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# Purification and characterization of two soluble acid invertase isozymes from Japanese pear fruit

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**Abstract**

Two isozymes (AIV I and AIV II) of soluble acid invertase (EC 3.2.1.26) were purified from Japanese pear fruit through procedures including $(NH_4)_2SO_4$ precipitating, DEAE-Sephacel column chromatography, Concanavalin A (ConA)-Sepharose affinity chromatography, hydroxyapatite column chromatography and Mono Q HR 5/5 column chromatography. The specific activities of purified AIV I and AIV II were 2670 and 2340 (nkat/mg protein), respectively. AIV I was a monomeric enzyme of 80 kDa, while AIV II may be also a monomeric enzyme, which is easy to be cleaved to 52 kDa and 34 kDa polypeptide during preparation by SDS-PAGE. The *K*m values for sucrose of AIV I and AIV II were 3.33 and 4.58 mM, respectively, and optimum pH of both enzyme activities was pH 4.5.


*Keywords:* Japanese pear (*Pyrus serotina*) fruit; Soluble acid invertase (AIV; EC 3.2.1.26); Enzyme purification; Isozymes of AIV

## 1. Introduction

Invertase (β-D-fructofuranosidase) cleaves sucrose to glucose and fructose. In general, higher plants contain a family of invertases, which can be discriminated into three types of enzymes, namely vacuolar, cell-wall and cytosolic (Tymowska-Lalanne and Kreis, 1998) ones. Invertase with acidic pH optima (acid invertase; AIV) is localized either in the vacuole or in the cell-wall. The former is a soluble protein in the vacuole, which is soluble AIV (EC 3.2.1.26). In storage organs, soluble AIV was purified from taproot of beet (Leigh et al., 1979), kernel of corn (Doehlert and Felker, 1987), shoot of jerusalem airtichoke (Goupil et al., 1988), potato tuber (Burch et al., 1992) and peach fruit (Moriguchi et al., 1991). Furthermore, soluble AIV has two or more isozymes, which were purified and characterized from some plants (Arai et al., 1991; Obenland et al., 1993; Tang et al., 1996). However, there are few reports that soluble AIV isozymes were purified from fruit (Yelle et al., 1991).

Soluble AIV plays important biological functions related to sucrose metabolism and would presumably hydrolyze sucrose to supply hexose for cell growth and development (Morris and Arthur, 1984; Pfeiffer and Kutschera, 1995; Tang et al., 1996; Woodson and Wang, 1987). Its activity was also reported by closely correlating the ratio of sucrose/hexose in sink organ of tomato fruit (Ohyama et al., 1995) and potato tubers (Zrenner et al., 1996) transformed by the antisense gene of soluble AIV. In addition, sucrose hydrolysis in vacuole by soluble AIV related closely to cell expansion throughout the regulation of osmotic pressure (Klann et al., 1996). In Japanese pear, soluble AIV activity was high in young fruit and decreased during fruit maturation in which the fruit accumulated a lot of sucrose (Moriguchi et al., 1992; Tanase and Yamaki, 2000). However, it is still unclear if a decrease of soluble AIV activity is related to sucrose accumulation as the fruit matures (Moriguchi et al., 1992). Perhaps, the presence of different isozymes is needed to achieve appropriate different roles, which may be regulated differentially. In

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order to test this hypothesis, it is first necessary to purify soluble AIV isozymes and compare their properties. The results of this endeavor are discussed below.

## 2. Results and discussion

### 2.1. Separation and purification of two AIV isozymes in Japanese pear fruit

Soluble AIV from Japanese pear fruit was partially purified through the processes of ammonium sulfate precipitation, DEAE-Sephacel chromatography and ConA-Sepharose chromatography (Table 1). Partially purified soluble AIV isozymes were separated and purified by the hydroxyapatite and Mono Q chromatography. As shown in Fig. 1, the hydroxyapatite chromatography (HA-1000) showed two large peaks of AIV activity. The first peak was eluted by low Na-phosphate buffer concentration, and defined as AIV I. The second peak, which was eluted by high Na-phosphate buffer concentration, was defined as AIV II. The occurrence of two isozymes was also reported in mung bean (Arai et al., 1991). This was a very effective step to separate and purify AIVs, although a large amount of activity was lost. After hydroxyapatite chromatography, each peak of activity was further purified individually using Mono Q anion exchange chromatography. AIV I and AIV II were purified to specific activities of 2670 and 2340 (nkat/mg protein), respectively, according to the procedures described in Table 1. The recovery of AIV I and AIV II was very low (1.38 and 0.62%, respectively) compared with other plant species. The purification levels of AIV I and AIV II were 351 and 307-fold, respectively, of the initial activity measured after ammonium sulfate precipitation. The specific activities of AIV I and AIV II were similar to those of mung bean seedlings (Arai et al., 1991), barley leaves (Obenland et al., 1993) and *Arabidopsis thaliana* leaves (Tang et al., 1996).

### 2.2. Molecular weight and subunit construction

The peak of activity in AIV I or AIV II appeared in the same fraction (66,000 daltons) on the Superose 12 gel filtration (data not shown). Analysis by SDS–PAGE of the purified AIV I protein revealed a single protein band with 80 kDa (Fig. 2A, lane 1). The $M_r$ of AIV I was nearly the same as the value obtained for the native protein by Superose 12 gel filtration. Therefore, AIV I is a monomeric protein similar to AIV I in barley leaves (Obenland et al., 1993) and AIV in potato tuber (Burch et al., 1992). On the other hand, the final preparation of AIV II contained a total of three bands (80 kDa, 52 kDa and 34 kDa) as revealed by SDS–PAGE (Fig. 2B, lane 1). Western blots using polyclonal antibodies raised against the 38 kDa polypeptide and 30 kDa polypeptide of mung bean AIV (Arai et al., 1991) revealed major polypeptides on SDS–PAGE to be different (Fig. 2A and B, lanes 2 and 3). The antibody against the 38 kDa polypeptide is monospecific, whereas the antibody against the 30 kDa polypeptide can cross-react with the glycoproteins. The 80 and 52 kDa polypeptides of AV II clearly cross-reacted with the antibody against the 38 kDa polypeptide, whereas the 34 kDa polypeptide of AIV II were recognised by only the antibody of 30 kDa polypeptide suggesting that the 52 and 34 kDa of polypeptides of AIV II of Japanese pear fruit corresponded to the 38 and 30 kDa polypeptides of mung bean AIV, respectively. Proteolytic cleavage has been reported for invertases of barley leaves (Obenland et al., 1993), carrot seedlings (Unger et al., 1992) and mung bean seedlings (Arai et al., 1991). Further, the recovery of AIV II activity is less than in AIV I as shown in Table 1, perhaps due to its lower stability.

### 2.3. Optimum pH

The sucrose cleavage activities of both AIV I and AIV II were observed in the acidic range. They were the highest at pH 4.5 and fell sharply at a higher pH (data not shown), implying that these invertases are acid invertases.

Table 1
Purification of soluble AIV from Japanese pear fruit

| Purification step | | Activity (nkat[a]) | Recovery (%) | Protein (mg) | Specific activity (nkat/mg protein) | Purification (-fold) |
|---|---|---|---|---|---|---|
| Crude extract | | | | 646 | | |
| 40–80% ammonium sulfate | | 1210 | 100 | 159 | 7.61 | 1.00 |
| DEAE-Sephacel | | 774 | 64.0 | 33.6 | 23.0 | 3.02 |
| ConA-Sepharose | | 280 | 23.1 | 4.30 | 65.1 | 8.55 |
| HA-1000 | AIV I | 19.1 | 1.58 | $6.80\times10^{-2}$ | 281 | 36.9 |
| | AIV II | 60.5 | 5.00 | $9.45\times10^{-2}$ | 640 | 84.1 |
| Mono Q | AIV I | 16.7 | 1.38 | $6.26\times10^{-3}$ | 2670 | 351 |
| | AIV II | 7.45 | 0.616 | $3.19\times10^{-3}$ | 2340 | 307 |

Two kilogram of flesh tissues of young fruit were homogenized in the extraction medium.
[a] 1 nkat = 1 nmol produced glucose/s.

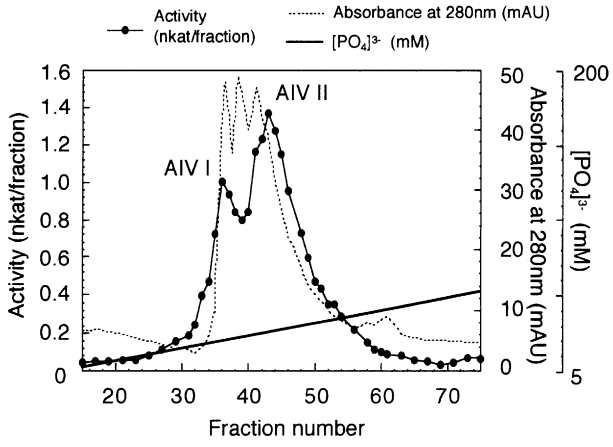


Fig. 1. Elution profile of AIV from hydroxyapatite (HA-1000) chromatography. Proteins were eluted with the elution medium of a linear gradient of 5–200 mM of Na-phosphate buffer. Fractions were collected in 0.42 ml each. AIVs were separated into AIV I and AIV II.

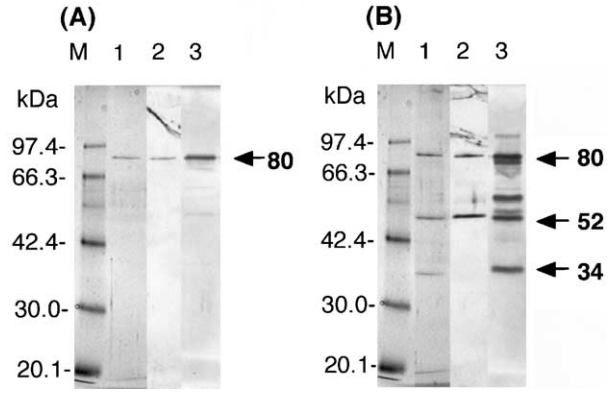


Fig. 2. SDS–PAGE and Western blot of AIV I (A) and AIV II (B). SDS-PAGE was carried out in 10% (W/V) polyacrylamide gels. AIV I (A) and AIV II (B) were stained with silver nitrate (lane 1), immunostained with anti-38-kDa antibody from mung bean AIV (lane 2) and immunostained with anti-30-kDa antibody from mung bean AIV (lane 3). Molecular markers are given on the left.

### 2.4. Kinetic parameters and specificity for substrate

Sucrose cleavage reactions of these enzymes nearly conformed to Michaelis–Menten kinetics (Fig. 3). Kinetic parameters by Lineweaver–Burk plot are shown in Table 2. For AIV I, the $K_m$ value for sucrose was 3.3 mM, which was almost the same as that for AIV II (4.6 mM), and also as that of AIV in peach fruit (4.2 mM, Moriguchi et al., 1991), AIV in carrot seedling (5.0 mM, Unger et al., 1992) and INV1 and INV3 in *Arabidopsis thaliana* leaves (5.0 mM, Tang et al., 1996). Substrate specificity known for other AIV (Pressey and Avants, 1980) were determined with Japanese pear AIV isozymes. These enzymes also hydrolyzed raffinose and stachyose, although the activities were about 20 and 18% of that of sucrose as substrate, respectively (Table 3). These enzymes were unable to hydrolyze lactose, maltose, trehalose, melezitose and melibiose. The

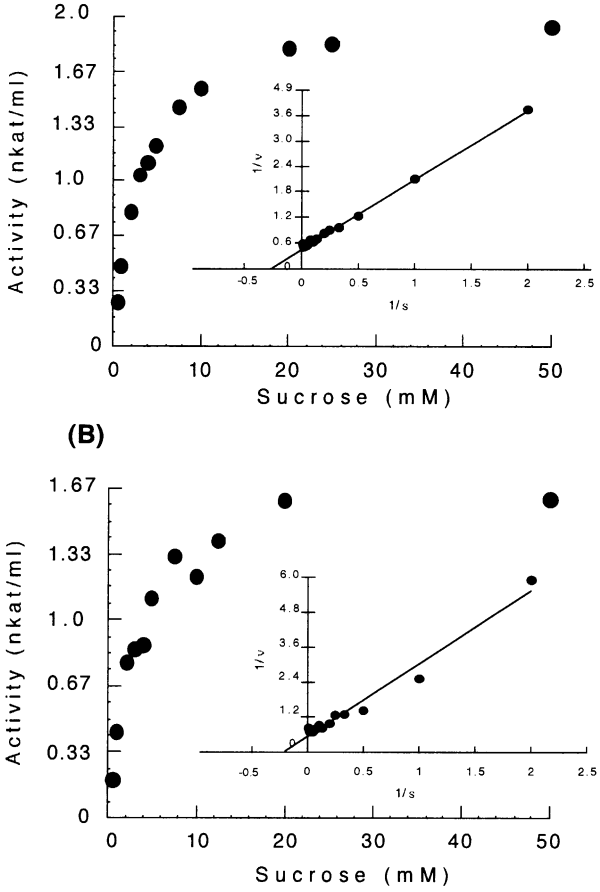


Fig. 3. Saturation curve of AIV I (A) and AIV II (B) for sucrose. The insets show the Lineweaver–Burk plot.

substrate specificity is consistent with that for a β-fructofuranosidase.

There are some reports about the gene expression of soluble AIV isoforms, although the functional difference of its isozymes is ambiguous. In grape, two cDNA (GIN 1 and GIN 2) were cloned from berry (Davies and Robinson, 1996). Expression of both genes was highly active in the young berry before accumulating much hexose, and decreased with maturation. Both showed the same expression pattern in berry. However, in leaf the GIN 1 was expressed highly in unexpanded leaf compared with the GIN 2. In carrot root, two isogenes sI and sII were cloned (Unger et al., 1994). sI was expressed mainly in primary root, while sII was expressed highly with the expansion of taproot. It was suggested that the expression of sII controlled the amount

Table 2
Kinetic parameters for sucrose of soluble AIV from Japanese pear fruit

| | $K_m$ (mM) |
|---|---|
| AIV I | 3.33 |
| AIV II | 4.58 |

and composition of sugar in vacuole, and related clearly to the growth of taproot (Sturm et al., 1995). In Japanese pear, soluble AIV activity was high in young fruit and decreased during fruit enlargement (Moriguchi et al., 1992). However, it is unclear how the two isozymes of soluble AIV change during fruit development. Further studies about the regulation of genes of AIV I and II will be needed to make clear the details of their physiological roles in Japanese pear fruit.

## 3. Experimental

### 3.1. Plant materials

Young fruits of Japanese pear (*Pyrus serotina* Rehder var. *culta* Rehder cv. 'Hosui') were harvested on June 19 and 21 in Nomura's orchard at Anjo in Aichi-pref. Japan. Flesh tissues were excised and stored at −80 °C after freezing them in liquid nitrogen.

### 3.2. Purification of AIV

All procedures were carried out at 4 °C. For purification of soluble AIVs, frozen flesh tissue (2 kg) from young Japanese pear flesh were homogenized in 6 l of 0.15 M Na-phosphate buffer (pH 7.0) containing 1 mM EDTA, 10 mM Na-ascorbate, 10 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10% insoluble PVPP. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 10,000*g* for 30 min. Solid ammonium sulfate was added to the supernatant and the precipitate formed between 40 and 80% salt saturation was collected by centrifugation at 10,000*g* for 30 min. The precipitate was resuspended in a small volume of 10 mM Na-phosphate buffer (pH 6.5) containing 1 mM DTT (buffer A), dialyzed against buffer A and adsorbed to DEAE-Sephacel (Amersham Pharmacia) (2.5 cm i.d.×11 cm) pre-equilibrated with buffer A. The gel was washed with buffer A, then the proteins were eluted with 400 ml of the elution medium which gave a linear gradient of 0–350 mM NaCl in buffer A. The active fractions were applied to a Con-A Sepharose (Amersham Pharmacia) affinity column (1 cm i.d.×13 cm) previously equilibrated with 10 mM Na-phosphate buffer (pH 6.5) containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM DTT (buffer B). The column was washed with 100 ml of buffer B and then eluted with buffer B containing 0.65 M α-methylmannoside. After centrifugation in a Centricon 50 microcentrifuge tube (Amicon), the active fractions were desalted by NAP10 (Amersham Pharmacia) column. The sample was adsorbed to a HA-1000 (Tosoh Co.) hydroxyapatite column (0.8 cm i.d.×7.5 cm) equilibrated with 5 mM Na-phosphate buffer (pH 6.5) containing 1 mM DTT (buffer C). The gel was washed with buffer C, then the proteins were eluted with a linear gradient of 5–200 mM Na-Phosphate buffer (pH 6.5) containing 1 mM DTT. The active fractions were collected and dissolved in a small volume of 20 mM BTP-MES (pH 6.5) buffer containing 10% glycerol and 1 mM DTT (buffer D). The sample was adsorbed to Mono Q (Amersham Pharmacia) (0.5 cm i.d.×5.0 cm) pre-equilibrated with buffer D. The gel was washed with buffer and then the proteins were eluted with a linear gradient of 0–500 mM NaCl in buffer D. The peak fractions were adjusted to 10% glycerol and stored at −80 °C.

Table 3
Substrate specificity of AIV I (A) and AIV II (B)

| Substrate | Activity (nkat/ml) | |
|---|---|---|
| | 50 mM (%) | 100 mM (%) |
| (A) | | |
| Sucrose | 2.05 (100) | 2.22 (100) |
| Raffinose | 0.48 (23.6) | 0.50 (22.5) |
| Stachyose | 0.37 (17.9) | 0.38 (17.3) |
| Lactose | n.d | n.d |
| Maltose | n.d | n.d |
| Trehalose | n.d | n.d |
| Melezitose | n.d | n.d |
| Melibiose | n.d | n.d |
| (B) | | |
| Sucrose | 1.70 (100) | 1.82 (100) |
| Raffinose | 0.37 (21.6) | 0.38 (21.1) |
| Stachyose | 0.32 (18.6) | 0.32 (17.4) |
| Lactose | n.d | n.d |
| Maltose | n.d | n.d |
| Trehalose | n.d | n.d |
| Melezitose | n.d | n.d |
| Melibiose | n.d | n.d |

n.d. = Not detected.

### 3.3. Estimation of molecular weight by Superose 12 gel filtration

The molecular weights of soluble AIVs were estimated using a column of Superose 12 (1.0 cm i.d.×30 cm; Amersham Pharmacia) gel filtration. The standard protein samples (alcohol dehydrogenase, 150,000; BSA, 66,000; Carbonic anhydrase, 29,000 and cytochrome c, 12,400) were applied to a Superose 12 column pre-equilibrated with buffer A containing 150 mM NaCl and 10% glycerol, and run with the same buffer. The fraction was collected in 0.5 ml each and assayed for the activity of AIVs and the absorbance of standard protein samples at 280 nm.

### 3.4. Assay of soluble AIV activity

The reaction mixture contained 30 mM acetate buffer (pH 4.5), 200 mM sucrose and enzyme solution, and incubated for 30 min at 30 °C. The reaction was stopped

by the addition of 400 mM Tris–HCl buffer (pH 8.5). The AIV activity was determined by measuring the amount of reducing sugar formed. Production of glucose was determined by glucose test kit (Wako Pure Chemical Co.). The production of fructose was determined by the enzyme-coupling method using ATP, $NADP^+$, hexokinase, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (Bernt and Bergmeyer, 1974). One nKatal is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product per sec.

### 3.5. Determination of protein content

Protein content was measured by Bradford's method (1976). BSA was used as the standard.

### 3.6. SDS–PAGE and immunoblotting

SDS–PAGE was carried out as described by the method of Laemmli (1970). The gel was stained with $AgNO_3$ using Silver Stain Plus (BIO-RAD). For immunoblotting after SDS–PAGE, proteins were transferred to a cellulose nitrate membrane using a semi-dry blotting apparatus (BIO-RAD) based on the modified procedure of Towbin et al., (1979). The peptides were cross-reacted with rabbit antibodies raised against mung bean soluble AIV (38 kDa and 30 kDa peptide) (Arai et al., 1992).

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