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Chromatographic properties of pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP) from leaves of a CAM plant, pineapple (*Ananas comosus*)

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Introduction

The most typical trait of crassuracean aci metabolism, CAM, is that massive atmospheric CO_2 is taken up via phosphoenolpyruvate (PEP) carboxylase (PEPC) using PEP produced in glycolysis. It appears that regulation of PEPC plays a central role in the control of CAM (Nimmo et al. 1990). PEP of the substrate for PEPC catalysis is supplied by glycolysis at night; by day, the glycolysis pathway works to convert the C_3-residues of malate decarboxylation into sugars. Glycolysis]is the metabolic pathway from glycogen to pyruvate in the living cell, and phosphofructokinase (PFK) has been recognized as a key enzyme in the control of the metabolic flow (Plaxton, 1996).

PFK has two isozymes, which use different energy donors for catalysis (Plaxton, 1996). Especially in pineapple CAM, PPi-PFK (PFP), using pyrophosphate (PPi), was more active than ATP-dependent PFK (Black et al. 1996). While plant PFP has a heteromeric arrangement composed by α- and β- subunits, Tripodi and Podesta (1997) showed that the pineapple leaf PFP possessed a homodimeric (β-type) quaternary structure. Although PFP was shown to be highly regulated by Fru-2,6-P_2 (Stitt, 1990), the glycolic activity of pineapple leaf PFP showed a larger change between day and night than the Fru-2,6-P2 did (Shaheen and Nose, 2000). This study examined the chromatographic properties of pineapple leaf PFP.

Materials and Methods

The plant materials. Pineapple, *Ananas comosus* (L.) Merr. cv. Smooth cayenne, were grown in a heated greenhouse at Saga University, Japan. The pineapple plants were 1.5 years old when used for this experiment. Experimental leaf materials, D leaves, were collected after the plants were kept for 10 days more in a growth chamber (KG-50HLA, Koito Co. Ltd., Japan). The conditions in the chamber were as follows: Day and night temperatures were kept at 30 and 20C, respectively; PAR on the sample leaves was about 330micro mol m^{-2} s^{-1}; and day length was 12hrs, from 8:00 to 20:00.

Isoelectric focusing of PFP: Approximately 7g fresh weight of experimental materials was collected from D leaves at 17:00 (day-form) and the same amount at 24:00 (night-form). The leaf materials were weighed just after collecting, fixed in liquid nitrogen and kept at –80ºC. Enzymes were extracted with 10mL buffer of 100mM HEPES-NaOH pH 8.0, 150mM CH_3COOK, 30mM β-mercaptoethanol, 5mM $MgCl_2$, 1mM EGTA-NaOH pH 8.2, 0.5mM monoiodo acetic acid (MIA), 2%(W/V) PEG 2000, 1%(W/V) PVP-40, and 3g sea sand. The debris was filtered through four layers of cheesecloth, then centrifuged for 15 min at 20000g. The supernatant was used for isoelectric focusing analysis (Standard Rotofer Cell, Biorad, USA).

Purification: Approximately 80g fresh weight of experimental materials was collected from D leaves, then cut into 2- to 5-mm-thick pieces in a cold room (4ºC) and homogenized for 20 min in 100mL cold extraction buffer with Cooking Mixer (MX-X1, National Electrical Ltd., Japan). The buffer was composed of 100mM HEPES-NaOH pH 8.0, 150mM CH_3COOK, 30mM β-mercaptoethanol, 5mM $MgCl_2$, 1mM EGTA-NaOH pH 8.2, 0.5mM MIA, 20%(V/V) glycerol, and 1%(W/V) PVP-40. The homogenate was filtered through four layers of cheesecloth, then centrifuged for 20 min at 38000g, 4ºC. The supernatant was fractionated with 55% saturated NH_4SO_4 (194g/L) at under 4ºC, kept on ice for 30 min, and then centrifuged for 20 min at 20000g. The pellet was resuspended in a minimal volume of the extraction buffer and kept at –20ºC. The resuspended solution was desalted with Sephadex G-25 equilibrated by buffer A; 20mM HEPES-NaOH pH 7.2, 1mM EGTA-NaOH pH 8.2, 2mM β-mercaptoethanol, 1mM $MgCl_2$, 5%(V/V) glycerol. The desalted solution was loaded onto a DEAE-cellulose column (Protein-Pak, DEAE 8HR, 5x100mm, Waters, USA) equilibrated with buffer A connected to a medium- pressure liquid chromatography system (626LC, Waters). After the solution was loaded, an elution was made by increasing the concentration of KCl in buffer A from 0 to 400mM. Active fractions were pooled and then concentrated into a given volume with a freeze-drying centrifuge (RD400,Yamato Sci. Co., Ltd., Japan). The concentrated active solution was size-excluded with buffer A by a Protein-Pak 300 column (8x300mm, Waters) connected to the Waters 626LC system.

Assay of activity: PFP was assayed spectrophotometrically in the glycolytic direction in a medium containing 100mM HEPES-NaOH pH 8.0, 2.5mM $MgCl_2$, 0.1mM NADH, 10mM Fru-6-P, 6 units aldolase, 6 units triosephosphate isomerase, [and] 6 units glycerophosphate dehydrogenase. The contents of chlorophyll and protein were determined by the method of Arnon (1949) and Bradford (1976), respectively.

Results and Discussion

Figure 1 show[s] differences in isoelectric points (IEP) of pineapple leaf PFP between daytime and nightime. High levels of activity of the day-form PFP were observed at fractions 8 and 9, with pH of 4.88 and 5.34, respectively. The night-form PFP showed the highest level of activity at fraction 8, pH 4.80, but the activity at fraction 9 was lower than that of the day-form. Tripodi and Podesta (1997) purified kept in 7hrs illumination and observed an IEP of 6.25. Carlisle et al. (1990) calculated the theoretical IEPs for the potato tuber α- and β-subunits from the sequence data, which were 6.9 and 6.2, respectively. These results suggested that the IEP of crude pineapple PFP was lower than that of the purified protein and showed a different behavior between daytime and nighttime.

Purification results on a DEAE-cellulose column are shown in Fig. 2. Pineapple PFP eluted as two peaks in KCl concentrations: 110mM and 120mM. The early eluted

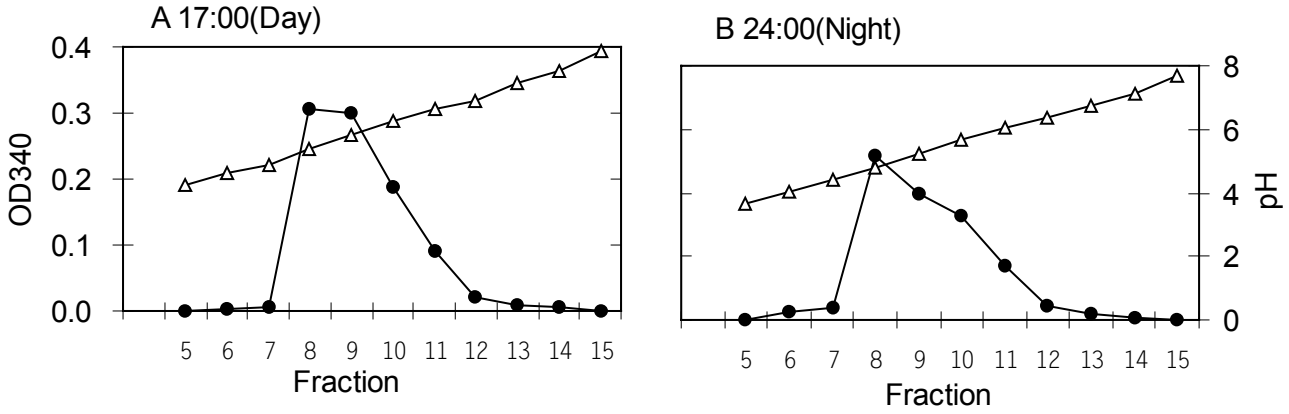

Fig. 1. Isoelectrophoreitic analysis on PFK of pineapple leaves. OD340 means the decreasing rate per min. of absorption at 340nm in activity assay.

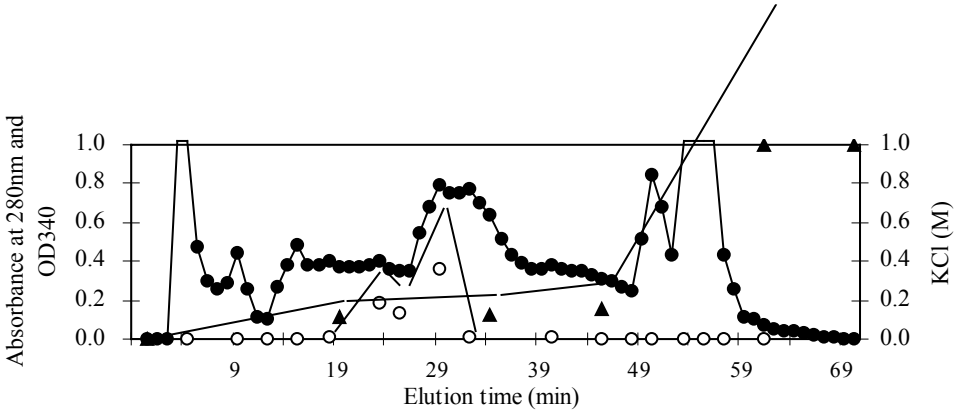

Fig.2. Purification of PFP by chromatography on DEAE-cellulose colum (Protein-Pak, DEAE 8HR, 5x100mm). OD340 means PFP activity shown by the decreasing rate of absorbance at 340nm.

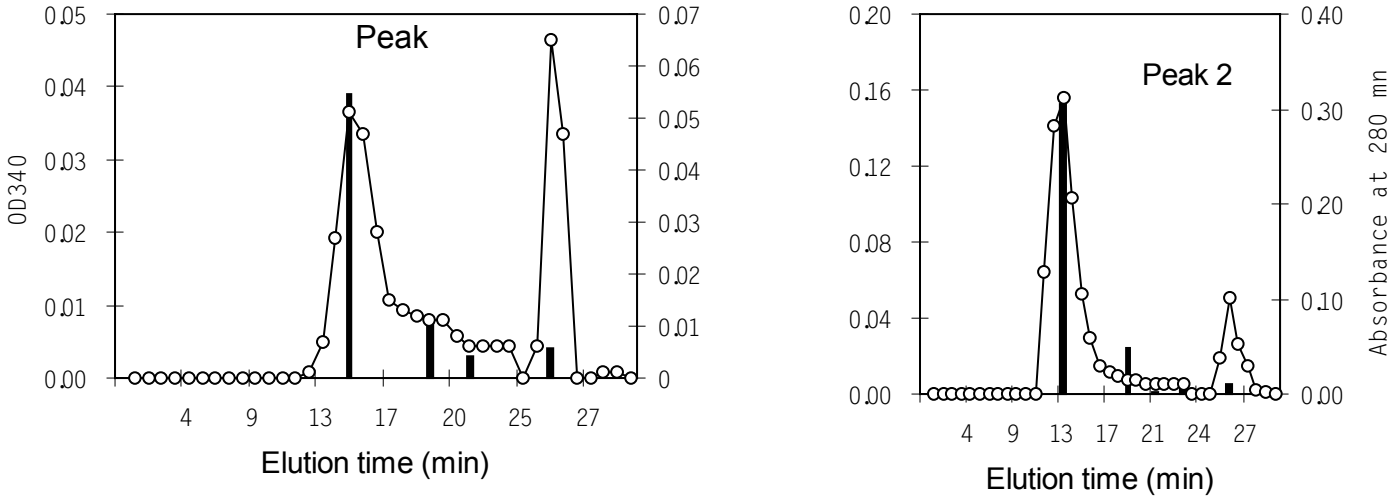

Fig. 3. Purification of two PFP proteins from DEAE 8HR column on molecular sieve chromatography Protein-Pak 300 column . OD340 is same as Fig. 2.

protein was named Peak 1 and the latter Peak 2. Each fraction pool was concentrated into a given volumn by a freeze-drying centrifuger, after which the concentrated fractions were purified by molecular-sieve chromatography (Fig. 3). The p]eak 1 fraction showed two protein peaks on the chromatogram. The first peak, eluted at 14.23 min, had a significant level of PFP activity. In a similar method, peak 2 was also analyzed and showed two protein peaks on the chromatogram. Active PFP protein was eluted at 12.51 min.

The results of this study suggest that the day-form PFP of pineapple was as two proteins with different properties of change balance and molecular weight. Tripodi and Podesta (1997) purified eectrophoretic homogeneity from illuminated leaves, and showed that the purified enzyme consists of a single subunit related to the potato tuber PFP β-subunit. They also suggested that their PFP was not subjected to partial proteolytic degradation during purification. Thus far, many studies have been done on the protein structure of plant PFP in various plant species, and most of these studies have shown that plant PFP is constructed of two subunits, α and β, with different molecular weights. Especially, it was suggested that the α-subunit is responsible for Fru-2,6-P_2 sensitivity (Nielsen, 1994).

The chromatographic properties of pineapple PFP in this experiment showed some differences from previous results, which used the purified protein. In pre-experiments of this study, we also examined the ability of protease inhibitors to conserve activity in extraction. MIA and leupeptin conserved the activity of pineapple PFP for more than 4 hrs, but phenylmethylsulfonyl fluoride (PMSF) did not prevent the decline of activity. Therefore, in this experiment, we used MIA as the protease inhibitor. Tripodi and Podesta (1997), however, used PMSF in their extraction. The difference in protein inhibitors might be one reason why different tendencies appeared.

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