extension at 72ºC for 1 min 30 sec, and a final extension at 72ºC for 5 min. The PCR fragment was digested with *Nde*I and *Xho*I, which was cloned into an expression vector, pHCXHD [19]. The resulting recombinant plasmid was designated as pHCBvAF. The entire nucleotide sequence was verified by SolGent (Korea). *Escherichia coli* MC1061 was used as a host for the gene manipulation and expression.

Gene expression and enzyme purification

E. coli harboring pHCBvAF was grown in LBA broth (0.5% bacto-tryptone, 1% yeast extract, 1% NaCl, 100 μg/ml of ampicillin) at 37°C for 14 h. *E. coli* BL21 (DE3) harboring pETBlABN [20] was also cultivated in LBA broth at 37°C with 0.1 mM IPTG induction. The recombinant *E. coli* cells were harvested and disrupted by ultrasonicator VCX750 (Sonics & Materials, USA). Each enzyme with C-terminal six-histidines was purified by using a Ni-NTA affinity chromatography (Qiagen, Germany). The purity and the molecular mass of each enzyme were determined by using 12% SDS-PAGE analyses. The protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, USA) with bovine serum albumin as a standard.

Enzyme activity assay

The 3,5-dinitrosalicylic acid (DNS) reducing sugar method [21] was employed for the determination of the hydrolyzing activity against arabinans or arabinoxylans. L-Arabinose/D-Galactose assay kit (Megazyme, Ireland) was applied for the activity assay towards AOS. The activity towards *p*-nitrophenyl arabinofuranoside (*p*-NPAf; Sigma-Aldrich, USA) was determined by measuring the amount of *p*-nitrophenol liberated from the reaction. The purified enzyme was reacted with 0.5% of each substrate (except for 2.5% of arabinoxylans) under the optimal condition for an appropriate reaction time. One unit of enzyme activity on each substrate was defined as the amount of enzyme producing 1 μmol/min of L-arabinose equivalent.

Time-course analysis of hydrolysis products

Thin layer chromatography (TLC) was applied to the analysis of hydrolysis products. An appropriate amount of enzyme was reacted with 0.5% of each substrate under the optimal condition. The resulting hydrolysis products were separated three times on a $60F_{254}$ silica gel plate (Merck, Germany) using chloroform:acetate:water (6:7:1) as a solvent. The spots were visualized by dipping the plate in a developing solution (0.3% *N*-1-naphthyl-ethylenediamine and 5% H_2SO_4 in methanol), and subsequently heating it at 110°C for 10 min. In order to determine the synergistic arabinan-degradation and hydrolysis yield, 0.5% of each substrate was reacted with 0.5 U/ml of BvAF or BlABN (*B. licheniformis endo*-arabinanase) [20] in 50 mM sodium acetate buffer (pH 6.0) at 45°C for 5 h. For the stepwise enzyme treatments, either of *exo*-BvAF and *endo*-BlABN was firstly reacted with a substrate for 150 min. After the heat inactivation by boiling for 3 min, the next enzyme was then treated for additional

150 min at the second step. For the simultaneous enzyme treatment, each substrate was reacted with 0.25 U/ml of BvAF and BlABN for 300 min. The liberated reducing sugar or L-arabinose was measured by DNS assay and L-Arabinose/D-Galactose assay, respectively. For the time-course hydrolysis of branched AOS, 3^2-α-L-arabinofuranosyl-1,5-α-L-arabinotriose (AA^3A), or the mixture of 3^2-α-L-arabinofuranosyl-1,5-α-L-arabinotetraose (AAA^3A) and 2^2,3^2-di-α-L-arabinofuranosyl-1,5-α-L-arabinotriose ($AA^{2+3}A$) was reacted with 0.05 U/ml of BvAF, respectively. For the complete hydrolysis of AOS, the excessive amount (0.5 U/ml) of BvAF was treated with a substrate for 12 h.

Results and Discussion

Gene cloning and expression of BvAF

The Gram-positive bacterium, *Bacillus velezensis* FZB42 (the synonym of *B. amyloliquefaciens* subsp. *plantarum* and *B. methylotrophicus*), was known as the model strain for the promotion of plant growth and the biocontrol of rhizobacteria [22, 23]. Recent comparative genome analyses among various *B. velezensis* spp. have revealed their potential as the degraders for lignocellulosic biomass [24]. An open reading frame encoding a novel AFase (BvAF) was found from the genome of *B. velezensis* FZB42 [25]. Approximately 1.5 kb of DNA fragment was amplified using a set of PCR primers, BvAF-N and BvAF-C. The resulting PCR-fragment was cloned into a constitutive expression vector of pHCXHD, which was designated as pHCBvAF. The DNA sequencing analysis verified that the corresponding open reading frame consists of 1,491 nucleotides encoding 496 amino acid residues. The gene encoding BvAF with C-terminal six-histidines was constitutively expressed from *E. coli* harboring pHCBvAF (Fig. 1). The recombinant BvAF was purified to an apparent homogeneity via a Ni-NTA affinity chromatography. The apparent molecular mass of

recombinant BvAF including six-histidines is approximately 56 kDa, which coincides with its calculated molecular mass of 56,857 Da.

Two genes encoding AFases GH51 with different substrate specificities, AbfA (WP_004398747.1) and Abf2 (WP_004398654.1), were previously found from the genome of *B. subtilis* 168T$^+$ [14]. The deduced amino acid sequence of BvAF shares the identities of 23.8% and 81.3% with AbfA and Abf2, respectively. The AFases from *Geobacillus* sp. KCTC3012 (ABM68633.1) [19], *Geobacillus stearothermophilus* T-6 (AAD45520.2) [11], and *Thermotoga maritima* MSB8 (NP_228093.1) [26] showed 23.3%, 23.8%, and 27.5% of amino acid sequence identities with BvAF, respectively. The overall structure of common AFases GH51 consists of both catalytic $(\beta/\alpha)_8$-barrel domain and C-terminal jelly-roll domain [11, 27]. Although the primary structure similarity of BvAF with the other AFases GH51 is less than 30%, the amino acid sequence alignment revealed that BvAF shares two putative catalytic amino acid residues, a general acid/base (Glu172) and a nucleophile (Glu295).

Enzymatic characterization of BvAF

To optimize the reaction conditions, the hydrolyzing activity of BvAF on sugar beet arabinan as a substrate was determined by using DNS reducing sugar and L-arabinose assay methods. BvAF exhibited the highest activity (41.3 U/mg) in 50 mM sodium acetate buffer (pH 6.0) at 45°C (Fig. 2). It also showed over 77% of relative activity at 35~55°C, but it was rapidly decreased to 16% at 70°C. The half-life of BvAF was determined as 15 h at 45°C and 4 min at 70°C, respectively. BvAF possesses relatively narrow optimum near pH 6.0, but it showed the relatively high pH stability at the broad pH range of 4.0~10.0 (data not shown). Most known microbial AFases have their reaction optima at pH 5.0~8.0 and 40~60°C [8, 9].

For example, the AFases (AbfA and Abf2) from *B. subtilis* showed their highest activity at 50~60ºC and pH 8.0 [14], whereas that from *Geobacillus* sp. (GAF) is also highly active at 60ºC and pH 5.0 [19]. The highly thermostable AFases were also reported from *Thermotoga maritima* [26] and *Caldicellulosiruptor saccharolyticus* [28].

The specific activities of BvAF were investigated on various substrates such as *p*-NPAf, AOS, arabinans, and arabinoxylans (Table 1). BvAF showed the highest activity (207.6 U/mg) on *p*-NPAf, the synthetic substrate for *exo*-AFase activity assay, which implies that BvAF is likely to be a typical *exo*-acting AFase. This enzyme could preferentially hydrolyze sugar beet arabinan, whereas it showed only negligible activities towards various arabinoxylans. Interestingly, BvAF could hardly hydrolyze debranched arabinan, a common substrate for AFases. Its much lower relative activity (7~9%) on linear AOS than sugar beet arabinan polymer suggested that BvAF is a novel *exo*-hydrolase exclusively cleaving α-(1,2)- and/or α-(1,3)-linked arabinosyl residues at the branches, not α-(1,5)-linkages in the backbone of sugar beet arabinan.

B. subtilis AbfA is the intracellular AFase being active towards both sugar beet arabinan and debranched arabinan, which can be expressed from the gene cluster for arabinan-degradation and utilization. On the contrary, the other intracellular Abf2 could hydrolyze only sugar beet arabinan, which is not included within the same gene cluster. As the detailed mode of debranching action of Abf2 has not been reported yet, BvAF was investigated as a model for debranching AFases in the present study.

Detailed mode of debranching action of BvAF

In order to investigate the hydrolysis patterns of BvAF, 0.5 U/ml of enzyme was reacted with 0.5% of each substrate, and the resulting hydrolysates were comparatively identified by TLC analysis (Fig. 3). As the reaction proceeded, BvAF could exclusively release

L-arabinose without any AOS intermediates from sugar beet arabinan. The time-course analyses revealed the stepwise degradation of AOS via *exo*-type enzymatic actions of BvAF. For example, BvAF firstly hydrolyzed arabinotetraose to arabinotriose and L-arabinose, and then the resulting arabinotriose was further hydrolyzed into arabinobiose and L-arabinose. However, its much low activity towards α-(1,5)-arabinofuranosyl linkages resulted in the slow and incomplete hydrolysis of linear AOS. When the excessive amount (5.0 U/ml) of BvAF was reacted with the substrates for 12 h, all the AOS substrates were finally hydrolyzed into only L-arabinose (data not shown).

An *endo*-α-(1,5)-L-arabinanase from *B. licheniformis* DSM 13 (BlABN) was known to have no detectable activity on sugar beet arabinan, due to its extremely low accessibility towards branched structure [20]. Therefore, the synergistic and efficient degradation of sugar beet arabinan was achieved via the simultaneous treatment with an AFase from *Geobacillus* sp. KCTC3012 (GAF) [7]. As GAF has almost same activities (4.52 U/mg) against both sugar beet and debranched arabinans, the simultaneous treatment of both *exo*-GAF and *endo*-BlABN on arabinans could maximize their cooperative and complementary actions to shorten the operation time and increase the conversion yield into L-arabinose. The synergistic production of L-arabinose by the thermostable *exo*- and *endo*-arabinanases was also reported from *Caldicellulosiruptor saccharolyticus* [6]. In Fig. 4, the single treatment of BlABN or BvAF (0.5 U/ml) showed no detectable activity against sugar beet arabinan or debranched arabinan, respectively. When sugar beet arabinan was pre-treated with BvAF for 150 min, total 13.3 μmol of L-arabinose was released. Interestingly, BlABN could attack the resulting BvAF-treated sugar beet arabinan to produce various AOS up to 28.6 μmol of arabinose equivalent. The simultaneous treatment of BvAF and BlABN resulted in more rapid hydrolysis of sugar beet arabinan at the early reaction step than the single or the stepwise

enzyme treatments. The pre-treatment of BvAF could remove α-(1,2)- and/or α-(1,3)-linked arabinofuranosyl branches of sugar beet arabinan to generate the debranched form of arabinan, which can be the more accessible substrate for the *endo*-actions of BlABN. Although the simultaneous treatment of BvAF and BlABN showed the highest hydrolysis yield of 38.1% from sugar beet arabinan, it is much lower than that with GAF and BlABN (91.5%) [7]. The incomplete degradation of arabinan into the residual short AOS was caused by the much low hydrolyzing activity of BvAF against the α-(1,5)-linked arabinan backbone.

To understand the detailed mode of debranching actions, three different branched AOS were reacted with 0.05 U/ml of BvAF, and the resulting hydrolysates were identified by time-course TLC analysis (Fig. 5). In case of AA^3A, BvAF firstly attacked α-(1,3)-linked arabinofuranosyl branch to produce arabinotriose. The resulting arabinotriose was very slowly degraded to L-arabinose and arabinobiose. Towards the mixture of AAA^3A and $AA^{2+3}A$, α-(1,3)-linked arabinofuranosyl branch of AAA^3A was rapidly removed via the debranching activity of BvAF at the early reaction stage. The resulting arabinotetraose was also very slowly hydrolyzed to produce L-arabinose and AOS intermediates. After the preferred hydrolysis of AAA^3A, the residual $AA^{2+3}A$ was gradually hydrolyzed into arabinotriose at the slow rate. When 0.5 U/ml of BvAF was reacted for 12 h, all three different branched AOS were completely saccharified into L-arabinose as a sole final product. The detailed mode of action of BvAF on sugar beet arabinan and branched AOS was schematically represented in Fig. 6.

Compared with common AFases including GAF, BvAF is the novel arabinan-specific *exo*-hydrolase having the high debranching activity towards α-(1,2)- and/or α-(1,3)-linked branches of sugar beet arabinan to generate the debranched arabinan. The debranching actions of BvAF can promote more rapid and efficient arabinan-degradation by the other *exo*-

and *endo*-arabinosyl hydrolases from *B. velezensis*. The deeper insight on the AFases with versatile mode of actions will expedite the enzyme engineering for the development of designer's prebiotics in future.

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Conflict of Interest

The Authors have no financial conflicts of interest to declare.

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Figure legends

Fig. 1. SDS-PAGE analysis for gene expression and enzyme purification of BvAF from recombinant *E. coli*. Lane M, protein size marker; 1, cell extract from *E. coli* harboring an empty vector (pHCXHD); 2, cell extract from *E. coli* harboring pHCBvAF; 3, recombinant BvAF purified by Ni-NTA chromatography.

Fig. 2. Effect of (A) temperature and (B) pH on the arabinan-hydrolyzing activity of BvAF. Relative activities of BvAF on sugar beet arabinan was determined at different temperatures and pH by using DNS reducing sugar assay.

Fig. 3. TLC analysis of the hydrolysates from various substrates reacted with BvAF. Each substrate (0.5%) was reacted with 0.5 U/ml of BvAF at pH 6.0 and 45ºC for 0, 10, 60, and 360 min. The arrow head indicates L-arabinose as a reaction product. AS, arabino-oligosaccharides standard; SA, sugar beet arabinan; DA, debranched arabinan; A2~A4, arabinobiose to arabinotetraose.

Fig. 4. Time-course TLC analysis of (A) arabinan-degradation and (B) hydrolysis yield via single, stepwise, or simultaneous treatment with BvAF (AF) and BlABN (AN). 0.5 U/ml of each enzyme was reacted with 0.5% of sugar beet arabinan (SA) or debranched arabinan (DA) for 300 min, respectively. For the stepwise treatment, the first enzyme was heat-inactivated after 150 min (vertical arrow), and the second enzyme was reacted for additional 150 min. For the simultaneous treatment, 0.25 U/ml of both AF and AN were reacted with arabinan for 300 min. The hydrolysis products were measured by DNS reducing sugar assay.

Fig. 5. TLC analysis of the hydrolysates from the branched arabinooligosaccharides. (A) 0.5% of AA^3A or (B) the mixture of AAA^3A and $AA^{2+3}A$ was reacted with 0.05 U/ml of BvAF at pH 6.0 and 45ºC for 0, 15, 30, 90, and 360 min, respectively. AS, arabinooligosaccharides standard; EX, the excessive treatment with 0.5 U/ml of BvAF for 12 h.

Fig. 6. Proposed mode of debranching actions of BvAF against sugar beet arabinan and branched arabinooligosaccharides. The solid and the dashed arrows indicate the high and the extremely low hydrolyzing activities of BvAF, respectively. The routes marked by asterisks are not clarified.

Table 1. Specific activities of BvAF on polymeric and oligomeric substrates

Substrate	Specific activity (U/mg)
p-Nitrophenyl arabinofuranoside	207.56 ± 9.70
Sugar beet arabinan	41.29 ± 4.13
Debranched arabinan	0.88 ± 0.14
Arabinoxylans[a]	ND[b]
Arabinobiose	3.10 ± 0.17
Arabinotriose	2.05 ± 0.22
Arabinotetraose	3.09 ± 0.26
Arabinopentaose	3.43 ± 0.16
Arabinohexaose	3.46 ± 0.21

[a] Oat-spelt, rye, and wheat arabinoxylans

[b] Enzymatic activity was not detected.

Fig. 1

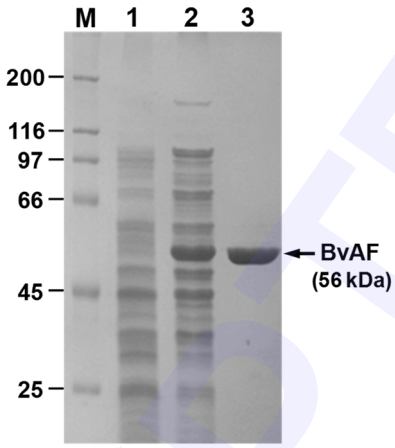


Fig. 2

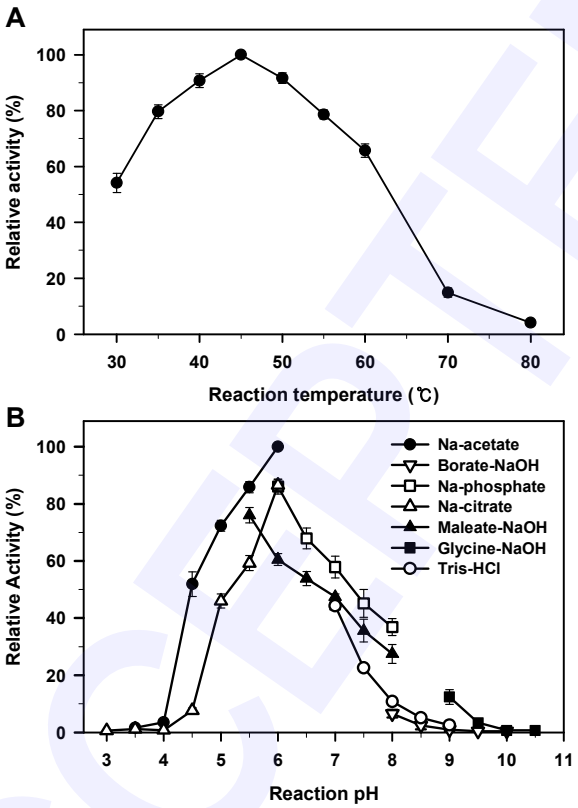
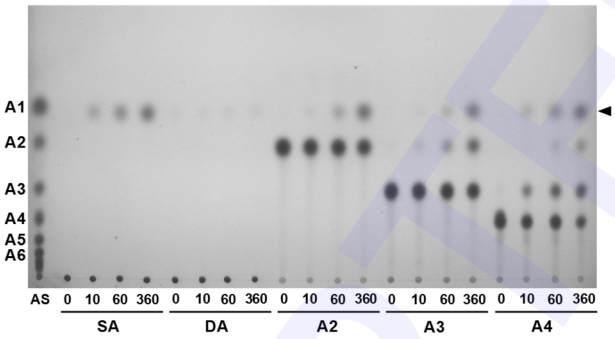


Fig. 3



446 **Fig. 4**

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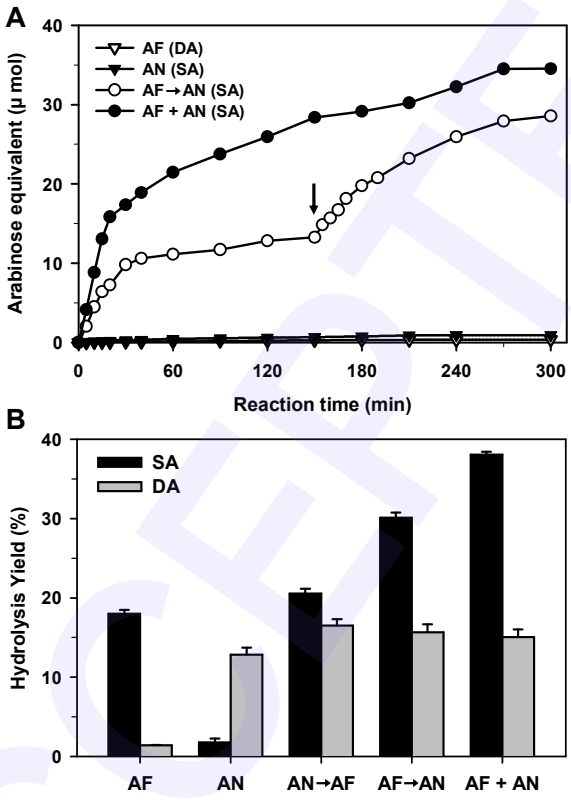

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Fig. 5

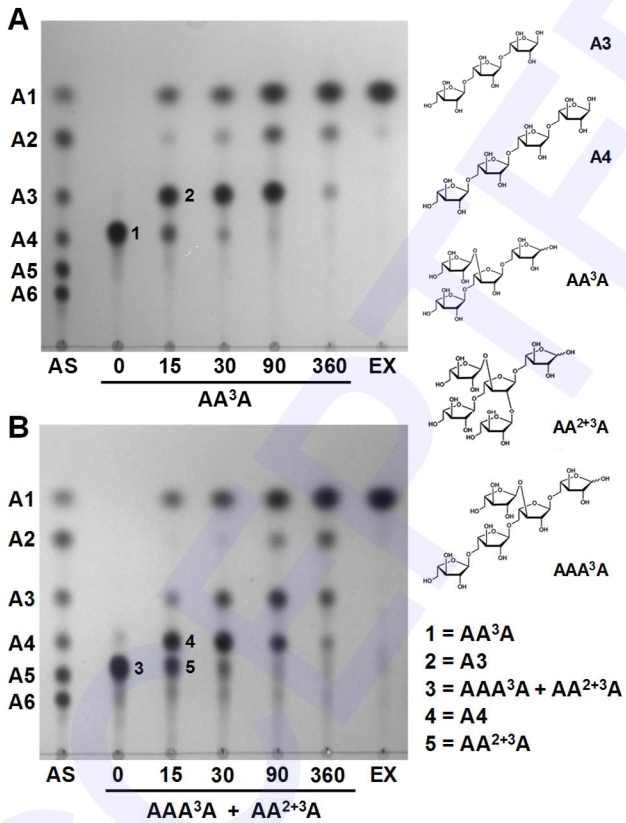

Fig. 6