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Synergy Between an α-L-Arabinofuranosidase from *Aspergillus oryzae* and an Endo-Arabinanase from *Streptomyces coelicolor* for Degradation of Arabinan

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An α-L-arabinofuranosidase gene of *Aspergillus oryzae* was expressed in *Pichia pastoris*. The recombinant enzyme released L-arabinose from arabinose-containing polysaccharides such as lupin pectic galactan, corn hull arabinoxylan, sugar beet arabinan, and potato pectic galactan. The enzyme displayed an optimum activity at 45℃ and pH 4.0. The enzyme was slowly inactivated above pH 6.0 and below pH 3.0, and was stable at temperatures up to 40℃. On the other hand, a putative endo-arabinanase gene of *Streptomyces coelicolor* was cloned and expressed in *Escherichia coli*. The recombinant enzyme hydrolyzed linear arabinans and produced α-1,5-arabinooligosaccharides. The enzyme displayed an optimum activity at 45℃ and pH 6.0. The enzyme was slowly inactivated above pH 10.0 and below pH 4.0, and it was stable at temperatures up to 35℃. Synergisms between the α-L-arabinofuranosidase and the endo-arabinanase for the degradation of arabinan and debranched arabinan were observed. The hydrolysis was most efficient when α-L-arabinofuranosidase and endo-arabinanase were in a ratio of 95: 5.

Keywords: arabinan, α-L-arabinofuranosidase, glycoside hydrolase family 43, glycoside hydrolase family 54, endo-arabinanase

Introduction

L-Arabinose residues are widely distributed in plant cell walls, where they are present in polymers such as arabinans, arabinoxylans, arabinogalactans and arabinogalactan proteins. The arabinans are composed mainly of α-L-arabinofuranosyl residues arranged into 1,5-linked chains. Varying numbers of these residues are substituted at the O-2 and/or O-3 position by additional α-L-arabinofuranosides (Bacic *et al.,* 1988). L-Arabinose is a major component of arabinan. Although it has a sweet taste, it is not readily absorbed by the body (Seri *et al.,* 1996). It has been reported that L-arabinose selectively inhibits the sucrase activity of pig intestinal mucosa in an uncompetitive mechanism and therefore suppresses the increase of blood glucose in a dose-dependent manner after the ingestion of sucrose (Seri *et al.,* 1996). It has been also reported that L-arabinose delays and decreases the digestion, absorption and energy production from sucrose when both are ingested simultaneously (Sanai *et al.,* 1997). Thus, L-arabinose is useful in preventing postprandial hyperglycaemia in diabetic individuals when foods containing sucrose are ingested.

Arabinan-degrading enzymes have been distinguished on the basis of their mode of action, i.e. *endo*-acting or *exo*-acting. The arabinan-degrading enzymes that act in an *endo*-fashion are called *endo*-1,5-α-L-arabinanases (EC 3.2.1.99) and those that act in an *exo*-fashion are called α-L-arabinofuranosidases [α-L-AFases (EC. 3.2.1.55)]. Depending on their amino acid sequences, α-L-AFases have been classified into five glycoside hydrolase (GH) families, namely families 3, 43, 51, 54 and 62 (Henrissat and Bairoch, 1993). The substrate specificity of some α-L-AFases has been characterized in detail and generally α-L-AFases belonging GH family 3 and family 54 show high specificity toward arabinose-containing polysaccharides. These enzymes preferentially remove side chain arabinose residues from arabinan (Hata *et al.,* 1992; Kaneko *et al.,* 1993; Yoshida *et al.,* 1994; Kaneko *et al.,* 1994; Kawabata *et al.,* 1995; Kaneko *et al.,* 1995; Kaneko and Kusakabe, 1995; Kaneko *et al.,* 1998a; Kaneko *et al.,* 1998b; Kaneko *et al.,* 1998c; Kaneko *et al.,* 1998d; Matsuo *et al.,* 2000; Miyanaga *et al.,* 2004). In contrast, endo-arabinanases have been classified into GH family 43 and it is known that they cleave more linear α-1,5-arabinan rather than branched arabinan.

In the present work, to determine the synergy effect of the substrate specificity of an α-L-AFase and an endo-arabinanase for the hydrolysis of arabinan, recombinant GH family 54 α-L-AFase from *Aspergillus oryzae* (AoAra54 A) and GH family 43 endo-arabinanase from *Streptomyces*

Abbreviations: AoAra54A, α-L-arabinofuranosidase from *Aspergillus oryzae* NFRI1599; ScAra43A, endo-arabinanase from *Streptomyces coelicolor* A3(2); α-L-AFase, α-L-arabinofuranosidase; PNP-α-L-Ara*f*, *p*-nitrophenyl α-L-arabinofuranoside; HPAEC-PAD, high-performance anion-exchange chromatography with a pulsed amperometric detection; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis, GH; glycoside hydrolase.

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coelicolor (ScAra43A) were obtained and the synergy between AoAra54A and ScAra43A for the degradation of arabinan was studied.

Materials and Methods

Substrates Arabinan is a polymer of 1,5-α-L-linked arabinofuranose which is highly substituted by 1,3-linked α-L-arabinofuranose residues. It also contains L-rhamnose, D-galactose and D-galacturonic acid in the pectic region. Arabinan was prepared from sugar beet by the method described previously (Kusakabe *et al.,* 1975). Debranched arabinan and linear arabinan were purchased from Megazyme International Ltd. (Wicklow, Ireland). According to the manual, debranched arabinan was prepared by the treatment of arabinan with α-L-arabinofuranosidase and linear arabinan was prepared by ion exchange chromatography of debranched arabinan to remove most of the charged pectic fraction. Sugar compositions of arabinan, debranched arabinan, and linear arabinan were determined as follows: arabinan (L-arabinose: D-galactose: L-rhamnose: D-galacturoic acid = 72.9: 17.1: 5.0: 5.0), debranched arabinan (L-arabinose: D-galactose: L-rhamnose: D-galacturoic acid = 61.2: 22.3: 10.0: 6.5), linear arabinan (L-arabinose: D-galactose: L-rhamnose: D-galacturoic acid = 61.2: 31.9: 4.4: 2.5). Sugar composition of the samples was analyzed by high-performance anion-exchange chromatography as described below.

α-1,5-Arabinobiose, α-1,5-arabinotriose, and pectic galactans from lupin and potato were also from Megazyme International Ltd. Gum arabic was from Nacalai Tesque Inc. (Kyoto, Japan). Nihon Shokuhin Kakoh Co. (Fuji, Japan) supplied corn hull arabinoxylan, whereas *p*-Nitrophenyl α-L-arabinofuranoside (PNP-α-L-Ara*f*) and larch arabinogalactan were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Sugar composition analysis of polysaccharide Polysaccharide sample was hydrolyzed with 2 M trifluoroacetic acid by incubation at 121℃ for 1 h, and then the acid was removed by N_2 gas stream. Sugar composition of the sample was analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detection (HPAEC-PAD) system using a CarboPac™ PA1 column (Dionex Corp., Sunnyvale, CA, U.S.A.) which eluted with 0.1 M NaOH (0–5 min), followed by a linear gradient (5–40 min) of sodium acetate (0–0.4 M) at a flow rate of 1 ml/min.

Expression of ScAra43A and AoAra54A genes To reveal ScAra43A (Genbank accession No. CAB92901), PCR was carried out using two oligonucleotide primers based on the nucleotide sequence of the mature proteins (5′ primer; 5′-AAG CTT GAT CCA AAT CCA GGC CGG GTC ACC-3′ and 3′primer; 5′-GAA TTC TTG TAC GCC ACT GGC CAG CCG CTG CT-3′). The product was digested with EcoRI and HindIII, and then ligated into the corresponding site of the expression vector pET30a (Novagen) and transformed into *Escherichia coli* BL 21(DE3). The procedures for transformation and culture to express proteins were performed by the methods described previously (Kaneko *et al.,* 2004).

To express AoAra54A (Genbank accession No. AB 073860), the following primers were designed: (5′primer; 5′-TCT AGA ATG CAT AGT ACC ACT CGT GCC CTA TAC-3′ and 3′primer; 5′-GAA TTC CAT GCA AAG CCA GTG CTG ACC ACC CA-3′). PCR was carried out using the primers, and the product was subcloned into the pGEM-T Easy vector. The vector was digested with EcoRI and SpeI, and the fragment was ligated into *Eco*RI and *Xba*I site of the pPICZαC and transformed into *Pichia pastoris* KM71H. The procedures for transformation and culture to express proteins were performed by the methods described previously (Ichinose *et al.,* 2005).

Purification of recombinant AoAra54A and ScAra43A Purifications of α-L-AFase (AoAra54A) and endo-arabinanase (ScAra43A) were performed at room temperature. The crude enzyme preparation of AoAra54A was dialyzed against 10 mM acetate buffer, pH 3.5, and applied to a SP-Sepharose Fast Flow (Amersham Biosciences) column (5×50 mm) equilibrated with the same buffer. The column was washed with the 10 mM acetate buffer at pH 3.5, at a flow rate of 1 ml/min. The elution of AoAra54A was achieved by using a linear gradient consisting of 0 to 1 M NaCl in the same buffer. Fractions containing α-L-AFase activity were pooled and concentrated by ultrafiltration (Millipore Corporation, MA, USA). During the process, the buffer was changed to 10 mM Tris-HCl buffer, pH 8.0. The solution pooled above was applied to a Q-Sepharose Fast Flow (Amersham Biosciences) column (5×50 mm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was washed with this buffer at a flow rate of 1 ml/min, and the elution was performed with a linear gradient consisting of 0 to 1 M NaCl in the same buffer. Fractions containing α-L-AFase activity were pooled, dialyzed against phosphate buffer at pH 7.0, and then added ammonium sulfate (4 M) to the enzyme solution to achieve a final concentration of 1 M. The mixture was applied to a Phenyl-Sepharose CL-4B (Amersham Biosciences) column (5×50 mm) that equilibrated with a solution containing 1 M ammonium sulfate in 50 mM phosphate buffer, pH 7.0. The column was washed with the same buffer at a flow rate of 1 ml/min. Elution of AoAra 54A was performed with a decreasing linear gradient consisting of 1 to 0 M ammonium sulfate in 50 mM phosphate buffer, pH 7.0. The fractions containing α-L-AFase activity were pooled and concentrated by ultrafiltration, and during the process the buffer was changed to deionized water. This preparation was stored at 4℃ and used as the purified enzyme.

The crude enzyme solution of ScAra43A was applied to a Ni^{2+}-NTA agarose (QIAGEN, USA) column (5×50 mm) equilibrated with 50 mM phosphate buffer, pH 7.0. The column was washed with this buffer to remove unbound materials, and the bound proteins were eluted with 250 mM imidazole in the same buffer. The fractions containing endo-arabinanase activity were pooled, dialyzed against 50 mM phosphate buffer, pH 7.0, and then loaded onto a DEAE-Sepharose Fast Flow (Amersham Biosciences) column (5×50 mm) equilibrated with the same

buffer. The column was washed with the buffer, and the enzyme was eluted with a linear gradient from 0 to 1 M NaCl in the same buffer, at a flow rate of 1 ml/min. The fractions containing endo-arabinanase activity were pooled, dialyzed against deionized water and stored at 4℃ as the purified enzyme.

Enzyme assay and measurement of protein The α-L-AFase activity was determined in a mixture containing 0.5 ml of 2 mM PNP-α-L-Ara*f* solution, 0.4 ml of McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid), pH 4.0 and 0.1 ml of enzyme solution. The reaction was performed at 45℃ for 10 min, and then stopped by the addition of 1.0 ml of 0.2 M Na_2CO_3. The amount of *p*-nitrophenol released was measured at 405 nm. One unit of enzyme activity is defined here as the amount of enzyme that releases 1 μmol of *p*-nitrophenol from PNP-α-L-Ara*f* per min under the conditions described above.

Endo-arabinanase activity was measured in a mixture containing 25 μl of 0.5% (w/v) debranched arabinan, 20 μl of McIlvaine buffer, pH 6.0, and 5 μl of enzyme solution at 45℃ for 10 min. Activity on debranched arabinan was assayed by measuring the released arabinose by the method of Somogyi-Nelson (Nelson, 1944). One unit of enzyme activity is defined here as the amount of enzyme that released 1 μmol of reducing power corresponding L-arabinose per min from debranched arabinan under the conditions described above.

Protein contents of the enzyme were measured by absorbance at 280 nm, assuming that the absorbance of 1.0 at 280 nm equals to the concentration of 1 mg per ml.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE was performed with a 12% gel as described by Laemmli (1970). The protein was stained with Coomassie Brilliant Blue R-250, and then destained with 30% methanol and 10% acetic acid solution. The molecular mass of the enzyme was measured by SDS-PAGE with a molecular mass marker (Bio-Rad SDS-PAGE standard low, Bio-Rad).

Enzymic properties The effects of pH on the activity and stability of α-L-AFase (AoAra54A) and endo-arabinanase (ScAra43A) were investigated with a series of McIlvaine buffers ranging in pH from 2.0 to 8.0 and Atkins-Pantin buffers (0.2 M boric acid/0.2 M KCl/0.2 M Na_2CO_3) ranging in pH from 8.0 to 11.0. The activities of AoAra54A and ScAra43A were assayed under the standard conditions described above. For determination of the pH stabilities of AoAra54A and ScAra43A, the enzymes were pre-incubated at various pH with 0.1% (w/v) BSA in the absence of substrate at 30℃ for 1 h, and the residual activities were assayed under the standard conditions. The effect of temperature on the activities of AoAra54A and ScAra43A were determined by using a series of water baths. With the exception of temperature, the assay conditions were the same as described for the standard method. For the temperature-stability measurements of AoAra54A and ScAra43A, the enzymes were pre-incubated at various temperatures at pH 4.0 and 6.0, respectively, for 1 h; and the residual activities were determined under the standard conditions.

Substrate specificity The substrate specificity of α-L-AFase (AoAra54A) toward arabinose containing polysaccharides was determined using luipin pectic galactan, potato pectic galactan, gum arabic, larch arabinogalactan, debranched arabinan, arabinan, linear 1,5-arabinan, and corn hull arabinoxylan as substrates. The reactions were performed in the McIlvaine buffer at pH 4.0, containing 1% (w/v) substrate and 0.2 units of enzyme. After incubation for 0 and 48 h, 2.5 volume of ethanol were added and the precipitates were collected by centrifugation. The precipitates were washed twice by 70% ethanol, dried, and then incubated at 121℃ for 1 h after the addition of 2 M trifluoroacetic acid. The acid was removed by N_2 gas stream, and sugar composition of the samples was analyzed by HPAEC-PAD system as described above.

The substrate specificity of endo-arabinanase (ScAra43 A) toward polysaccharide was determined using debranched arabinan, arabinan, and linear 1,5-arabinan as substrates. The reactions were performed in McIlvaine buffer, pH 6.0, containing 1% (w/v) substrate and 0.2 units of enzyme. After incubation for 0, 1, 3, 6, 12 and 24 h at 30℃, the reaction was stopped by boiling for 5 min. The increased reducing power was determined by the Somogyi-Nelson method. The hydrolysis products were also analyzed by HPAEC-PAD using the same conditions described above.

Synergism between AoAra54A and ScAra43A for the hydrolysis of arabinans was determined using debranched arabinan and arabinan as substrates. The reactions were performed in McIlvaine buffer, pH 5.0, containing 0.1% (w/v) substrate and 4.5 μg of enzymes with different activity ratio (AoAra54A: ScAra43A = 6: 94, 54: 46, 95: 5).

After incubation for 0, 1, 3, 6, 12 and 24 h at 37℃, the reaction was stopped by boiling for 5 min. The increased reducing power was determined by the Somogyi-Nelson method.

Results and Discussion

α-L-Arabinofuranosidase from A. oryzae (AoAra54A) The putative α-L-AFase gene without putative signal sequence from *A. oryzae* was constructed into the expression vector of pPICZαC. AoAra54A was expressed in *P. pastoris* KM71H strain as a secreted form with the aid of α-factor, which is a secretion signal of yeast. The purification steps of recombinant AoAra54A are summarized in Table 1. The purified enzyme gave a single band on SDS-PAGE when visualized by staining with Coomassie Brilliant Blue R-250 (Fig. 1A). The molecular mass of recombinant AoAra54A was estimated to be 61 kDa by SDS-PAGE. The specific activity of purified AoAra54A was 32 units/mg and overall purification was 97-fold (Table 1).

The pH and temperature activity and stability profiles of purified AoAra54A were investigated. The recombinant AoAra54A showed the maximal activity at pH 4.0. The enzyme retained almost full activity at pH values ranging from 3.0 to 6.0 after incubation 1 h at 30℃; however, the activity was reduced by 80% at pH 7.0. AoAra54A

exhibited the maximal activity at 45℃ and full activity below 40℃; however, its activity was significantly decreased above 50℃.

The substrate specificities of AoAra54A were investigated by using arabinose-containing polysaccharides (Table 2). AoAra54A released arabinose from sugar beet arabinan, corn hull arabinoxylan, and pectic galactan (potato and lupin) with rather high hydrolysis rates. On the other hand, the enzyme was ineffective against gum arabic, larch wood arabinogalactan, linear 1,5-arabinan and debranched arabinan.

Endo-arabinanase from S. coelicolor (ScAra43A) A putative gene of *S. coelicolor* A3 (2), which classified into GH family 43, was cloned and expressed in *E. coli*. ScAra 43A was also successfully expressed in *E. coli*. The purification procedures are summarized in Table 3. The specific activity of the purified enzyme for debranched arabinan was 18.2 units/mg. The overall purification of the enzyme was 48-fold, and the recovery of the enzyme was 25%. The purified ScAra43A could be resolved as single band on SDS-PAGE when visualized by staining with CBB R-250 (Fig. 1B). As expected, the molecular mass of ScAra43A as estimated from SDS-PAGE was found to be 40 kDa.

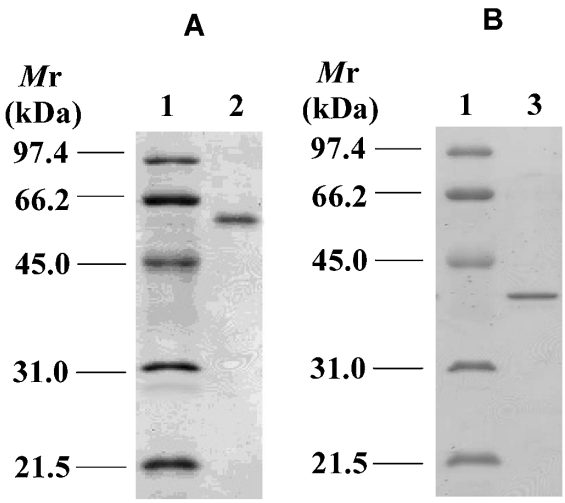

Fig. 1. SDS-PAGE of purified recombinant AoAra54A and ScAra43A.
Lane 1 contained the following molecular weight marker: rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), 1 μg each. Line 2 contained 1 μg purified AoAra54 A. Line 3 contained 1 μg purified ScAra43A.

The enzyme achieved maximal activity at pH 6.0 and was stable between pH 4.0 and 10.0. The enzyme achieved maximal activity at 45℃ and more than 80% activity was retained after incubation at 35℃ and pH 6.0 for 1 h.

For determination of the substrate specificities of ScAra43A, various arabinose-containing polysaccharides such as arabinan, debranched arabinan, and linear arabinan were used as substrates (Fig. 2). ScAra43A was hydrolyzed with these substrates in the following decreasing order of reactivity: linear arabinan > debranched arabinan > arabinan. The result corresponds with the proportion of α-1,3-linked arabinofuranosyl side chains. The reaction products of linear arabinan by ScAra43A were analyzed by HPAEC-PAD (Fig. 3). As seen from the figure, at the early stage of reaction (1 h), arabino-oligosaccharides (indicated with arrows) were detected, whereas at the later stage (12 h and 24 h), α-1,5-L-arabinobiose and α-1,5-L-arabinotriose were accumulated in addition to arabinose. The result indicates that ScAra 43A is a typical endo-acting enzyme.

Synergism between AoAra54A and ScAra43A for the hydrolysis of arabinan The synergistic effects between α-L-AFase (AoAra54A) and endo-arabinanase (ScAra43A) in the degradation of arabinan and debranched arabinan (mainly α-1,5-linked arabinan) were investigated. The time course of the hydrolysis of arabinan by AoAra54A and ScAra43A is shown in Fig. 4A. The hydrolysis rates after 24h-treatment were 23.4% by AoAra54A and 4.9% by ScAra43A, indicating α-1,3-linked side chain arabinose residues of arabinan are effectively removed by AoAra54 A.

Figure 4B shows the degree of hydrolysis of debranched arabinan by AoAra54A and ScAra43A versus time. After 24 h-treatment, the hydrolysis rates by AoAra54A and ScAra43A were 1.5% and 57.1%, respec-

Table 1. Summary of Purification of Recombinant AoAra54A.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude enzyme	15.6	48	0.33	1	100
SP-Sepharose	4.1	0.55	7.5	23	26
Q-Sepharose	2.3	0.11	21	64	15
Phenyl-Sepharose	1.9	0.06	32	97	12

Table 2. Substrate Specificities of AoAra54A towards L-Arabinose-containing Polysaccharides.

Substrate	Arabinose content (%, w/w)*	Hydrolysis rate (%)**
Pectic galactan (lupin)	15.6	79
Arabinogalactan (larch wood)	11.7	1
Arabinoxylan (corn hull)	31.1	17
Gum arabic	26.8	1
Arabinan (sugar beet pulp)	72.9	48
Pectic galactan (potato)	4.9	43
Debranched arabinan (sugar beet pulp)	61.2	7
Linear 1,5-arabinan (sugar beet pulp)	61.2	8

*Arabinose content was analyzed as described in Materials and Methods and expressed as %(w/w).

** The content of arabinose in the residue after enzymatic reaction was determined and hydrolysis rate was estimated by assuming that all arabinose was released from the substrate as 100%.

Table 3. Summary of Purification of Recombinant ScAra43A.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude enzyme	81	212	0.38	1	100
Ni^{2+}-chelating affinity	28	6.1	4.6	12	35
DEAE-Sepharose	20	1.1	18.2	48	25

tively, indicating that debranched arabinan is easily hydrolyzed by endo-arabinanase (ScAra43A) but is scarcely hydroloyzed by α-L-AFase. These results indicate that the combination of these two enzymes is useful for the hydrolysis of arabinan.

As expected, when both enzymes were used together to hydrolyze arabinan and debranched arabinan, the degree of hydrolysis was higher than that obtained with the individual enzymes (Fig. 4). The hydrolysis rate of arabinan by ScAra43A was improved greatly by the dose-dependent addition of AoAra54A.The best performance of arabinan hydrolysis was obtained (>50%) when the proportion of AoAra54A and ScAra43A was 95: 5. This suggests that the rate limiting step for the arabinan degradation is removal of α-1,3-linked side chain arabinose residues.

In conclusion, we demonstrated that a good combination of α-L-AFase and endo-arabinanase leads to efficient hydrolysis of arabinan by the effect of their substrate specificities. We hope the data provided in this paper

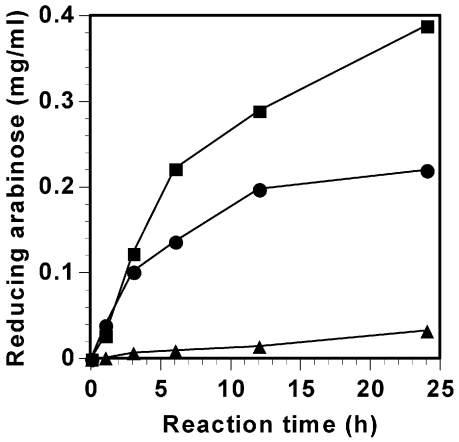

Fig. 2. Substrate specificity of ScAra43A.
Arabinan, debranched arabinan, and linear arabinan were from sugar beet and used as substrates. ■: 1% (w/v) linear arabinan, ●: 1% (w/v) debranched arabinan, ▲: 1% (w/v) arabinan.

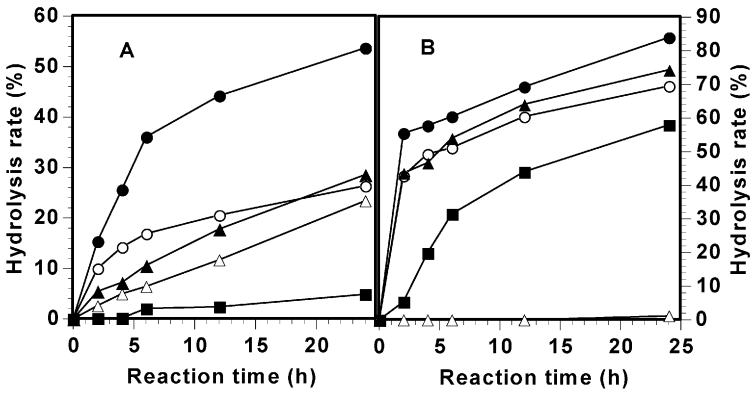

Fig. 4. Synergistic degradation of arabinan and debranched arabinan by AoAra54A and ScAra43A.
The conditions were described in Materials and Methods section. (A) Arabinan. (B) Debranched arabinan. The ratios of the activities of AoAra54A and ScAra43A were: ■: 0/100; △: 100/0; ▲: 6/94; ○: 46/54, ●: 95/5.
After the determination of the amount of arabinose released during the enzyme reaction, hydrolysis rate was estimated by assuming that all arabinose was released from the substrate as 100%.

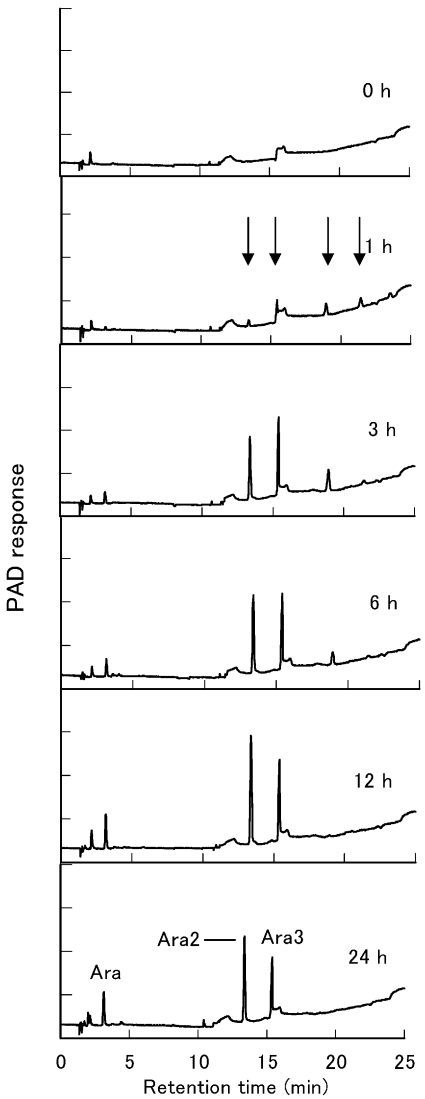

Fig. 3. HPAEC-PAD analysis of hydrolysis products of linear arabinan by ScAra43A.
The experimental conditions performed were described in the Materials and Methods section. The abbreviations Ara, Ara2 and Ara3 are L-arabinose, α-1,5-L-arabinobiose and α-1,5-L-arabinotriose, respectively.

will be useful for the effective production of L-arabinose in the food industry.

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