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Separation of a Phloem Specific 42.5 kDa Protein with Two-Dimensional Gel

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ABCTRACT: Phloem exudates leaking from *Luffa cylindrica* fruits by a simple razor blade cutting were mixed with the extraction medium containing antioxidant and rapidly separated by 2-dimensional gel, *i. e.*, native and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). A major protein of 85 kDa occupying 28.2% of total protein had 5 bands on denatured gel. One of them with molecular weight at 42.5 kDa was complementally identified by reversed-phase high performance liquid chromatography and silver stained-band on SDS-gel.

KEY WORDS: P-protein, Native-PAGE, SDS-PAGE, HPLC, Silver staining, *Luffa cylindrica*.

INTRODUCTION

P-protein is a group of proteins occurring in phloem tissues of higher plants, especially monocotyledons and dicotyledons (Behnke and Sjolund, 1990). Morphological forms of p-proteins in amorphous, filamentous, tubular and crystalline forms have been observed in phloem exudate and phloem tissues *in situ*. Their diversity in structure varies with plant species and their developmental stages (Cronshaw, 1975; Kollmann *et al.*, 1970).

Phloem exudates obtained from *Cucurbita* and other plant species displayed that their total protein concentration in phloem exudate was much higher than that of non-phloem tissues (Cronshaw and Sabnis, 1990; Chang, 1995). Analysis of p-proteins by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has shown that p-proteins consist of various kinds of phloem specific proteins and their conformation changes clearly differ from species to species (Cronshaw and Sabnis, 1990). Their molecular weights ranging from 14 to 220 kDa were reported (Kleinig *et al.*, 1971; Weber *et al.*, 1974). Most of p-proteins shown on major band of SDS-PAGE and affinity chromatography were glycoproteins and lectins (Sabnis and Hart, 1978). Phloem lectins had specific affinity for N-acetylglucosamine oligomers(Gietl *et al.*, 1979). PP1 (80 kDa) and PP2 (26 kDa) were identified as a filamentous p-protein and lectins, respectively. Affinity-purified polyclonal antibodies of PP2 from *C. moschata* cross-reacted strongly with PP1 from *C. maxima* (Read and Northcote, 1983). The mRNA of PP2 was detected in companion cells (Bostwich *et al.*, 1992) and PP2 gene sequence containing 858 base pairs was determined (Wang *et al.*, 1995).

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Trypsin inhibitor isolated from phloem exudate of *C. maxima* has been progressed on its cDNA cloning (Murry and Christeller, 1995).

In the present study, a 2-D gel combing native- and SDS-PAGE was provided a rapid separation of p-protein in phloem exudate. The purity of 42.5 kDa protein was examined with high performance liquid chromatography (HPLC) and silver staining of SDS-PAGE.

MATERIALS AND METHODS

Plant materials

Pepos of *Luffa cylindrica* were obtained from Taiwan Provincial Agriculture Experiment Institute, Wu-Feng, Taiwan. Seeds were germinated and grown in an open field. Fruits were collected for further studies.

Extraction and purification of 42.5 kDa protein

Fresh and edible pepos were transversely cut off the outer pericarp with a razor blade. Phloem exudates from natural bleeding were collected with pipetman and mixed well with same volume of collection buffer containing 10 mM Tris (pH 6.8) and 0.13 M β-mercaptoethanol. Meanwhile, collecting buffer were also applied on surface of wounding cut for further bleeding of phloem exudates.

The isolation and purification of 42.5 kDa phloem protein were according to the 2-dimensional gel method of Hames and Rickwood (1981) with minor modification. Brief descriptions were as follows: collected phloem exudate was first run on 10 % native-PAGE at 100 V; An 85 kDa protein were collected after staining with Coomassie Brilliant Blue R 250, immersed in equilibrium solution containing 2% SDS and 2% mercaptoethanol for 30 min, heated at 90°C for 10 min and run second dimensionally on 0.1% SDS-PAGE at 110 V; collected 42.5 kDa protein was eluted with SDS-free electrophoretic buffer in ISCO Little Blue Tank at 50V for 4 h; the anodic protein solution was pooled, dialyzed at 4°C overnight and vacuumly dried by a Savant Speed Vac concentrator. Dried samples were stored at -20 °C for further use.

HPLC separation of 42.5 kDa proteins

Dialyzed and dried samples were dissolved in 300 μl of 0.05% phosphoric acid and filtered with 0.45 μm millipore filter. The filtrate was further run on a Beckman reverse phase-HPLC. Samples were injected into the 0.05% phosphoric acid pre-equilibrium column, separated by using a gradient of 0-90% acetonitrile as a carrier at a flow rate of 0.75 min for 15 min, and finally ran strait with 90% acetonitrile for another 5 min. Protein content was measured at 280 nm and its molecular weight was determined by its retention time in HPLC profile.

Staining of proteins following PAGE

Silver staining of protein on gel was followed the method of Oakley *et al.* (1980) with minor modification. The PAGE gel was gently fixed in 50% methanolic solution (v/v) containing 8% acetic acid (v/v) and 0.0185% formaldehyde (v/v) for overnight, stirred in the same solution except that 50% methanol was substituted by 50% ethanol for 20 min and

washed with fresh 50% ethanol three times (each for 20 min); incubated shakingly in 0.2% $Na_2S_2O_3$ (w/v) for 1 min and rewashed three times with distilled water (each 20 sec); stained in 2% $AgNO_3$ (w/v) staining solution containing 2.8% formaldehyde (v/v) for 20 min and washed three times with distilled water (each 20 sec); mordanted in 6% Na_2CO_3 solution containing 0.185% formaldehyde and 0.0016% $Na_2S_2O_3$ about 10 min, washed with distilled water three times (each for 2 min); fixed in 50% methanolic solution containing 8% acetic acid for 10 min and stored in 50% methanol solution for 10 min; photographed and dried for keeping permanently.

Protein contents

The protein content of phloem exudate was determined with Coomassie Brilliant Blue G 250-binding method (Bradford, 1976). The optical density of protein-dying binding products were measured at 595 nm using bovine serum albumin as a standard. Relative amounts of protein bands on denatured SDS-gel were detected with demsitometry and transformed into percentage.

RESULTS

As shown in Table 1, the volumes of phloem exudates collected through natural bleeding were 2-4 ml per pepo. Protein content of phloem exudate was higher up to 69.2 ± 11.5 mg/ml. The density of phloem exudate is 1.45-1.55. The total protein content in phloem exudate is much higher than that of non phloem tissues or homogenized tissues of leaves (2.46 mg/g), stem (1.86 mg/g) and roots (1.35 mg/g), on the milligram of protein content per milliliter of homogenized tissue basis. Total proteins of phloem exudate in fruits of *L. cylindrica* varied with seasonal changes (Chang, 1995)

Table 1. Protein content of phloem exudate collected from *L. cylindrica* fruits in different season.

Date	Protein content* (mg/ml)
04-21-94	60.30 ± 0.14
04-24-94	72.90 ± 8.91
07-04-94	67.30 ± 0.99
09-10-94	77.80 ± 3.96
09-20-94	81.40 ± 1.13
10-07-94	75.10 ± 2.12
02-05-95	48.20 ± 1.70

* Measured in triplicate

Phloem exudate contained more than ten distinct groups of proteins ranging from 10 to 700 kDa in their molecular weight (Fig. 1A).There were ten major bands on the first dimension gel. Most of them identified as glycoproteins were well separated on a 10% discontinuous native-gel. A major band of 85 kDa occupying the relative amount at 26.76% of total proteins (Table 2) was cut from first dimensional gel, denatured, and run on SDS-gel

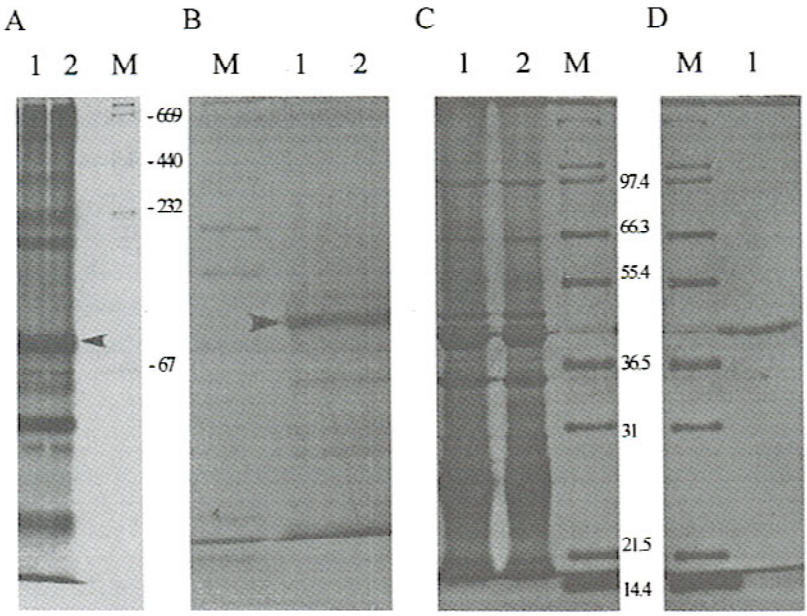

Fig. 1. Electrophoretogram of phloem proteins from fruits of *L. cylindrica* Seven Beauty. Lines M are protein markers. A: Total phloem proteins on native-gel stained with Coomassie blue. B: 42.5 kDa protein further separated on SDS-PAGE stained with Coomassie blue. C: Toatal proteins on SDS-PAGE gel with silver staining. D: HPLC-purifed 42.5 kDa protein on SDS-PAGE gel with silver staining.

(12.5%). There were five distinct bands on second dimensional gel with their molecular weight of 25, 28, 32, 42.5 and 48 kDa. The 42.5 kDa protein was the major band and it occupied 75.68% of total protein on SDS-PAGE (Table 2 and Fig. 1B). Purified protein of 42.5 kDa was used for further study and antibody production. The 42.5 kDa protein was also identified in one dimensional gel of denatured SDS-PAGE (Fig. 1C).

Table 2. Relative protein content of different bands obtained from 85 kDa separated on second-dimensional SDS-gel*.

Peak number	Area	Height	Percent
1	0.183	0.028	3.484
2	0.087	0.024	1.662
3	3.821	0.249	72.586
4	0.758	0.060	14.401
5	0.414	0.039	7.868

* Protein bands and their contents were detected with densitometry.

There were twelve major protein bands on SDS-gel detectable by densitometer in phloem exudate of *L. cylindrica* fruits and they belonged to four major groups of p-proteins (Fig. 1C). Brief description as follows: group I had one band and its molecular weight was 97 kDa; group II contained proteins in their weight ranging from 32-46 kDa and had three major bands of 44, 40 and 34 kDa; group III proteins ranging from 21.5-31 kDa had two

major bands of 30 and 24 kDa; group IV containing proteins with their molecular weight were smaller than 21.5 kDa had six major bands. The relative amounts of group I, II, III and IV p-proteins on gel were 0.75%, 4.47%, 24.2% and 64.57% , respectively.

As shown in Table 2, the 42.5 kDa protein collected from second dimensional gel was eletrotransfered at the recovery of 16% total protein on gel. Dialyzed and dried samples were further separated by a reverse phase-HPLC profile. As shown in Fig. 2A, there were two peaks with retention times at 2.07 min and 10.79 min, respectively, in the blank test. However, there was one additional peak with retention time at 3.81 min in 42.5 kDa protein-containing sample (Fig. 2B). Active fraction collected at 3.81 min retention time was further identified with silver staining on 12% denature gel and it was identical to 42.5 kDa protein (Fig. 1D).

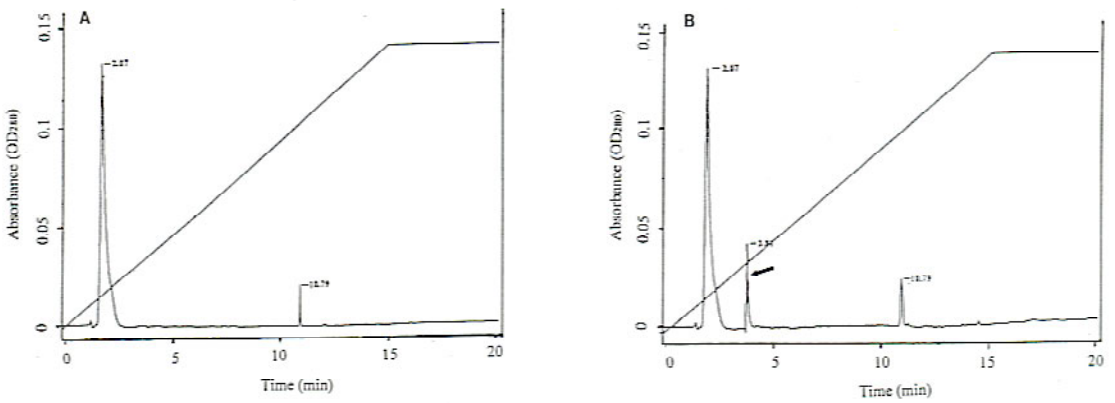

Fig. 2. Profile of HPLC separation of 42.5 kDa protein cut and eluted from SDS-gel. A: Blank profile with two peaks in buffer. B: Sample profile with three peaks. The 42.5 kDa protein with a retention time at 3.31 min was arrowed and two other peaks (retention times at 2.07 and 10.79 min, respectively) were identical to those in blank profile of A.

DISCUSSION

The protein concentrations in phloem exudate vary with plant species. Phloem exudate containing usually high protein content ranging from 10 to 60 mg/ml was reported in most cucurbits (Cronshaw and Sabnis, 1990). In the present study, the protein content of phloem exudate of *L. cylindrica* pepo was also around 60 mg/ml. However, protein contents in phloem exudate from non-cucurbit species were typical low. The sieve-tube exudate protein of *Ricinus communis* seedling was 0.1-0.2 mg/ml (Sakuth *et al.*, 1993). Similar low protein contents in phloem exudate were also found in *Oryza* (0.15-0.2 mg/ml, Ishiwatari *et al.*, 1995), *Tilia* (0.3 mg/ml, Kennecke *et al.*, 1971) and sugar beet (2 mg/ml, Fife *et al.*, 1962).

The protein concentration of phloem exudate was much higher than that of nonphloem tissues in *L. cylindrica* (Wang, 1996). Similar results were also observed in phloem exudate of *Nicotinana* (Easu and Cronshaw, 1967), *Ricinus* (Hall and Baker, 1972) and *Cucurbita* (Eschrich *et al.*, 1971). Meanwhile, phloem exudate of pepos harvested in September has higher protein content than that of pepos harvested in April (Chang, 1995). Low temperature effect on loading and unloading of the phloem related to partitioning of nutrients in sink and source has been paid much attention (Clarkson and Gerlofff, 1980; Farrar, 1988). Only few

papers concerning protein and isozyme patterns of phloem exudate in low temperature-growing plants different from that of plants normal growing plants were reported (Hallgren and Oquist, 1990).

Phloem proteins are abundant proteins in phloem exudate (Cronshaw and Sabnis, 1990). One dimensional SDS-PAGE provides a simple and inexpensive method for analysis of this kind of protein mixture, but the protein bands too closed to be distinguished from each other (Chang, 1995; Schulz *et al.*, 1989). Recent studies on p-proteins were often employed with higher resolution of O'Farrell's 2-D gel consisting isoelectric focus and SDS-PAGE to discrete protein spots on the gel (Ishwatari *et al.*, 1995; Wang *et al.*, 1995). In the present study, a modified 2-D gel consisting of native and denatured SDS-PAGE was adequately employed resolution of 42.5 kDa protein in phloem exudate of pepo. The purity of 42.5 kDa was further identified by reversed phase-HPLC and silver staining of SDS-PAGE. Studies on peptide mapping with cyanogen bromide and analysis of amino acid composition showed the purified 42.5 kDa protein was useful in N-terminal amino acid determination and polyclonal antibody production (Wang, 1996).

There were two peaks in reverse-phase HPLC profile of the blank. The first peak at 2.07 min retention time was eluted at high polarity (87.30% aquous solution containing 0.05% phosphoric acid; Hancock, 1984) and it might be the complex of EDTA-derivatives. The second peak at 10.79 min retention time was eluted at nonpolar region (63.38% acetonitrile). It may originate from the micelle formation of SDS in long duration of low temperature storage (Volkin and Keivano, 1989). Their absorbance at 280 nm might relate to chelating formation.

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利用兩段式電泳分析分離絲瓜韌皮部 42.5 kDa 蛋白質

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摘　　要

絲瓜果實經刀片輕切裂口，塗上含抗氧化劑的萃取液後，其與韌皮部溢流物一起收集。以混合液經活性與變性 (SDS) 的兩次電泳分析。一維分離獲得 85 kDa 活性蛋白質，其約佔全蛋白質的 28.2%，其在二維電泳膠上呈五條帶，其中的 42.5 kDa 的變性蛋白質在高效能電泳色層分析顯示單一峰帶，而在變性銀染色膠上僅呈一蛋白質帶。

關鍵詞：P-蛋白質，原態電泳，變性電泳，高效能色層分析，銀染色，絲瓜。

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