

Isolation, Characterization, and Cloning of α -L-Arabinofuranosidase Expressed during Fruit Ripening of Japanese Pear

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α -L-Arabinofuranosidase (α -L-arafase) was purified from fruit of Japanese pear (*Pyrus pyrifolia*). The enzyme solubilized from the cell wall by NaCl and Triton X-100 had the homogeneity of a single 62-kD polypeptide on SDS-PAGE after purification through the steps of hydroxyapatite, anion-exchange chromatography, and size-exclusion chromatography. A related cDNA clone was isolated (*PpARF2*). The transcript and related protein were detected solely in the ripening fruit corresponding to the increase of α -L-arafase activity. Transcripts of *PpARF2* were not detected in buds, leaves, roots, or shoots of the Japanese pear. The deduced amino acid sequences of *PpARF2* had low identity with those of other plants or bacteria. This α -L-arafase belonged to glycoside hydrolase family 3, which includes some β -xylosidases. The purified enzyme hydrolyzed mainly *p*-nitrophenyl α -L-arabinofuranoside and also reacted bifunctionally with *p*-nitrophenyl β -D-xylopyranoside. However, it released only arabinose from native cell wall polysaccharides prepared from Japanese pear and from sugar beet arabinan. The enzyme did not release xylose from arabinoxylan and xylan. The only activity of the α -L-arafase presented here was hydrolyzing the arabinosyl residue from native polysaccharides, whereas it showed bifunctional activity against artificial substrates. According to the expression pattern and properties of the enzyme, it is a new member of the glycoside hydrolase family 3 isolated from fruit, and it may be responsible for modification of the cell wall architecture during fruit softening.

The modification of cell wall architecture is involved in plant growth, development, and formation of shape. The cooperative biosynthesis and degradation of several cell wall components are necessary, and numerous cell wall-related enzymes are implicated in these processes. Fruit softening or textural changes are important factors that decide fruit quality, and they are caused by modification of cell wall polysaccharide architecture during fruit ripening. Several cell wall-metabolizing enzymes contribute to the changes in cell wall architecture (Fischer and Bennett, 1991). During fruit ripening, pectic and some hemicellulosic polysaccharides become increasingly soluble and depolymerize with the release of neutral sugar residues from side chains of matrix polysaccharides (Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997; Sakurai and Nevins, 1997; Brummell and Harpster, 2001). It seems that the loss of the neutral sugar residues of the side chains occurs during the early stage of ripening associated with pectin solubilization (Sakurai and Nevins, 1997; Rose et al., 1998). The initial solubilization of pectic polysaccharides during ripening occurs without changes in the degree of polymerization (Dawson et al., 1992; Redgwell et al., 1992).

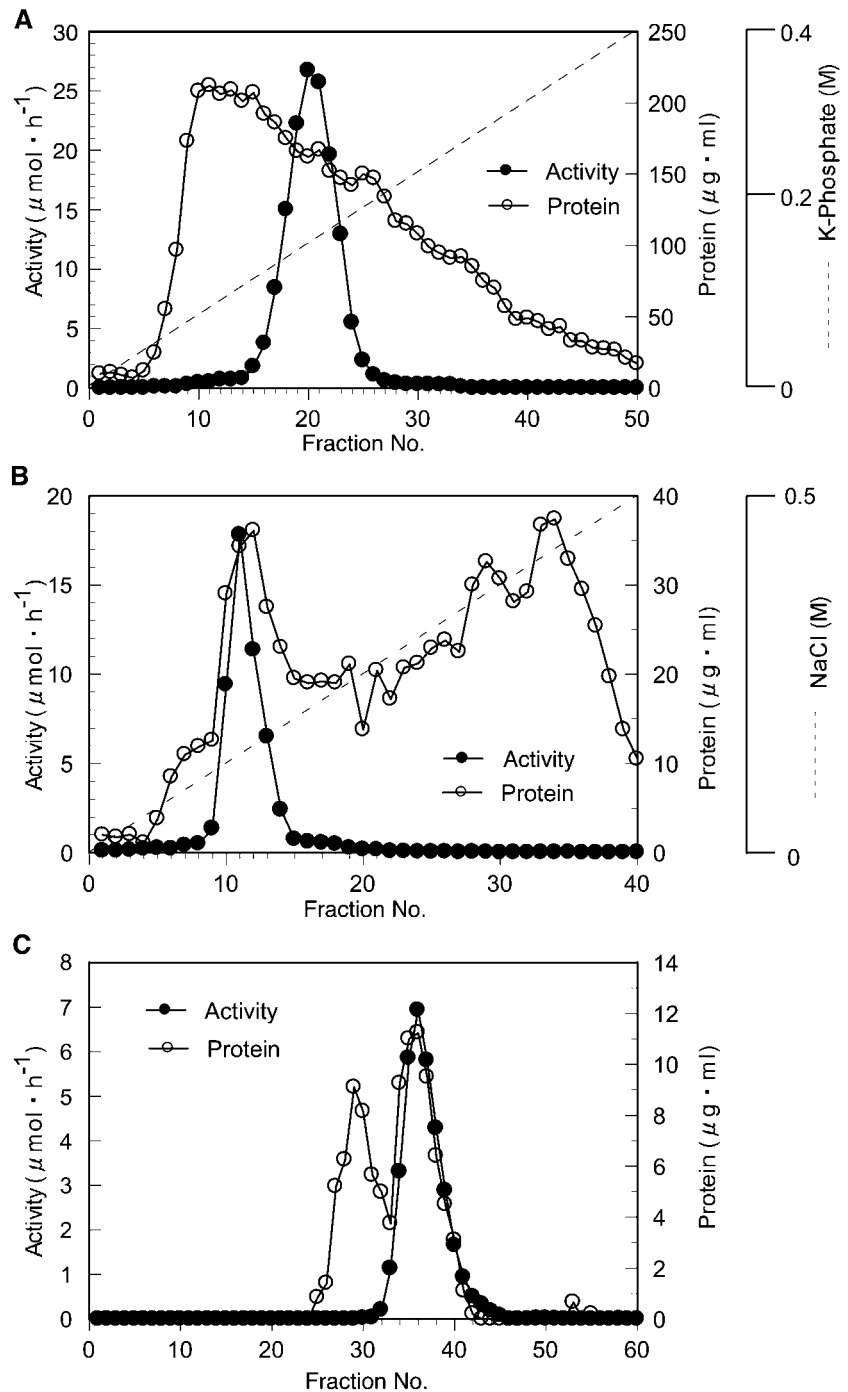
Side chains of matrix polysaccharides are removed by the actions of several glycosidases (Fry, 1995). The release of neutral sugar residues during fruit ripening was assumed to increase the sensitivity of enzymatic degradation or accessibility of other glycan hydrolases because almost all pectic or hemicellulosic polysaccharide backbones constituting the cell wall are associated with branched side chains, except for homogalacturonan. For example, β -galactosidase II, one of the glycosidases isolated from tomato (*Lycopersicon esculentum*) fruit, possesses the ability to release galactosyl residues from pectic side chains (Pressey, 1983). Recently, Smith et al. (2002) demonstrated that β -galactosidase II, which is encoded by *TBG4*, had a significant effect on fruit softening by using antisense suppression of a related gene. Therefore, the release of galactosyl residues from side chains of pectic polysaccharides, at least, is involved in fruit softening in addition to the pectin depolymerization caused by polygalacturonase activity at the later stage of ripening. However, the actual role of other glycosidases *in vivo* remains to be established.

Besides the release of galactosyl residues, the loss of arabinosyl residues during fruit softening was also observed in many kinds of fruits (Gross, 1984; Gross and Sams, 1984). Terminal arabinosyl residues are widely distributed in pectic and hemicellulosic polysaccharides such as arabinan, arabinogalactan, arabinoxylan, arabinoxyloglucan, and glucuronoarabinoxylan (Beldman et al., 1997; Saha, 2000; Sozzi et al., 2002b). α -L-Arabinofuranosidase (α -L-arafase; α -L-arabinofurano-

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.056655.

Figure 1. Elution profiles of α -L-arafase activity by hydroxyapatite (A), Q-Sepharose (B), and Sephacryl S-200 (C) chromatography. The activity was represented by a black circle (●) and the protein by a white circle (○). Fractions eluted from hydroxyapatite were collected (fraction nos. 18–23; 36 mL), dialyzed, and loaded onto a Q-Sepharose anion-exchange column. Then fraction numbers 10 to 12 (15 mL) were collected, concentrated, and then loaded onto a Sephacryl S-200 gel-filtration chromatograph. Fractions were collected in 2-mL aliquots.



side arabinofuranohydrolase, EC 3.2.1.55) is an enzyme that is able to hydrolyze nonreducing arabinofuranosyl residues. Yamaki et al. (1979) have reported the loss of arabinosyl residues associated with fruit ripening in Japanese pear (*Pyrus pyrifolia*). Indeed, α -L-arafase activity increased during ripening of Japanese pear fruit (Tateishi et al., 1996) and its rise was also observed in ripening fruits of apple (*Malus domestica*; Yoshioka et al., 1995), avocado (*Persea americana*; Tateishi et al., 2001b), tomato (Sozzi et al., 2002a),

persimmon (*Diospyros kaki*; Xu et al., 2003), and peach (*Prunus persica*; Brummell et al., 2004a). Recently, Sozzi et al. (2002b) characterized α -L-arafase isoforms from tomato fruit and reported their different hormonal regulation and contribution to fruit development and ripening. One of the α -L-arafase isoforms is regulated by ethylene and releases arabinosyl residues from the pectic fraction. This indicates that the isoform plays an important role in arabinose (Ara) metabolism during ripening of tomato fruit.

Table 1. Typical purification steps of α -L-arafase from the cell wall of Japanese pear fruit

Solubilized enzyme extract from the cell wall was defined as the crude enzyme.

Step	Activity	Protein	Specific Activity	Fold	Yield
	$\mu\text{mol h}^{-1}$	mg	$\mu\text{mol h}^{-1} \text{ mg protein}^{-1}$		%
Crude enzyme	150.4	50.4	2.99	1.0	100
Hydroxyapatite	121.3	5.79	20.9	7.0	80.6
Q-Sepharose FF	38.58	0.49	77.9	26.1	25.6
Sephacryl S-200	6.95	0.02	315	105.7	4.62

To date, we have found α -L-arafase cDNA sequences from higher plants in the DDBJ/EMBL/GenBank databases; however, except for arabinoxylan arabinofuranohydrolase (AXAH; Ferré et al., 2000; Lee et al., 2001) and α -L-arafase (Lee et al., 2003) purified from barley (*Hordeum vulgare*), they appear to be defined as α -L-arafase on the basis of low sequence homology to bacterial α -L-arafase. There is no evidence that the protein translated from them actually possesses α -L-arafase activity. We have already reported the increase in α -L-arafase activity with the ripening of Japanese pear fruit and the purification of the enzyme (Tateishi et al., 1996). However, it was difficult to further analyze the enzyme properties due to low recovery of the enzyme protein after purification. In this study, we repurified α -L-arafase from ripening Japanese pear fruit, characterized its properties against native cell wall polysaccharides, and, moreover, isolated a related cDNA clone by elucidating the primary structure of the enzyme.

RESULTS

Purification of α -L-Arafase from Cell Walls of Japanese Pear Fruit

In a previous study, approximately 30% of α -L-arafase activity was solubilized from cell walls using trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) for 38 h (Tateishi et al., 1996). Using the buffer containing NaCl and Triton X-100 for solubilization, 20 times the activity was recovered compared to that solubilized by CDTA (Tateishi and Inoue, 2000). The condition also contributed to stabilization of the enzyme. α -L-Arafase was purified by hydroxyapatite, Q-Sepharose anion-exchange chromatography, and Sephacryl S-200 gel-filtration chromatography (Fig. 1; Table I). Through the purification steps, we could not use ammonium sulfate or ultrafiltration membrane to purify or concentrate the enzyme because the recovery was too low (data not shown). The enzyme was eluted by gel media with a delay, compared to ideal elution position, regardless of the addition of 0.2 M NaCl (data not shown); this is similar to a case previously reported by Sozzi et al. (2002b). After size-exclusion chromatography, α -L-arafase was purified 106-fold compared to the activity of the crude enzyme, and

the activity against *p*-nitrophenyl α -L-arabinofuranoside (*p*NPA) became $6.95 \mu\text{mol h}^{-1}$. The purified α -L-arafase appeared as a single protein with a molecular mass of 62 kD by staining with silver nitrate after SDS-PAGE (Fig. 2). It was larger than that obtained in the previous study (Tateishi et al., 1996). Proteolytic degradation may have occurred during long-term incubation with CDTA. The pH optimum of α -L-arafase against *p*NPA was 4.5, and the activity was detected at pH 4.0 to 6.5 (data not shown). Cu^{2+} and Hg^{2+} were found to be potent inhibitors (data not shown). The exact K_m value of α -L-arafase against *p*NPA was not determined because *p*NPA could not be dissolved at concentrations higher than approximately 20 mM. All the kinetic parameters assayed were almost the same as those of a previous study (Tateishi et al., 1996).

Isolation of a *PpARF2* cDNA Clone

We determined the amino acid sequences of several tryptic peptide fragments generated from purified α -L-arafase (Fig. 3). Degenerate primers were designed based on the obtained amino acid sequences and PCR was performed using a combination of all primers. The primer set of ARA-Fr39 and ARA-Fr57C gave a single cDNA fragment of 350 bp (data not shown). The

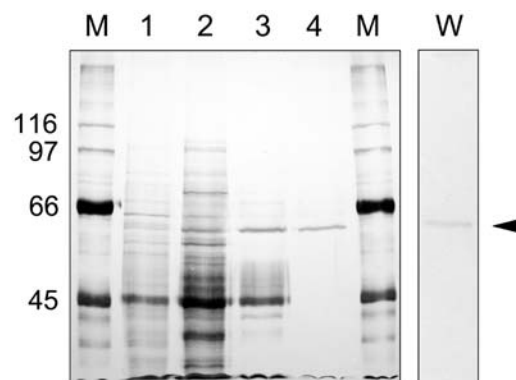


Figure 2. SDS-PAGE of each purification step of α -L-arafase and western-blot analysis. Marker (lane M), crude enzyme (lane 1), hydroxyapatite (lane 2), Q-Sepharose FF (lane 3), and Sephacryl S-200 gel filtration (lane 4). The gel was stained with silver nitrate. Lane W is a western-blot of α -L-arafase protein using anti-Japanese pear α -L-arafase IgG raised from recombinant protein.

Figure 3. Amino acid sequence of α -L-arafase (*PpARF 2*) from Japanese pear fruit. The shade-boxed amino acids are the sequences of N-terminal amino acids and tryptic peptide fragments generated from purified α -L-arafase. White-boxed amino acid sequences exhibited possible N-glycosylation sites. The cleaved signal peptide is indicated by an underline. The putative catalytic nucleophile (amino acid position 292) and catalytic acid/base (amino acid position 498) are circled.

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1                                     60
MAYNIAKLSL LSLLFLFSSL CSMAVVHARP PFACDPRNPI TRTLKFCRVR VPIHVRVQDL

61                                     120
IGRLTLQEKI GLLVNNAIAV PRLGIQGYEW WSEALHGVSN VGPGTKFGTF LGATSFPQVI

121                                    180
TTAASFNESL WEEIGRVVSD EARAMYNGGA AGLTFWSPNV NIFRDPRWGR GOETPGEDPV

181                                    240
LAAKYGARYV KGLQGDGAGN RLKVAACCKH YTAYDLDNWN GVDRFHFNAR VSKQDLEDTY

241                                    300
NVPFKACVVD GNVASVMCSY NQVNGKPTCA DPDLLKGTIR GQWKLNGYIV SDCDSVGVYY

301                                    360
DNQHYTKTPE AAAAYAIKGA LDLDCGPFLG IHTEAAIRTG QVNEIDINYA LANTITVQMR

361                                    420
LGMFDGEPST QRYGNLGLAD VCKPSSNELA LEAARQGIVL LENRGNSLPL STIRHRTVAV

421                                    480
IGPNSDVTET MIGNYAGIAC GYTTPLQGIA RYTRTIHQAG CTDVHCNGNQ LIGAAEVAAR

481                                    540
QADATVLVIG LDQSIEAEFR DRTGLLLLPGH QOELVSERVAR ASRGPTILVI MSGGPIDVTF

541                                    600
AKNDPCIGAI IWVGYPGQAG GTAIADVLFG TTNPSGKLPM TWYPQNYVAN LPMTDMAMRA

601                                    660
DPARGYPGRT YRFYKGPVVF PFGMGLSYTR FSHSLAQGPT LVSVPLTSLV AAKNTTMLSN

661                                    720
HGVRVSHTNC DSLSLDFHID IKNTGTMDGT HTLLVFATQP AGKWAPNKQL VGFHKVHIVA

721                                    774
GSERRVRVGV HVCKHLSIVD KLGIRRIPLG QHKLEIGDLK HYVSIEANLG EIKF

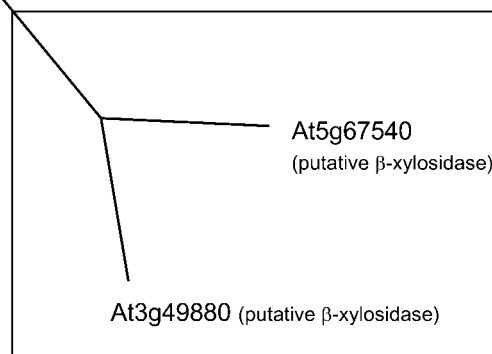
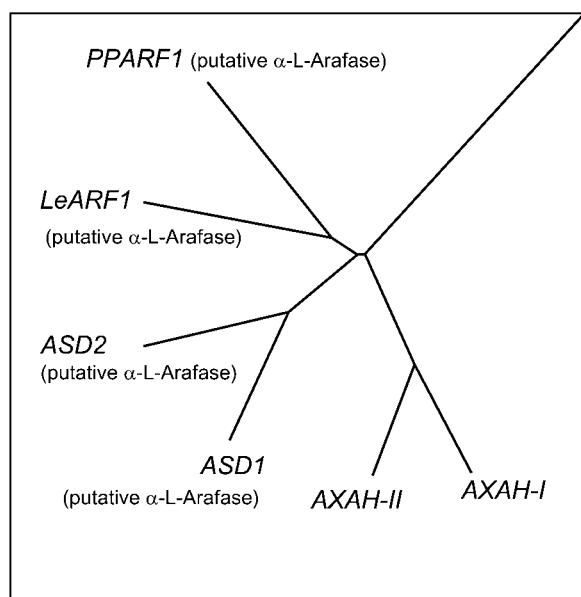
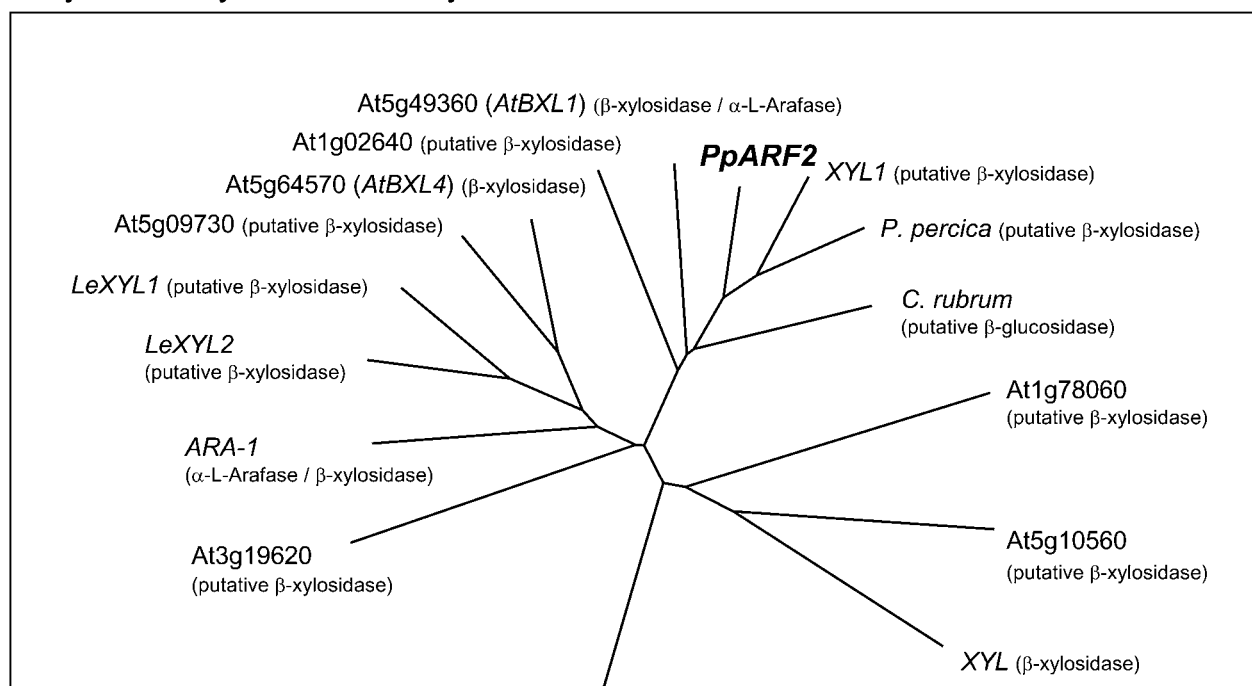
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deduced amino acid sequence from the clone obtained matched the internal amino acid sequence of purified α -L-arafase. Based on the sequence obtained, the 5'- and 3'-ends of the cDNA fragment were determined using RACE-PCR (see "Materials and Methods"). Finally, we obtained a full-length cDNA clone encoding Japanese pear α -L-arafase (named *PpARF2*; accession no. AB195230; Fig. 3). The cloned cDNA fragment (2,575 bp) contained an untranslated region at both the 5'- and the 3'-ends, and a presumptive coding sequence of 2,322 nucleotides. The molecular mass calculated from the deduced amino acid sequence was 84 kD, consisting of 774 amino acids (nucleotides 37–2,358), which had a calculated pI of 8.29. The N-terminal amino acid sequence from purified α -L-arafase was Arg-Pro-Pro-Phe-Ala-Cys-Asp-Pro-Arg-Asn, which corresponds to the amino acid sequence of 29 to 38 deduced from *PpARF2*. This indicates that a signal sequence existing in the N terminus of *PpARF2* protein would be cleaved in the mature protein. The sequences of a total of 41 amino acid residues from three different tryptic peptides exactly matched the sequence deduced from *PpARF2* (Fig. 3). *PpARF2* was subcloned into an expression vector and

protein expression was induced by the addition of isopropylthio- β -galactoside. Under our conditions, the induced protein was recovered as an inclusion body without activity (see "Materials and Methods"). Therefore, we dissolved the protein and used it as an antigen to raise an antibody against α -L-arafase. The prepared antibody recognized a single polypeptide in the crude extract and also reacted with purified α -L-arafase after gel-filtration chromatography (Fig. 2).

Two N-glycosylation sites were predicted from the deduced amino acid sequence of *PpARF2*. PSORT (Nakai and Kanehisa, 1991) and TargetP (Emanuelsson et al., 2000) programs predicted that the encoded α -L-arafase would be exported to the apoplast, indicating an enzyme would act on cell wall substrates. Both nucleotide and deduced amino acid sequences showed low identity with those of other plant and bacterial α -L-arafases. However, they showed relatively higher similarity with putative β -glucosidase and β -xylosidase cDNA clones isolated from *Chenopodium rubrum* and Arabidopsis (*Arabidopsis thaliana*), respectively, and extremely high identity with a Japanese pear cv Nijisseiki β -xylosidase-like gene expressed during fruit ripening and senescence (Itai et al.,

Glycoside Hydrolase Family 3

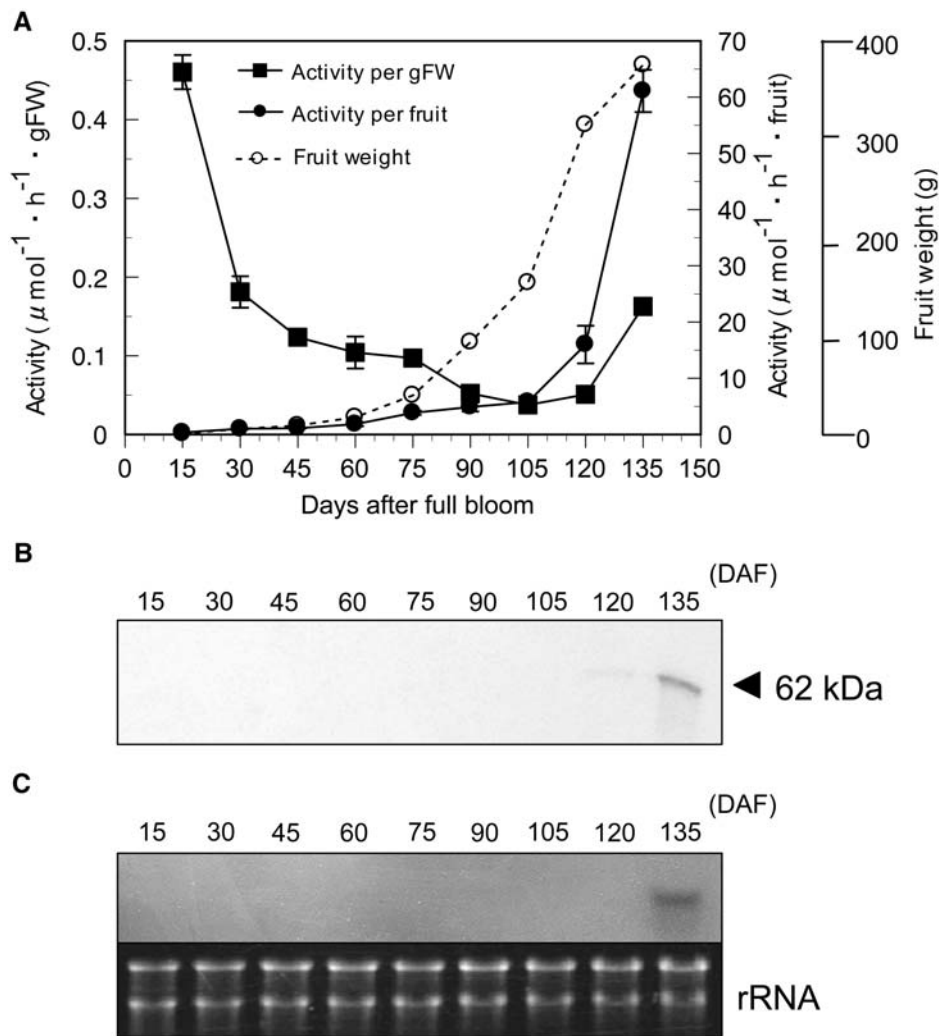


Glycoside Hydrolase Family 43

Glycoside Hydrolase Family 51

Figure 4. Comparison of deduced amino acid sequence of *PpARF2* and several α -L-arafases, β -xylosidases, and β -glucosidase. Using the ClustalW program, amino acid sequences were aligned and an unrooted phylogenetic tree was generated. Their GenBank accession numbers are *ASD1* and *ASD2* from Arabidopsis (AY243509 and AY243510), *PPARF1* from Japanese pear (AB073311), *LeARF1* from tomato (AB073310), *AXAH-I* and *AXAH-II* from barley (AF320324 and AF320325), β -glucosidase from *C. rubrum* (AY320257), β -xylosidase from peach (partial, AF362990), *XYL1* from *Fragaria* \times *ananassa* (partial, AY486104), *ARA-1* and *XYL* from barley (AY029259 and AY029260), and *LeXYL1* and *LeXYL2* from tomato (AB041811 and AB041812). At1g02640, At5g49360 (*AtBXL1*), At5g10560, At1g78060, At3g19620, At5g64570 (*AtBXL4*), and At5g09730 were β -xylosidases from Arabidopsis.

Figure 5. Changes in α -L-arafase activities, accumulation of protein, and expression of mRNA during fruit development and ripening. A, Activity of α -L-arafase solubilized from the cell wall using *p*NPA as substrate. The activity on a per gram fresh weight basis is indicated by a black box (■) and the activity on a whole-fruit basis is indicated by a black circle (●). Fruit growth was indicated by a white circle (○) with a dashed line. The values are the means of three independent experiments. Vertical bars indicate the SE. B, Accumulation of α -L-arafase protein. C, Ripening-specific expression of *PpARF2*. The probe was prepared from mainly the 3'-untranslated region of *PpARF2*. The hybridization temperature was 68°C and the final washing condition was 0.1× SSC, 0.1% SDS at 68°C. rRNAs stained with ethidium bromide were used as a loading control.



1999). Bioinformatics analysis of deduced amino acid sequences of *PpARF2* also revealed that purified α -L-arafase was grouped into the glycoside hydrolase (GH) family 3 (<http://afmb.cnrs-mrs.fr/CAZY>; Fig. 4), which contains β -glucosidase, β -xylosidase, and α -L-arafase cDNA clones.

The Activity and Gene Expression of α -L-Arafase with Fruit Development and Ripening

Western-blot analysis revealed that α -L-arafase protein accumulated as ripening progressed in fruit (120 and 135 days after full bloom [DAF]; Fig. 5B). RNA gel blot analysis also detected accumulation of *PpARF2* transcripts only in the ripening stage (Fig. 5C). We also assayed α -L-arafase activity during fruit development and ripening (Fig. 5A). The highest activity on a per gram fresh weight basis was detected in both young fruit (15 DAF) and fully ripened fruit (135 DAF). To exclude the dilution of the activity with fruit enlargement, the fluctuation of activity on a whole-fruit basis is also described in the same figure. The activity

increased vigorously with fruit ripening after fruit enlargement (Fig. 5A). Moreover, *PpARF2* transcripts were detected solely in ripening fruit, but not in buds, young and mature leaves, and shoots (Fig. 6). The increase of the activity, accumulation of the enzyme protein, and expression of *PpARF2* were strongly specific to ripening fruit.

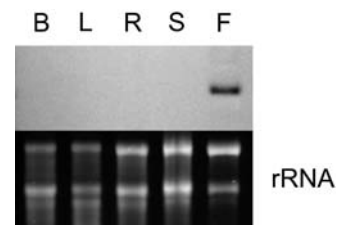


Figure 6. Fruit-specific expression of *PpARF2*. RNA samples were prepared from buds (lane B), leaves (lane L), roots (lane R), shoots (lane S), and ripe fruit (lane F). Ethidium bromide-stained gel was used as a loading control (indicated rRNA).

Activities against Native Cell Wall Polysaccharides and Substrates Containing Xylose

We assayed the activity against several Ara-containing polysaccharides because the activity against an artificial substrate does not necessarily reflect that against polysaccharides in vivo (Fig. 7). α -L-Arafase released only Ara as a consequence of hydrolysis of alcohol-insoluble substances (AIS) prepared from the cell wall of Japanese pear fruit. α -L-Arafase also possessed the activity of hydrolyzing sugar beet arabinan to Ara. However, no Ara could be detected in the reaction mixture incubated up to 72 h with wheat (*Triticum aestivum*) arabinoxylan. α -L-Arafase also hydrolyzed an arabino-oligosaccharide, arabinohexaose, to Ara within 72 h (Fig. 7). The relative activity against arabinan and/or arabinohexaose seems to be lower than that against *p*NPA estimated from the Ara spot of thin-layer chromatography.

Bioinformatics analysis revealed that purified α -L-arafase is grouped into GH family 3 involving mainly

plant β -xylosidases. Therefore, we also assayed the activity against substrates containing xylose (Xyl). α -L-Arafase had not hydrolyzed xylan to Xyl after a long incubation (72 h; Fig. 8), and we also did not detect Xyl in the reaction mixture with Japanese pear AIS and wheat arabinoxylan (Fig. 7). On the other hand, α -L-arafase also hydrolyzed xylohexaose to Xyl; however, some hydrolysis reactions were still in progress after a 96-h incubation (Fig. 8). As an enzymatic property, α -L-arafase possessed hydrolysis activity against *p*-nitrophenyl β -D-xylopyranoside (*p*NPX) that was 31% of that against *p*NPA. It was clear that the rate of hydrolysis of xylohexaose to Xyl was slower than that of arabinohexaose to Ara.

DISCUSSION

We purified an α -L-arafase to the homogeneity of a single polypeptide on SDS-PAGE. We designed

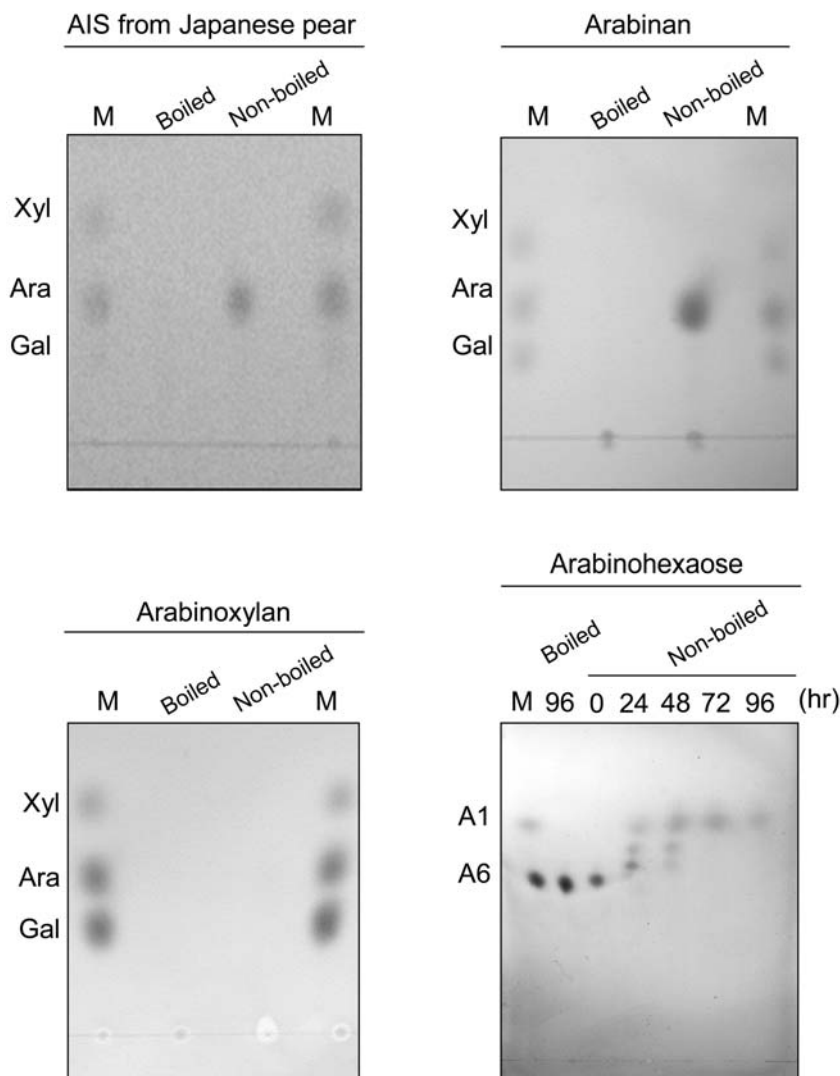
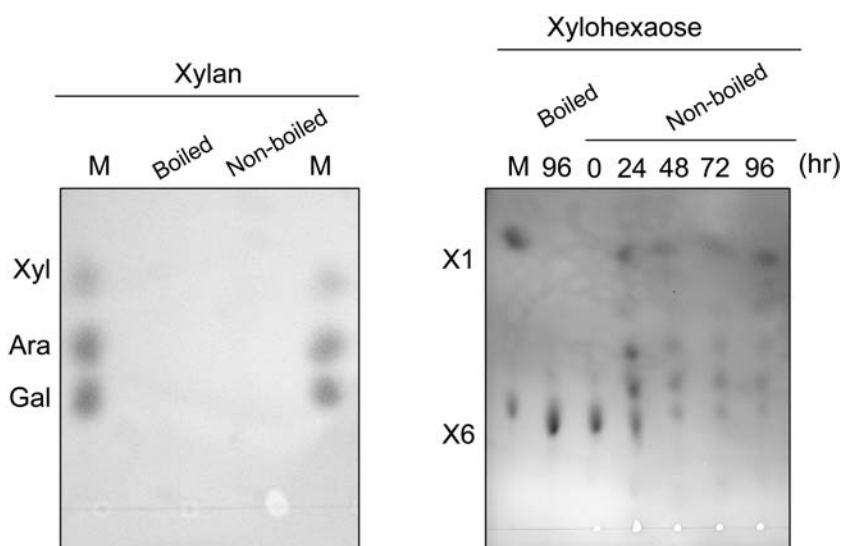


Figure 7. Hydrolysis of polysaccharides and arabinohexaose by purified α -L-arafase. Released monosaccharides were separated using thin-layer chromatography. AIS isolated from Japanese pear fruit, arabinan from sugar beet, and arabinoxylan from wheat were used as polysaccharide substrates. Lane M indicates standard sugars.

Figure 8. Hydrolysis activity against Xyl-containing substrate. Xylan from birchwood and xylohexaose were used as substrates. Released monosaccharides were separated using thin-layer chromatography. Lane M indicates standard sugars.



degenerate primers based on internal amino acid sequences and then obtained the cDNA clone encoding α -L-arafase (*PpARF2*; Fig. 4). The sequences of the three independent peptide fragments were actually found in the deduced amino acid sequence of *PpARF2*. The N-terminal amino acid sequence also exactly matched the deduced amino acid sequence of *PpARF2* (below amino acid position 29). Furthermore, the antibody raised against recombinant α -L-arafase from *PpARF2* recognized purified α -L-arafase (Fig. 2). Thus, we determined the complete primary structure of α -L-arafase from corresponding cDNA. The calculated molecular mass of *PpARF2* was 84 kD, which is considerably larger than that of the α -L-arafase obtained on SDS-PAGE, 62 kD. A single 62-kD polypeptide was detected by immunoblot analysis when the frozen sample was inactivated immediately in SDS-denatured sample buffer. Therefore, it is hard to consider that proteolytic degradation of the protein was caused by isolation. This discrepancy may be due to a process of the C-terminal region of the protein during maturation, besides cleaving of the N-terminal signal peptide. A similar case has been reported in barley α -L-arafase (Lee et al., 2003). A hydrophobic signal peptide is present at the N terminus, and both PSORT and TargetP programs predicted that the protein would be exported to the outside of the plasma membrane. These results imply that α -L-arafase works actively in cell wall degradation.

Based on amino acid sequences of active sites rather than their substrate specificities, α -L-arafases were classified into five GH families (family 3, 43, 51, 54, and 62), and we can find α -L-arafase sequence information for several plants in families 3, 43, and 51 (Fig. 4). Almost all α -L-arafases were found in family 51 and indeed were cloned from fruit of Japanese pear (*PpARF1*, AB073311), tomato (*LeARF*; Itai et al., 2003), and Arabidopsis (*ASD1* and *ASD2*; Fulton and

Cobbett, 2003). Ferré et al. (2000) and Lee et al. (2001) reported that α -L-arafases belonging to family 51 encoded AXAH protein. According to the substrate specificity, the α -L-arafase purified from Japanese pear should be separated in other α -L-arafase groups. Indeed, Japanese pear α -L-arafase (*PpARF2*), which was not classified in family 51, could not hydrolyze arabinoxylan (Figs. 4 and 7) and it was isolated from ripening fruit.

Japanese pear α -L-arafase belongs to GH family 3, which includes β -glucosidase and β -xylosidase as well as α -L-arafase. Due to limits in the availability from plants, it has not been elucidated whether the proteins encoded by the clones possessed β -xylosidase or β -glucosidase activity or not, except for β -xylosidases from barley and Arabidopsis (Goujon et al., 2003; Lee et al., 2003; Minic et al., 2004). Recently, Lee et al. (2003) reported that α -L-arafase purified from barley was classified into GH family 3 and that it possessed β -xylosidase activity besides α -L-arafase activity. Moreover, most recently, it was reported that one of the β -xylosidases of Arabidopsis was classified into GH family 3 and also possesses α -L-arafase activity (Goujon et al., 2003; Minic et al., 2004). Therefore, we determined the properties of Japanese pear α -L-arafase as β -xylosidase and β -glucosidase. The purified enzyme did not possess β -glucosidase activity; however, through all the purification steps, elution profiles of β -xylosidase activity corresponded with that of α -L-arafase (data not shown). Purified α -L-arafase also hydrolyzed xylohexaose at a relatively slow rate compared to arabinohexaose (Figs. 7 and 8). This indicates that the enzyme possesses bifunctional activity as α -L-arafase/ β -xylosidase as an enzymatic property. Bifunctional activity has been reported in plant α -L-arafases purified from radish (*Raphanus sativus*) seeds (Hata et al., 1992) and spinach (*Spinacia oleracea*) leaf (Hirano et al., 1994) besides barley (Lee

et al., 2003). It is not clear whether these α -L-arafases belong to GH family 3 or not; however, it may be reasonable that several β -xylosidases grouped family 3 were called α -L-arafase rather than β -xylosidase. It should be noted that the activities of bifunctional α -L-arafase against *p*NPA are 3-fold higher than those of β -xylosidase activity against *p*NPX.

α -L-Arafase isolated from Japanese pear actually released Ara and Xyl hydrolyzed bifunctionally from both *p*-nitrophenyl (*p*NP) aglycon and oligosaccharides. Surprisingly, only Ara was detected in hydrolysates from AIS prepared from Japanese pear fruit, which contains xylosyl-polysaccharides (Yamaki et al., 1979). Moreover, the α -L-arafase did not release Xyl from xylan (Fig. 8). The case of the barley bifunctional α -L-arafase was different (Lee et al., 2003). The bifunctional β -xylosidase from *Arabidopsis* released Ara and Xyl from arabinoxylan polysaccharides (Minic et al., 2004), but our α -L-arafase did not. One of the possible reasons is that there are few xylosyl sites, which would be cleaved by α -L-arafase, in native substrates such as AIS or xylan compared to those in *p*NPX or xylohexaose. However, Japanese pear α -L-arafase did not release Xyl from xylan that had been previously partially hydrolyzed by trifluoroacetic acid (data not shown). According to substrate specificity, we consider that the α -L-arafase we isolated from pear fruit was a distinct type of bifunctional α -L-arafase/ β -xylosidase and may release only arabinosyl residues from cell wall polysaccharides in situ. In fact, *PpARF2* is expressed highly in ripening fruit (Figs. 5 and 6), whereas barley *ARA-1* is expressed in grain, root, and leaf, and *AtBXL1* is expressed highly in stem. Moreover, during fruit ripening, including Japanese pear, the loss of arabinosyl residues from the fruit cell wall was generally observed, but loss of xylosyl residues was not. The release of arabinosyl residues from Japanese pear was observed in an acid-soluble hemicellulose fraction where uronic acids were also detected (Yamaki et al., 1979) during the over-ripening stage. α -L-Arafase may act in the hydrolysis of the highly branched side chain of pectic and hemicellulosic polysaccharides and contribute to a part of the fruit-softening process.

In this study, we purified α -L-arafase from the cell wall of ripening pear fruit and determined that its mRNA is expressed highly only in ripening fruit. It was also determined that *PpARF2* is a new member of the GH family 3, according to the primary structure of the enzyme and its activity against native substrates. However, we did not elucidate how the loss of arabinosyl residues during fruit ripening affects fruit softening. The existence of arabinosyl residues in wall polysaccharides seems to affect the adhesion of each polysaccharide or cell. In apple fruit, Ara content decreased during the over-ripening stage (Peña and Carpita, 2004) and a decrease of arabinosyl residues from cell wall polysaccharides was observed in the development of mealiness of apple fruit during post-harvest storage (Nara et al., 2001). On the other hand,

in peach fruit, extensive loss of arabinosyl residues from both loosely and tightly bound matrix glycans was observed in normal ripening fruit, but did not occur in mealy fruit, which showed a decline in the loss of arabinosyl residues containing polysaccharides firmly attached to cellulose (Brummell et al., 2004a, 2004b). A larger amount of tightly bound arabinosyl-containing polysaccharides was observed in softening-suppressed, colorless, nonripening (*Cnr*) mutant tomato fruit, which has a mealy texture (Thompson et al., 1999; Orfila et al., 2001, 2002). It was also reported that the existence of arabinosyl residues in pectic side chains affected cell-to-cell adhesion in cultured cells (Iwai et al., 2001). Fruit softening is a complex process and several cell wall-metabolizing enzymes contribute to modification of cell wall architecture. Therefore, α -L-arafase, in particular, may play an important role in alteration of fruit texture during softening.

MATERIALS AND METHODS

Plant Material

Japanese pear (*Pyrus pyrifolia*) cv Housui fruit was harvested from Shinohara Orchard in Yokohama City, Kanagawa Prefecture, Japan. Fruit was picked 15, 30, 45, 60, 75, 90, 105, 120, and 135 DAF. The fruit harvested on 135 DAF was at optimum maturity for eating. Buds, leaves, roots, and shoots were sampled to use in RNA gel-blot analysis. Peeled and cored fruit and the other organs were immediately frozen in liquid nitrogen and then stored at -85°C .

Enzyme Extraction and Purification

The extraction of α -L-arafase was carried out at 4°C , and all buffers contained 10 mM 2-mercaptoethanol, unless stated otherwise. The cell wall-bound enzyme fraction was prepared by the method described previously (Tateishi et al., 1996). To solubilize this enzyme from the cell wall, the cell wall fraction was resuspended in 10 mM borate buffer, pH 9.0, containing 1.0 M NaCl and 0.1% (v/v) Triton X-100, and gently stirred for 1 h. The suspension was centrifuged at 15,000g for 30 min, and the resultant supernatant was defined as the crude enzyme. The crude enzyme was then dialyzed against 10 mM potassium phosphate buffer, pH 8.0, containing 0.1% (v/v) Triton X-100 (buffer A). It was then applied to a hydroxyapatite (Wako Pure Chemical Industries, Osaka) column (2.5 \times 6.1 cm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of 0 to 400 mM potassium phosphate buffer, pH 8.0, containing 0.1% (v/v) Triton X-100. Active fractions were pooled and dialyzed against 10 mM Tris-HCl buffer, pH 8.5, containing 0.1% (v/v) Triton X-100. After dialysis, the dialysate was applied to a Q-Sepharose FF (Amersham-Pharmacia Biotech, Uppsala) column (1.6 \times 10 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.5, containing 0.01% (v/v) Triton X-100 (buffer B). The column was washed by the same buffer and the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer B. The active fractions were inserted into a dialysis tube and concentrated to 3 mL using polyethylene glycol (average M_r 20,000) surrounding the tube. Finally, the concentrated protein was loaded onto a Sephacryl S-200 (Amersham-Pharmacia Biotech) size-exclusion chromatography column previously equilibrated with buffer B containing 0.2 M NaCl. Assay of α -L-arafase activity using *p*NPA (Sigma, St. Louis) was performed according to the method described previously (Tateishi et al., 1996).

Protein Determination and SDS-PAGE

The protein concentration of the fractions was determined using the Coomassie Brilliant Blue dye-binding method (Bradford, 1976) with bovine serum albumin as a standard. SDS-PAGE was carried out by the method of

Laemmli (1970) on 8.5% separation gel, and the protein bands were stained with silver nitrate (Wray et al., 1981).

Activity against Native Substrate and Oligosaccharides

Cell wall polysaccharides from Japanese pear fruit were prepared according to the method previously described (Tateishi et al., 2001b). Briefly, fruit (105 DAF) was homogenized in ice-cold 80% ethanol, and enzymes included in precipitates were inactivated in Tris-buffered phenol (Huber and O'Donoghue, 1993). The ethanol-insoluble materials were washed by ethanol and reprecipitated by 80% (v/v) ethanol. The resultant cell wall precipitate was freeze dried and defined as AIS. Xylan from birchwood was purchased from Sigma. Sugar beet arabinan, wheat arabinoxylan, arabinofuranohexaose, and xylopyranohexaose were purchased from Megazyme (Bray, Ireland).

Purified α -L-arafase was dialyzed against 5 mM sodium-citrate buffer, pH 4.5, and then concentrated using polyethylene glycol surrounding the dialysis tube. Reaction mixtures consisting of 0.5% (w/v) substrate, 5 mM sodium-citrate buffer (pH 4.5), and purified α -L-arafase, equal to 1 μ mol h⁻¹ activity against pNPA were incubated at 37°C for 72 h for native substrates, and 96 h for oligosaccharides. Toluene (20 μ L) was added to each reaction mixture to prevent bacterial effects.

The monosaccharide product released from native substrates during enzymatic hydrolysis by α -L-arafase was determined by using thin-layer chromatography on silica plates (silica gel 64 F254; Merck, Rahway, NJ). After incubation, the reaction mixture was centrifuged at 15,000 rpm for 15 min. Then ethanol was added to the supernatants (200 μ L) to the final concentration of 80% (v/v), and the mixture was centrifuged at 15,000 rpm for 30 min. The supernatant was collected and evaporated to dryness with a rotary vacuum evaporator, then dissolved again in 5 μ L of water. A silica plate was sprayed with 0.2 M NaH₂PO₄, dried in air for more than 12 h, and dried completely at 110°C. Concentrated samples (0.5 μ L) were spotted onto the plate and developed in *n*-propanol:water (85:15). Sugars were visualized by heating the plate at 95°C after spraying ethanol:sulfuric acid:*p*-anisaldehyde (95:2.5:2.5) on the plate. For analysis of the products of oligosaccharide substrates, 1.0 μ L of the reaction mixture was spotted on a silica plate (no pretreatment) and developed in ethyl acetate:acetic acid:water (2:1:1) for arabinohexaose or in *n*-propanol:ethanol:water (7:1:2) for xylohexaose.

Determination of Internal Amino Acid Sequence

Protein purified by SDS-PAGE was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) and the N-terminal amino acid residues were sequenced with an ABI automated protein sequencer using Edman degradation chemistry. In addition, purified α -L-arafase was extracted from the gel and digested by trypsin. The peptide fragments generated were separated with reversed-phase HPLC and the sequences of the peptide fragments were also determined.

Cloning of α -L-Arafase cDNA

Total RNA was extracted from ripe Japanese pear fruit using the hot borate extraction method (Wan and Wilkins, 1994); the extraction buffer contained 1% Nonidet P-40, 2% polyvinylpyrrolidone, and 1% sodium deoxycholate (Tateishi et al., 2001a). First-strand cDNA was synthesized from 1 μ g total RNA using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase with a *NotI*-oligo d(T)18 primer (5'-ACCTGGAAGAATTCGCGGCCGAG-GAA(T)18-3'). The degenerate primers were designed based on amino acid sequences from purified Japanese pear α -L-arafase fragments; ARA-Fr39 (5'-CCNGGNCAVCARCARGARTTRGC-3', upstream) and ARA-Fr57C (5'-CCCATNCCRAANGGRAANCANAC-3', downstream) were used. The temperature program was 1 cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; 1 cycle of 20 min at 70°C. PCR products were analyzed on 1% agarose gel and the cDNA fragments were recovered from the gel using QIAEX II (Qiagen, Valencia, CA) followed by cloning into a pGEM-T easy vector (Promega, Madison, WI), according to the manufacturer's instructions. After the sequence was confirmed, the 5'-end of the *PpARF2* cDNA was amplified using the 5'-RACE system (version 2.0; Invitrogen); ARA-GSP95C (5'-TCACATCAATAGGGCCGCT-3') was used as the gene-specific primer. After incubating oligo-dC with terminal deoxynucleotidyl

transferase, the dC-tailed cDNA was purified and amplified by PCR using the Japanese pear-specific nested primer ARA-GSP44C (5'-AAGCCCTGGC-CACCCTAGAC-3') and the abridged anchor primer supplied with the kit. An amplified DNA fragment was cloned into a pGEM-T easy vector and sequenced. The full-length *PpARF2* cDNA was synthesized using high-fidelity DNA polymerase (Platinum *Pfx* DNA polymerase; Invitrogen). The primers, ARA-START2 (5'-GGGAGAAAAATACACAATATTCC-3', sense) designed from the obtained sequence of 5'-RACE and *NotI*-oligo d(T)18 (antisense) were used, incubated with a temperature program of 1 cycle of 2 min at 94°C; 30 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 68°C, and then incubated at 70°C for 20 min after addition of 1 unit of *Taq* DNA polymerase to the reaction tube. An approximately 2.6-kb band was recovered from the agarose gel and cloned into a pGEM-T easy vector.

DNA Sequencing and Sequencing Analysis

Sequencing was carried out by the primer walking method with an ALF Express II DNA sequencer (Amersham-Pharmacia Biotech). BLAST programs were used for searching homology against nucleotide sequence databases and deducing amino acids. The targets of the deduced protein were analyzed with PSORT (Nakai and Kanehisa, 1991) and TargetP (Emanuelsson et al., 2000), and the motifs were subjected to the PROSITE program. The ClustalW program was used for alignment of the deduced amino acid sequences and drawing the unrooted phylogenetic tree.

RNA Gel-Blot Analysis

Total RNA (1.5 μ g per lane) was separated by electrophoresis on 0.8% formaldehyde denaturing agarose gel and blotted onto a positively charged nylon membrane. The blotted membrane was prehybridized at 68°C for 1 h in a solution containing 5 \times SSC, 50% formamide, 1% (w/v) blocking reagent (Roche Diagnostics, Mannheim, Germany), 0.1% (v/v) *N*-lauroylsarcosine, and 0.02% (w/v) SDS. Then overnight hybridization followed in the same solution containing a digoxigenin (DIG)-labeled RNA probe that was prepared from the 3'-untranslated region of a full-length α -L-arafase cDNA clone (nucleotide sequence corresponding to 2,265–2,575) with a DIG RNA-labeling kit (Roche Diagnostics). After hybridization, the membrane was washed twice in 2 \times SSC containing 0.1% (w/v) SDS for 5 min, followed by two washes in 0.1 \times SSC containing 0.1% (w/v) SDS at 68°C for 15 min. The hybridized DIG-labeled probe was detected by a DIG nucleic acid detection kit using CDP-Star (Tropix, Bedford, MA).

Expression of *PpARF2* and Western Blotting

The DNA fragment excluding signal peptides of *PpARF2* was amplified using the primers of *SalI* ARA103 (5'-ACCGCTCGCATGGCAGTTG-TGCATGCTCG-3', sense) and *NotI*-oligo d(T)18 (antisense) with *Pfx* DNA polymerase. The temperature program was 1 cycle of 2 min at 94°C; 30 cycles of 1 min at 94°C, 2 min at 57°C, 3 min at 68°C, and incubation at 70°C for 5 min. The PCR fragment was digested by *SalI* and *NotI* and subcloned into a pPROEX expression vector (Gibco-BRL, Cleveland) previously digested by the same endonuclease. The subcloned insert was sequenced to confirm that no nucleotide error had occurred during PCR amplification. The subcloned *PpARF2* was cultured and the protein was induced with isopropylthio- β -galactoside, according to the manufacturer's instructions. The induced protein obtained as an inclusion body was solubilized with urea and purified using a HisTrap kit (Amersham-Pharmacia Biotech). The purified protein was injected into rabbit to raise the antibody that was used for immunoblot analysis. Proteins separated in the gel by SDS-PAGE were electroblotted on PVDF membrane, and the membrane was blocked in 1% blocking reagent in Tris-buffered saline plus Tween buffer. The blocked membrane was incubated for 1 h with the generated polyclonal antibody (1,000-fold dilution) in the same buffer. After washing steps, the membrane was incubated with anti-rabbit IgG-alkaline phosphatase-conjugated goat antibody. The signal was visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development after the washing steps.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank data libraries under accession number AB195230.

ACKNOWLEDGMENTS

The authors are very grateful to Prof. Naoki Sakurai, Hiroshima University, for helpful discussions and advice. We also thank Mr. Minoru Shinohara, Yokohama City, Kanagawa Prefecture, for assisting in the cultivation of Japanese pear.

Received November 16, 2004; revised February 24, 2005; accepted March 14, 2005; published June 17, 2005.

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