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Synergistic Action Modes of Arabinan Degradation by *Exo*- and *Endo*-Arabinosyl Hydrolases

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Two recombinant arabinosyl hydrolases, α-L-arabinofuranosidase from *Geobacillus* sp. KCTC 3012 (GAFase) and *endo*-(1,5)-α-L-arabinanase from *Bacillus licheniformis* DSM13 (*Bl*ABNase), were overexpressed in *Escherichia coli*, and their synergistic modes of action against sugar beet (branched) arabinan were investigated. Whereas GAFase hydrolyzed 35.9% of L-arabinose residues from sugar beet (branched) arabinan, *endo*-action of *Bl*ABNase released only 0.5% of L-arabinose owing to its extremely low accessibility towards branched arabinan. Interestingly, the simultaneous treatment of GAFase and *Bl*ABNase could liberate approximately 91.2% of L-arabinose from arabinan, which was significantly higher than any single *exo*-enzyme treatment (35.9%) or even stepwise *exo*- after *endo*-enzyme treatment (75.5%). Based on their unique modes of action, both *exo*- and *endo*-arabinosyl hydrolases can work in concert to catalyze the hydrolysis of arabinan to L-arabinose. At the early stage in arabinan degradation, *exo*-acting GAFase could remove the terminal arabinose branches to generate debranched arabinan, which could be successively hydrolyzed into arabinooligosaccharides *via* the *endo*-action of *Bl*ABNase. At the final stage, the simultaneous actions of *exo*- and *endo*-hydrolases could synergistically accelerate the L-arabinose production with high conversion yield.

Keywords: Synergistic effect, α-L-arabinofuranosidase, *endo*-(1,5)-α-L-arabinanase, arabinan degradation, L-arabinose

Introduction

L-Arabinose is one of the common components in hemicellulosic plant polysaccharides such as arabinan and arabinoxylan [7]. It has been reported that L-arabinose can inhibit intestinal sucrase activity to prevent the obesity caused by a high level of sucrose absorption. Therefore, L-arabinose is highly focused as a food additive such as a low-calorie sweetener [21]. Arabinan is a pectic polysaccharide with a backbone of α-(1,5)-linked L-arabinofuranosyl units, which are substituted for α-(1,2)- and/or α-(1,3)-linked arabinofuranosides [20]. For more efficient hydrolysis of arabinan towards L-arabinose, the side chains and the backbone of arabinan should be simultaneously degraded by a couple of hydrolases.

Arabinan-degrading enzymes can be classified into the α-L-arabinofuranosidases (AFases; E.C. 3.2.1.55) and the *endo*-1,5-α-L-arabinanases (ABNases; E.C. 3.2.1.99) on the basis of their modes of action. AFases are typical *exo*-acting enzymes, which hydrolyze the terminal nonreducing L-arabinose residues from arabinose-containing polysaccharides. This enzyme works in concert with other hemicellulases to completely degrade the hemicellulose backbone, which makes it an essential enzyme for the industrial production of L-arabinose [18]. AFases belong to glycoside hydrolase (GH) families 3, 43, 51, 54, and 62. A variety of AFases have been purified from fungi, bacteria, and plants [2-4, 6, 15, 17, 19]. The three-dimensional structure of AFase GH 51 from *Geobacillus stearothermophilus* revealed that it is a hexamer with a $(\beta/\alpha)_8$-barrel structure [5]. Meanwhile, ABNases are

endo-acting hydrolases that can cleave the internal α-(1,5)-L-arabinofuranosidic linkages of arabinan [1]. Microbial arabinanases have been studied from several *Bacillus*, *Cellvibrio*, and *Aspergillus* spp. [1, 9, 11, 16]. Most ABNases GH 43 are known to possess the monomeric structure with the 5-bladed β-propeller topology [14, 23].

In general, AFases are known to act as the accessory enzymes necessary for the complete hydrolysis of hemicellulosic biomass including arabinoxylan. These enzymes can synergistically hydrolyze the glycosidic bonds of polymers in combination with other hemicellulases [10, 22]. Some AFases with both *exo*- and *endo*-activity on arabinan have been reported [11, 13]. The same effect has been shown when these enzymes act synergistically on arabinoxylan. AFases also act synergistically with *endo*-arabinanase and cinnamoyl esterase from *Aspergillus niger* [8]. Compared with xylan degradation, however, the synergistic effects of *exo*- and *endo*-arabinosyl hydrolases on arabinan degradation have not been studied well. The synergistic L-arabinose production from arabinan was recently reported by using the combined treatment of thermostable enzymes from *Caldicellulosiruptor saccharolyticus* [10]. However, their detailed modes of action in synergism have not been studied yet.

In the present study, the synergistic effect of both *exo*- and *endo*-acting arabinosyl hydrolases on the degradation of arabinan was investigated on the basis of their modes of action. Moreover, we proposed the cost-effective enzymatic production of L-arabinose as a functional five-carbon sugar.

Materials and Methods

Substrates and Chemicals

Sugar beet (branched) arabinan, debranched (linear) arabinan, and arabinooligosaccharides were purchased from Megazyme (Wicklow, Ireland). Oat spelt xylan was obtained from Sigma-Aldrich (MO, USA). Other chemicals were supplied by Duchefa Biochemie (Haarlen, The Netherlands), Sigma-Aldrich, or Merck (Germany).

Gene Expression and Enzyme Purification

In order to produce *Geobacillus* sp. AFase (*G*AFase), *Escherichia coli* MC1061 harboring plasmid pHCXGAF [15] 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, encoding the *Bacillus licheniformis* ABNase gene [16], was cultivated in LBA broth at 37°C for 4 h, and IPTG at the final concentration of 0.1 mM was added to induce the gene expression. After additional cultivation for 12 h, the grown cells were harvested and disrupted by ultrasonication (VCX750; Sonics & Materials, Newtown, CT, USA). The recombinant enzymes with the C-terminal six-histidines were purified by using an AKTA Prime system with a HisTrap-FF column (GE Healthcare, Uppsala, Sweden). The protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as a standard.

Enzyme Activity Assay

Purified enzyme was reacted with 0.5% of each substrate in 50 mM sodium acetate buffer (pH 6.0) at 55°C for an appropriate time. The 3,5-dinitrosalicylic acid (DNS) reducing sugar method [12] was employed for the determination of the hydrolyzing activity against sugar beet arabinan, debranched arabinan, or oat spelt xylan (2.5%). One unit of enzyme activity on each substrate was defined as the amount of enzyme producing 1 μmol equivalent of L-arabinose per minute.

Analysis of Enzyme Reaction Products

Thin-layer chromatography (TLC) and high-performance anion-exchange chromatography (HPAEC) analyses were performed to determine the hydrolysis products generated from various substrates. The appropriate amount of *Bl*ABNase was reacted with 0.5% of each substrate at its optimal reaction conditions. For TLC analysis, the resulting products were separated on the $60F_{254}$ silica gel plate (Merck, Darmstadt, Germany) with the solvents of ethyl acetate/acetic acid/water (2:1:1 (v/v/v)). The plate was visualized by dipping it into a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol, and then heating it for 10 min at 110°C. A CarboPac PA1 column (0.4 × 25 cm; Thermo Fisher Scientific, Sunnyvale, CA, USA) was used for HPAEC analysis (Bio-LC ICS-3000; Thermo Fisher Scientific, Rockford, IL, USA) with an electrochemical detector (ED40; Thermo). Samples were eluted with a linear gradient from 100% buffer A (150 mM NaOH in water; Thermo) to 50% buffer B (600 mM of sodium acetate in buffer A; Sigma-Aldrich) over 50 min. The flow rate of the mobile phase was maintained at 1.0 ml/min.

Enzymatic Degradation of Arabinan

The reaction mixture (1 ml) containing 0.5% of sugar beet arabinan was reacted with a single enzyme (0.26 U) or in combination of dual enzymes (0.13 U each). The hydrolysis of arabinans in use of *G*AFase and *Bl*ABNase were incubated in 50 mM sodium acetate buffer (pH 6.0) at 55°C for 6 h. The released reducing sugars and L-arabinose were measured as L-arabinose equivalents by DNS assay and HPAEC analysis, respectively. For the stepwise enzyme treatments, either *endo*- or *exo*-acting enzyme was incubated with a substrate for 3 h at the first stage. The first enzyme (0.26 U) was inactivated by boiling for 3 min, and the other enzyme (0.26 U) was newly added and reacted for an additional 3 h at the second stage. In order to determine the hydrolysis yield and L-arabinose yield, the resulting products at

Table 1. Summarized characteristics of *exo*- and *endo*-arabinosyl hydrolases used in this study.

	GAFase	*Bl*ABNase
Enzyme type	α-L-Arabino furanosidase	*endo*-(1,5)-α-L-Arabinanase
Microbial origin	*Geobacillus* sp. KCTC 3012	*Bacillus licheniformis* DSM13
GH family[a]	GH 51	GH 43
Amino acids	504	328
Molecular mass	58.5 kDa	36.4 kDa
Optimal temp.	60°C	55°C
Optimal pH[b]	5.0	6.0
Reference	[15]	[16]

[a]Glycoside hydrolase (GH) family from Carbohydrate-Active enZYmes (http://www.cazy.org).

[b]Both enzymes showed the highest activity in 50 mM sodium acetate buffer.

each time interval were quantitatively analyzed by the DNS reducing sugar assay and HPAEC analysis, respectively.

Results and Discussion

Gene Expression and Purification of Arabinosyl Hydrolases

Both *exo*- and *endo*-arabinosyl hydrolase genes had been cloned previously [15, 16]. In this study, the recombinant GAFase and *Bl*ABNase with the C-terminal six-histidines were produced in *E. coli via* the constitutive and the inducible expression systems, respectively. By using Ni-NTA column chromatography, each enzyme was purified to apparent homogeneity. Gel permeation chromatography analyses revealed that GAFase (58.5 kDa of monomer) and *Bl*ABNase (36.4 kDa) exist as a hexamer and a monomer, respectively (data not shown). GAFase and *Bl*ABNase showed the highest activity at 60°C and 55°C in 50 mM sodium acetate buffer (pH 5.0 and 6.0), respectively (Table 1). These arabinosyl hydrolases shared similar optimal reaction conditions, which could make them good candidates to be treated simultaneously for efficient L-arabinose production.

Enzymatic Characteristics of GAFase and *Bl*ABNase

GAFase showed similar activities on both sugar beet and debranched arabinans, which proposed that this hydrolase is able to attack the α-(1,5)-linked arabinofuranosyl backbone as well as α-(1,2)- or α-(1,3)-linked arabinosyl branches (Table 2). GAFase can weakly hydrolyze arabinoxylan from oat spelt as well. Meanwhile, *Bl*ABNase could exclusively hydrolyze debranched arabinan, but not sugar beet arabinan. These results imply that *Bl*ABNase has an extremely lower accessibility towards the branched structure of sugar beet arabinan than the α-(1,5)-linked arabinofuranosyl backbone of debranched arabinan.

In order to confirm the modes of action, each enzyme was reacted with 0.5% (w/v) of arabinan substrate under the optimal reaction conditions for 6 h. At any given time interval, the resulting reaction products were taken and analyzed by TLC. *Exo*-acting GAFase could exclusively release L-arabinose from sugar beet and debranched arabinans through the reaction (Fig. 1A). On the contrary, *Bl*ABNase could hydrolyze only debranched arabinan to produce a series of arabinooligosaccharides (AOs) as intermediates longer than arabinotetraose, as the same as typical *endo*-acting enzymes. Even though arabinotetraose and arabinotriose are the major products from debranched arabinan hydrolysis, the excess amount (5.0 U/ml) of *Bl*ABNase could catalyze the further degradation of AOs into arabinobiose and arabinotriose (Fig. 1B). Time-course hydrolysis of debranched arabinan by *Bl*ABNase was quantitatively monitored by HPAEC analysis (Fig. 2). At the early stage, *Bl*ABNase mainly produced a series of AOs longer than arabinohexaose from debranched arabinan. As the reaction proceeded, however, the long-chain AOs were gradually degraded into arabinotriose and arabinotetraose as major products.

Table 2. Specific activities of GAFase and *Bl*ABNase on different substrates.

Substrates	Specific activity (U/mg)[a]	
	GAFase	*Bl*ABNase
Arabinan (sugar beet)	4.52 ± 0.46	0.13 ± 0.01
Debranched arabinan	4.52 ± 0.12	76.84 ± 2.49
Arabinoxylan (oat spelt)	0.18 ± 0.03	N.D.[b]

[a]Enzyme activity on 0.5% of arabinan or 2.5% of xylan substrate was determined by the DNS reducing sugar assay. One unit of activity was defined as the amount of enzyme to release 1 μmol of L-arabinose equivalent per minute under the optimal reaction conditions.

[b]Enzyme activity was not detected.

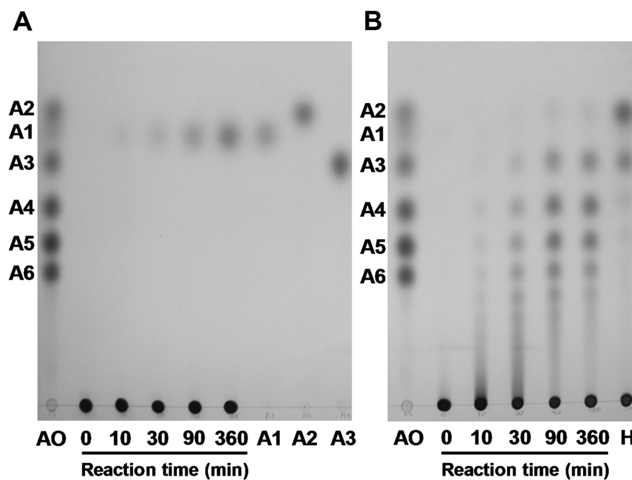

Fig. 1. TLC analyses of the enzymatic hydrolysates from sugar beet (branched) arabinan by GAFase (**A**) and from debranched arabinan by *Bl*ABNase (**B**).

Each substrate (0.5% (w/v)) was reacted with 0.1 unit of the corresponding enzyme. AO, arabinooligosaccharide standards from arabinose (A1) to arabinohexaose (A6); H, the complete hydrolysate reacted with 5.0 unit of *Bl*ABNase for 6 h.

Arabinan Degradation by Single Enzyme Treatments

In order to examine the detailed hydrolysis pattern and efficacy in arabinan degradation, 0.26 U of GAFase or *Bl*ABNase was reacted with 0.5% (w/v) of sugar beet arabinan under their co-optimal conditions at 55°C in 1 ml of 50 mM sodium acetate buffer (pH 6.0) for 6 h. The sugar beet arabinan (Megazyme) used in this study has an approximately 95% purity, and consists of L-arabinose (88%), galactose (3%), rhamnose (2%), and galacturonate (7%). The DNS reducing sugar assay was chosen for the simultaneous detection of both actions of *exo*- and *endo*-hydrolases, compared with L-arabinose as a standard. The resultant reaction products were taken at appropriate time intervals, and the changes of L-arabinose equivalent value were monitored by measuring the reducing sugar content.

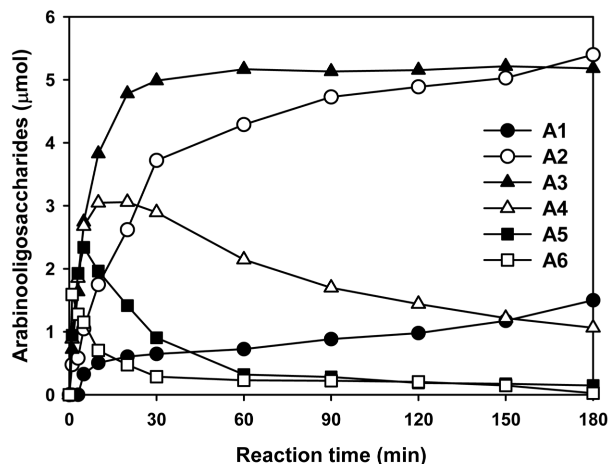

Fig. 2. Time-course analysis of the hydrolysis of 0.5% (w/v) debranched arabinan by 1.0 unit of *Bl*ABNase.

The concentration of each arabinooliosaccharides (AOs) was quantitatively determined by HPAEC analysis. AOs are shown as abbreviations from arabinose (A1) to arabinohexaose (A6).

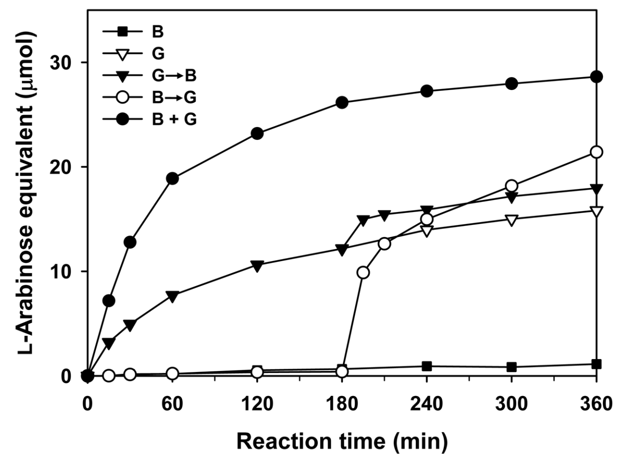

Fig. 3. Time-course hydrolysis of 0.5% (w/v) sugar beet arabinan *via* single, stepwise, or simultaneous enzymatic treatment.

G, treated with 0.26 U/ml of GAFase; B, with 0.26 U/ml of *Bl*ABNase; G+B, simultaneous treatment with 0.13 U/ml of GAFase and *Bl*ABNase, respectively; G→B or B→G, stepwise treatment of GAFase and *Bl*ABNase for 3 h each, or vice versa.

*Bl*ABNase rapidly hydrolyzed only debranched arabinan, but showed an extremely low activity on sugar beet arabinan with branches. As expected, *endo*-acting *Bl*ABNase could convert only 3.9% of arabinan into L-arabinose for 6 h, due to its poor activity towards the highly branched α-(1,5)-arabinofuranosyl backbone of arabinan. Meanwhile, *exo*-acting GAFase could consistently increase the release of reducing sugars, and its hydrolysis yield reached up to 54.0% (Fig. 3). As shown in Table 2, GAFase has similar preferences to branched and debranched arabinans. It means that this *exo*-hydrolase can simultaneously attack both arabinofuranosyl linkages in the backbone and the branches of sugar beet arabinan. The cooperative actions between *exo*- and *endo*-hydrolases should be considered for the improvement of the hydrolysis yield from arabinan to L-arabinose.

Synergistic Arabinan Degradation by Dual Enzymes

The cooperative reactions in use of *exo*-acting GAFase and *endo*-acting *Bl*ABNase were comparatively studied

here for the hydrolysis of sugar beet arabinan to arabinose units. The types of treatment with dual enzymes were designed as the stepwise (or sequential) or the simultaneous treatment. The stepwise treatment with dual enzymes, *endo-Bl*ABNase after *exo-G*AFase, could liberate 61.3% of L-arabinose from sugar beet arabinan. At the first stage for 3 h, 12.2 μmol of L-arabinose equivalent was produced by 0.26 U of *G*AFase treatment. However, very limited further hydrolysis, up to 18.0 μmol of L-arabinose equivalent, was observed during the second stage treated with 0.26 U of *Bl*ABNase for 3 h. It suggests that the structure of *G*AFase-treated arabinan is not suitable for the efficient *endo*-action of *Bl*ABNase during the second stage. On the other hand, the treatment of *exo-G*AFase after *endo-Bl*ABNase resulted in an enhanced L-arabinose equivalent (21.4 μmol) and hydrolysis yield (73.1%), respectively. This result reveals that the pretreatment of *endo*-hydrolase on arabinan can significantly facilitate the saccharifying action of *exo*-hydrolase. Although *Bl*ABNase treatment could not release any detectable short-chain AOs or free L-arabinose, the resultant hydrolysates at the first stage would be more favorable for the successive *G*AFase treatment (Fig. 3).

Interestingly, the simultaneous treatment of *exo*- and *endo*-hydrolases could remarkably increase both the reaction rate at the early stage and the hydrolysis yield (97.7%) at the end point. This result proposed that the simultaneous treatment of *exo*- and *endo*-arabinosyl hydrolases can highly accelerate the complete hydrolysis of branched arabinan *via* their synergistic actions, compared with the single enzyme treatments or the stepwise dual enzyme treatments.

In order to verify the synergistic effects of the cooperative enzyme actions on arabinan degradation, the exact amount of L-arabinose was quantitatively analyzed by the HPAEC method (Table 3). The single treatment with *G*AFase and *Bl*ABNase was able to liberate 35.9% and 0.5% of L-arabinose from sugar beet arabinan, respectively. The stepwise treatments of *endo*- after *exo*-hydrolase and *exo*- after *endo*-hydrolase showed 43.4% and 75.5% of hydrolysis yield, respectively. The simultaneous treatment gave the highest hydrolysis yield (91.2%) by the synergistic actions of *exo*- and *endo*-hydrolases. Even though HPAEC analysis showed a relatively low L-arabinose content, the tendency of the synergism among the enzyme treatments was almost the same as the L-arabinose equivalent values obtained from the DNS assay (Fig. 4).

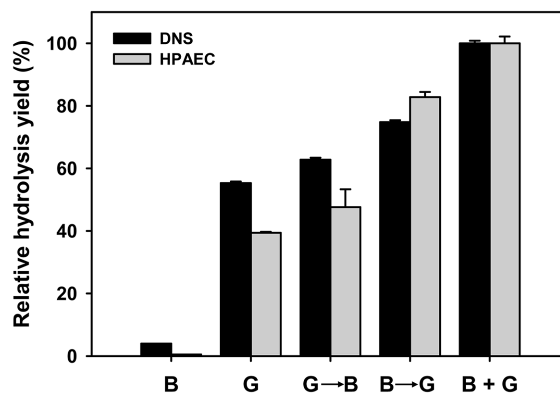

Fig. 4. Synergistic effects of enzymatic treatments on sugar beet arabinan degradation.

The relative yield (%) for arabinan hydrolysis was comparatively determined by the DNS reducing sugar assay and HPAEC analysis. The simultaneous treatment with the highest hydrolysis yield was considered as 100%. G, *G*AFase; B, *Bl*ABNase; G+B, simultaneous treatment with *G*AFase and *Bl*ABNase; G→B or B→G, stepwise treatment of *G*AFase and *Bl*ABNase.

Synergistic Modes of Action in Arabinan Degradation

When polymeric substrates are degraded by enzymes,

Table 3. Hydrolysis of sugar beet arabinan to L-arabinose *via* single or dual enzymatic treatments.

Enzymatic treatment		L-Arabinose (μmol)[d]	Hydrolysis yield (%)[e]
Single[a]	*endo-Bl*ABNase	0.1 ± 0.0	0.5 ± 0.0
	*exo-G*AFase	10.5 ± 0.1	35.9 ± 0.3
Stepwise[b]	*exo-G*AFase → *endo-Bl*ABNase	12.7 ± 1.5	43.4 ± 5.2
	*endo-Bl*ABNase → *exo-G*AFase	22.1 ± 0.4	75.5 ± 1.5
Simultaneous[c]	*endo-Bl*ABNase + *exo-G*AFase	26.7 ± 0.6	91.2 ± 2.0

[a]Each hydrolase (0.26 U/ml) was reacted with 0.5% sugar beet arabinan at 50°C for 6 h.

[b]Each hydrolase (0.26 U/ml) was reacted with sugar beet arabinan for 3 h each.

[c]Both hydrolases (0.13 U/ml each) were simultaneously treated on sugar beet arabinan for 6 h.

[d]The amount of L-arabinose liberated from sugar beet arabinan was quantitatively determined by HPAEC analysis.

[e]L-Arabinose (29.3 μmol) in 0.5% sugar beet arabinan was considered as 100%.

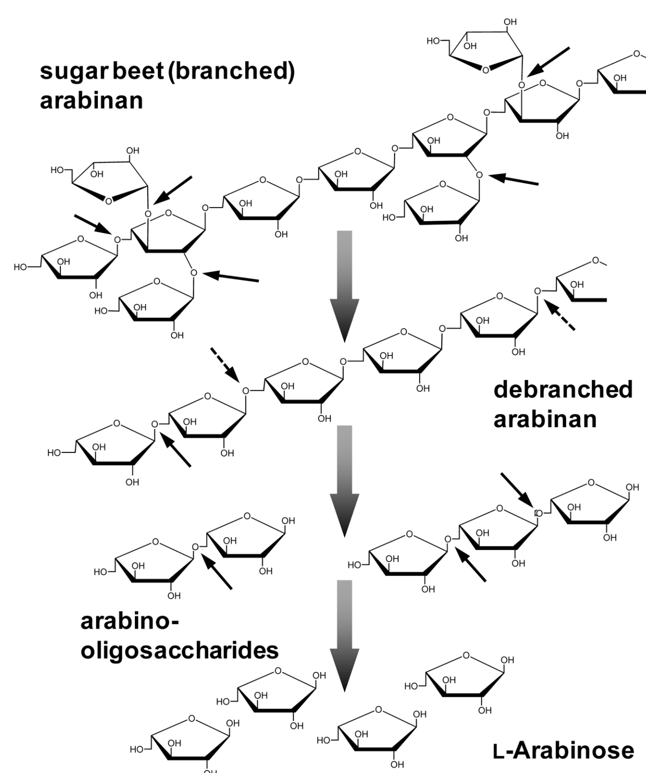

Fig. 5. Schematic model of the synergistic degradation of sugar beet arabinan *via* the simultaneous treatment with *exo*- and *endo*-arabinosyl hydrolases, GAFase (solid arrows) and *Bl*ABNase (dashed arrows).

treatment of both *exo*- and *endo*-hydrolases commonly results in their efficient saccharification. Specifically, the industrial enzymatic sccharification of starch materials to glucose can be a well-known example for the synergism of *exo*- and *endo*-hydrolases. Liquefying α-amylase randomly cleaves the internal α-(1,4)-glycosidic linkages in starch, which is followed by successive saccharification of the resulting dextrins *via* treatment of *exo*-acting glucoamylase or α-glucosidase.

However, the very short and abundant L-arabinosyl side chains can make sugar beet arabinan distinguished from starch with longer side chains. Although a single-enzyme process with GAFase or *Bl*ABNase showed the detectable hydrolytic activity on arabinan polymer, the simultaneous treatment of both the hydrolases released 2.5 times more L-arabinose (26.7 μmol) than the total sum of L-arabinose (10.6 μmol) produced from single-enzyme treatments (Table 3). It revealed that the simultaneous use of both *endo*-acting *Bl*ABNase and *exo*-acting GAFase could work synergistically with a much higher conversion of L-arabinose from sugar beet arabinan than that obtained from any single-enzyme process. It was recently reported that the combined use of thermostable arabinanases could synergistically produce L-arabinose with 80% of hydrolysis yield from sugar beet and debranched arabinan [10]. However, their synergistic modes of action have not been investigated on the basis of enzymatic characteristics in detail.

In the present study, therefore, both the stepwise and the simultaneous treatments on arabinan degradation were intensively compared with each other, and their synergistic modes of action were schematically proposed (Fig. 5). At the first stage of simultaneous treatment on arabinan, *endo*-*Bl*ABNase can cleave any specific region of branched arabinan to generate very limited amounts of branched long-chain AOs. Meanwhile, GAFase can remove L-arabinofuranosyl residues from the nonreducing ends of sugar beet arabinan and long-chain AOs. As GAFase continues to produce more debranched AOs and arabinan, the resulting debranched substances at the second stage are able to become more suitable substrates for the *endo*-action of *Bl*ABNase to produce various short-chain AOs. At the final stage, the debranched short-chain AOs can be rapidly degraded into L-arabinose *via* the *exo*-action of GAFase.

In conclusion, the simultaneous treatment of both *endo*-*Bl*ABNase and *exo*-GAFase could maximize their cooperative and complementary modes of action, which could shorten the operation time and enhance the conversion yield from sugar beet arabinan to L-arabinose.

Acknowledgments

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