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α-L-ARABINOFURANOSIDASE FROM CELL WALLS OF JAPANESE PEAR FRUITS

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Key Word Index—*Pyrus serotina*; Rosaceae; Japanese pear; α-L-arabinofuranosidase; purification; cell walls; ripening.

Abstract—Cell wall-bound glycosidase activities were measured in pre-ripe and ripe fruits of Japanese pears (*Pyrus serotina* Rehd. var. *culta*. cv. Hosui). α-L-Arabinofuranosidase (EC. 3.2.1.55) activity increased dramatically with fruit ripening and its activity was assayed during fruit development and ripening. After the fruit enlargement stage, cell wall-bound α-L-arabinofuranosidase activity increased 15-fold with fruit ripening. The enzyme was solubilized from cell walls using the chelator *trans*-1,2-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid and the solubilized enzyme purified using DEAE-cellulose, hydroxyapatite, Mono Q and Sephadex G-100 chromatography. The purified enzyme was a M_r 42 000 monomer on SDS-PAGE. Optimum pH activity was 5.0 and the K_m value was 34 mM for *p*-nitrophenyl-α-L-arabinofuranoside.

INTRODUCTION

Fruit-softening is closely connected with changes in cell wall polysaccharides and this cell wall modification depends on some cell wall-degrading enzymes [1]. Although polygalacturonase has been one of the most investigated enzymes in relation to tomato fruit-ripening, some recent studies have suggested that it is not the primary determinant of tomato fruit-softening [2, 3].

The loss of arabinose and galactose from cell wall fractions during fruit-ripening has been observed in apples [4, 5], tomatoes [6], Japanese pears [7] and kiwifruits [8]. In kiwifruit and nectarine, it has been suggested that the solubilization of pectic polymers during ripening occurs without changes in the degree of polymerization [8, 9]. It was proposed that the loss of arabinose and galactose from the side-chains of pectic polymers is an important event in pectin solubilization associated with fruit-softening.

Recently, β-galactosidase was isolated from muskmelon, avocado and kiwifruit, and its role in fruit ripening discussed [10–12]. However, α-L-arabinofuranosidase (α-L-Arafase) has not been purified from the cell wall of fruits and there is little information about its role in fruit-ripening. In the present study, α-L-Arafase activity was assayed during the development of Japanese pears and the enzyme solubilized and purified from the cell walls.

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RESULTS AND DISCUSSION

Changes in glycosidase activities during ripening

There are many kinds of glycosidase activity in the cell wall fraction of fruits. α-L-Arafase, α and β-galactosidase, β-glucosidase, α- and β-mannosidase and α- and β-xylosidase activities were measured in order to investigate the relationship between glycosidases and ripening of Japanese pears (Table 1). Among these eight glycosidases, α-L-Arafase showed the most change in its activity. The activities of β-glucosidase and β-xylosidase also increased during ripening. The increase in α-L-Arafase activity thus may be related to the loss of arabinose from the cell wall fraction of Japanese pears. In contrast, although galactose is also released from the cell wall, only a little change in the activity of β-galactosidase was observed between the pre-ripe and ripe stages. It is reported that one of the isoforms of β-galactosidase plays a role in the release of galactose from pectic polymers of Japanese pears [13].

Seasonal changes in α-L-Arafase activity

α-L-Arafase activity in the cell wall-bound and buffer-soluble fraction was measured at different stages (Fig. 1). The cell wall-bound activity was highest on a fresh weight basis in young small fruits on 25 May and then decreased gradually as the fruit expanded. During this period, the activity per fruit remained constant and the number of cells per fruit was also constant. Therefore, a decrease in the activity per gram fresh weight is due to cell expansion. After the fruit enlargement stage,

Table 1. Changes in cell wall-bound glycosidase activities in pre-ripe and ripe fruits of Japanese pear

Enzyme	Activity (pkat^{-1} g fr. wt)	
	Pre-ripe	Ripe
α-Arabinofuranosidase	3.8	18.2
α-Galactopyranosidase	41.4	50.6
β-Galactopyranosidase	167	173
β-Glucopyranosidase	17.5	51.4
α-Mannopyranosidase	117	120
β-Mannopyranosidase	10.1	11.7
α-Xylopyranosidase	ND*	ND
β-Xylopyranosidase	6.8	12.4

*ND = not detected.
Values are means of two separate experiments.

Table 2. α-L-Arafase activities solubilized from cell walls of Japanese pear fruits

Treatment	pH	Activity (pkat^{-1} g fr. wt)	%
Control*		17.8	100
1M LiCl	6.5	1.22	6.8
2M LiCl	6.5	0.63	3.5
3M LiCl	6.5	0.15	0.8
0.5% EDTA	6.5	1.43	8.0
1% EDTA	6.5	3.21	18.0
1% EDTA	7.5	3.54	19.9
1% EDTA	8.5	5.11	28.7
3% EDTA	8.5	0.83	4.7
5% EDTA	8.5	0.50	2.8
1% EGTA	8.5	3.47	19.5
1% CDTA	8.5	5.95	33.4

*Activity of cell wall-bound fraction.

activity increased with fruit-ripening. The activity per gram fresh weight in ripe fruits on 4 September was *ca* 15-fold of that in pre-ripe fruit at 15 August. This increase in α-L-Arafase activity in the cell wall-bound fraction during ripening suggests a role for this enzyme in fruit-softening. On the other hand, α-L-Arafase activity in the buffer-soluble fraction also increased slightly during fruit development and ripening. The function of this type of activity could not be elucidated.

Solubilization of α-L-Arafase from cell walls

For purification and further characterization, α-L-Arafase was solubilized from cell walls of ripe fruits. Although LiCl was usually used for enzyme extraction from the cell wall, it did not solubilize α-L-Arafase very effectively (Table 2). Changes in solubilization period, solution pH and LiCl concentration and the use of NaCl instead of LiCl also did not improve solubility (data not shown). In comparison with LiCl, EDTA solubilized this enzyme much better (Table 2). Other chelating reagents, such as ethyleneglycol bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) and *trans*-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid (CDTA), were also tested for their solubilization capacity; CDTA was the most effective (Table 2). CDTA is now often used for solubilization of pectic components from cell walls. Thus, α-L-Arafase might be solubilized in association with the pectic polysaccharides released by the chelating reagent. Another possibility is that the enzyme conjugates with the cell wall via a divalent cation, such as a Ca^{2+}-mediated bond.

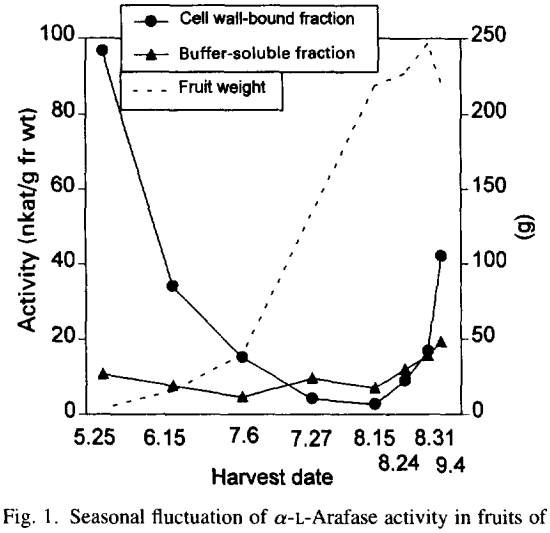

Fig. 1. Seasonal fluctuation of α-L-Arafase activity in fruits of Japanese pears.

Purification of α-L-Arafase

Typical purification steps are shown in Table 3. After solubilization of α-L-Arafase using 1% CDTA for 36 hr from the fruit cell walls, the supernatant was salted out with 25% saturated ammonium sulphate. Although 40% saturation of ammonium sulphate precipitated *ca* 50% of the activity, no more activity could be recovered by additional ammonium sulphate (data not shown). At the step of Mono Q column chromatography, when eluted by a linear gradient of 0–1 M NaCl, α-L-Arafase was obtained in the fraction from *ca* 0.4 M NaCl; no other peak of α-L-Arafase activity was detected (Fig. 2). The specific activity at this step was 56-fold from the CDTA extraction; recovery was 1.9%. When the enzyme preparation was subjected to SDS-PAGE, some bands were detected (data not shown). Active fractions obtained were therefore analysed by Sephadex G-100 gel filtration (Fig. 3). Fractions 22–33 were concentrated and subjected to SDS-PAGE. The reduced recovery of α-L-Arafase activity during the purification steps may result from instability after dissolving it from the cell wall or on proteolytic degradation. α-L-Arafase from fruit cell walls of Japanese pears was a monomer polypeptide with a M_r of 42 000 (Fig. 3). The M_r would appear to be smaller than the values found for α-L-Arafase from lupin cotyledons [14], soybean seedlings [15], radish seeds [16] and cell suspension cultures of carrot [17].

Table 3. Typical purification steps for α-L-Arafase from cell walls of Japanese pear fruits

Step	Activity (nkat)	Protein (mg)	Specific activity (nkat mg^{-1} protein)	Purification factor	Yield (%)
CDTA extract	9.59	29.9	0.32	1.0	100
0–20% $(NH_4)_2SO_4$	3.19	9.41	0.34	1.1	33.3
DEAE cellulose	1.31	0.65	2.02	6.3	13.7
Hydroxyapatite	0.57	0.16	3.58	11.2	5.9
Mono Q	0.18	0.01	18.0	56.3	1.9
Sephadex G-100	0.05	ND	ND	ND	0.5

*ND = not determined.

Properties of α-L-Arafase

α-L-Arafase activity was detected at pH 3.5–6.5 and the optimum was 5.0. α-L-Arafase activity was assayed from 1.2 to 15 mM of *p*-nitrophenyl-α-L-arabinofuranoside to determine a kinetic parameter since the solubility of this substrate was not more than 20 mM. The K_m value for pear fruit α-L-Arafase was 34.7 mM if the reaction showed typical Michaelis–Menten kinetics. This kinetic parameter is higher than those of other plant α-L-Arafase [14–17].

Effects of metal ions and EDTA on enzymic activity are summarized in Table 4; Cu^{2+} and Hg^{2+} were found to be potent inhibitors. This suggests the SH-group is essential for activity. Other divalent cations influenced activity within ±21%.

To our knowledge, this is the first report on the purification of α-L-Arafase from cell walls of a fruit. The M_r and kinetic parameters obtained in this report are different from those of α-L-Arafases from other sources. Interestingly, α-L-Arafase from Japanese pear was strongly associated with the cell wall and its activity increased dramatically during fruit-ripening. This suggests that the enzyme is related to the loss of arabinose from cell walls of this fruit during ripening. Further studies are required to clarify the physiological role of α-L-Arafase in fruit-softening.

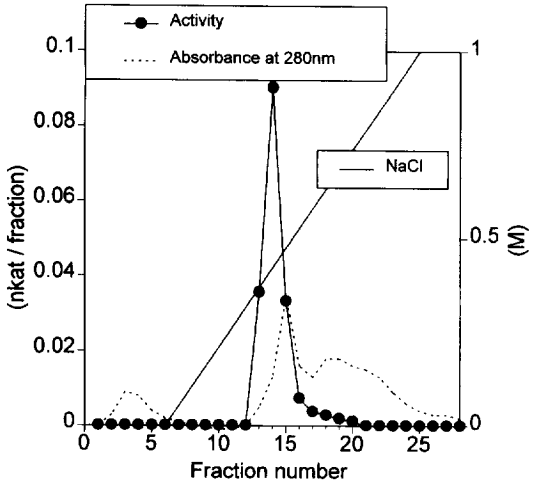

Fig. 2. Elution profile of α-L-Arafase by Mono Q chromatography. One ml fractions were collected.

EXPERIMENTAL

Preparation of buffer-soluble and cell wall-bound enzyme fractions. Enzyme extraction and purification were carried out in a chamber at 4° or on an ice-bath; all buffers contained 5 mM 2-mercaptoethanol, unless stated otherwise. Fruit flesh was homogenized with two vols of 100 mM K-Pi buffer (pH 6.5) containing 30 mM 2-mercaptoethanol and 0.1% (w/v) Na-L-ascorbate. The homogenate was centrifuged at 2500 *g* for 15 min. The supernatant was dialysed overnight against 10 mM K-Pi buffer (pH 6.5) (buffer A) giving a buffer-soluble fr. The ppt. obtained was suspended in buffer A

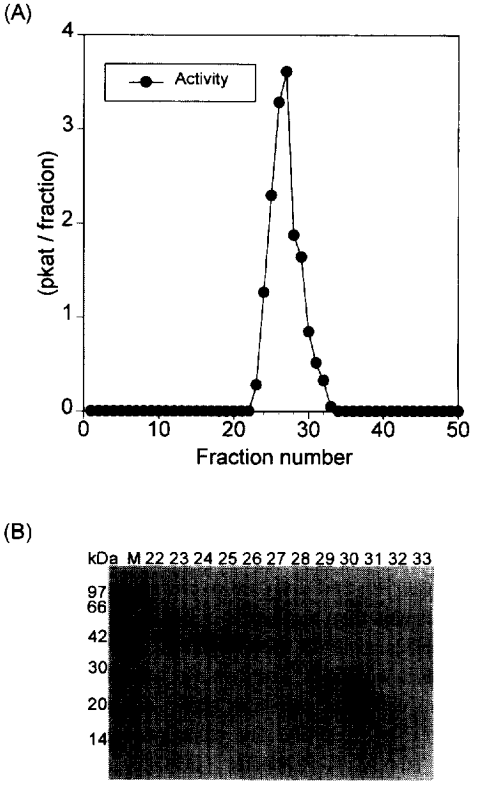

Fig. 3. Elution profile of α-L-Arafase by Sephadex G-100 gel filtration chromatography (A) and SDS-PAGE (B). One ml fractions were collected. Each fraction (22–33) was concentrated and loaded on to the gel for SDS-PAGE. The M_r markers used (lane M) were phosphorylase b (97 400), BSA (66 267), aldolase (42 400), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and lysozyme (14 400).

Table 4. Effects of metal ions on activity of α-L-Arafase, from Japanese pear fruits

	Concentration (mM)	%
$CaCl_2$	1	115
	5	104
$CoCl_2$	0.3	101
	1	102
$CuCl_2$	0.3	51.3
	1	45.9
$FeCl_2$	0.3	85.9
	1	93.2
$HgCl_2$	0.3	14.7
	1	3.9
$MgCl_2$	1	119
	5	121
$MnCl_2$	0.3	98.1
	1	117
$ZnSO_4$	0.3	105
	1	117
EDTA	1	99.6
	10	105

and centrifuged at 2500 *g* for 15 min. This step was repeated twice to avoid contamination with buffer-soluble materials. The ppt. was resuspended and dialysed overnight against the same buffer. This was the cell wall-bound enzyme fr.

Effects of solvents on solubilization of α-L-Arafase from cell walls. Ion order to solubilize α-L-Arafase from the fruit cell wall, LiCl (pH 6.5), EDTA, EGTA and CDTA in 10 mM K-Pi buffer (pH 6.5 and 7.5) or Tris–HCl buffer (pH 8.5) containing 0.1% (w/v) NaN_2 were tested. The cell wall-bound enzyme fr. was suspended in each solvent and stirred for 24 hr. The frs obtained were centrifuged at 12 000 *g* for 30 min and the resulting supernartant assayed for α-L-Arafase activity.

Purification of α-L-Arafase. The enzyme was extracted from cell walls using 1% (w/v) CDTA in 10 mM Tris–HCl buffer (pH 8.5) containing 0.1% (w/v) NaN_2 for 36 hr. After solubilization, it was centrifuged at 12 000 *g* for 30 min. To the supernatant was added solid $(NH_4)_2SO_4$ to 25% satn and the ppt. collected by centrifuguation at 12 000 *g* for 30 min. The ppt. was dissolved in 10 mM Tris–HCl buffer (pH 8.5) (buffer B) and dialysed overnight against the same buffer. It was then applied to a DEAE-cellulose (Whatman DE52) column (2.5 × 10 cm) previously equilibrated with buffer B. The column was washed with the same buffer and eluted with buffer B, which contained 250 mM NaCl. Active frs were pooled and dialysed against 10 mM K-Pi buffer (pH 8.0). After dialysis, protein was adsorbed on a hydroxyapatite (Wako) column (1 × 6.5 cm) that had been equilibrated with the same buffer. After washing with the same buffer, proteins were eluted with 150 mM K-Pi buffer (pH 8.0). Active frs were pooled and dialysed against buffer B. The dialysate was loaded onto a Mono Q (Pharmacia) column previously equilibrated with buffer B and eluted with a linear gradient of 0–1 M NaCl in buffer B. Active frs obtained were finally applied to a Sephadex G-100 (Pharmacia) column that had been equilibrated with buffer B containing 100 mM NaCl.

Assay of enzymic activity. Glycosidase activities were determined by the colorimetric measurement of *p*-nitrophenol released from *p*-nitrophenylglycosides. The reaction mixt., which contained 100 mM sodium-citrate buffer (pH 4.5), 1.2 mM substrate and the enzyme soln, was incubated at 34° for 40 or 60 min. The reaction was stopped by addition of 133 mM Na_2CO_3 and *p*-nitrophenol released measured at 400 nm.

SDS-PAGE. SDS-PAGE was carried out by the method of ref. [18]. Protein bands were stained with $AgNO_3$.

Protein measurement. Protein content was determined by the method of ref. [19] as modified in ref. [20].

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