# Analysis of Phloem Exudate Collected from Fruit-Bearing Stems of Coconut Palm: Palm Trees as a Source of Molecules Circulating in Sieve Tubes

Shin-ichi Nakamura[1], Akio Watanabe, Praphasri Chongpraditnun*, Nobuo Suzui**[,2], Hiroaki Hayashi**[,3], Hiroyuki Hattori, and Mitsuo Chino

*Department of Biological Production, Faculty of Bioresource Sciences, Akita Prefectural University, Akita, 010–0195 Japan; *Soil Science Division, Department of Agriculture, Phahonyothin Road, Chatuchak, Bangkok, 10900 Thailand; and **Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, 113–8657 Japan*

Received March 11, 2004; accepted in revised form June 18, 2004

**Sieve tubes have been attracting widespread research interest because of their possible role in mediating physiological signals within the whole plant. However, progress in research into the function of sieve tubes has been limited by the low volume of sap available. To overcome this problem, we attempted to collect phloem exudate from tropical coconut palm trees (*Cocos nucifera* L. cv. Namhom). As much as 3 to 15 mL of exudate per hour was collected from the cut surface of the plant's fruit-bearing stem. Our analyses revealed that the characterized profiles of sugars (sucrose: 339 mM), amino acids (total concentration: 17.1 mM), cations (potassium: 48.3 mM), and proteins (total concentration: 0.1 μg μL$^{-1}$) in the exudate were mostly consistent with those of phloem sap or phloem exudate collected from rice plants, castor bean plants, etc. This exudate was assumed to reflect the composition of the phloem sap from the source organs of coconut palm trees. The large volume of exudate collected contributed significantly to the analyses of the various compounds in the stream of sieve tubes.**

***Key Words:*** **coconut palm, *Cocos nucifera*, phloem sap, sieve tube.**

Sieve tubes, which form a network within the plant body, play an important role in the transport of various compounds including sugars, amino acids, inorganic cations, organic acids, proteins, etc. Because these tubes connect the source and sink organs, it is possible that plants use the sieve tube network not only for the transport of such compounds but also as a multifunctional conduit. Recent findings of signal molecules such as protein kinases (Nakamura et al. 1995; Avdiushko et al. 1997; Yoo et al. 2002) and RNAs (Sasaki et al. 1998; Ruiz-Medrano et al. 1999; Xoconostle-Cázares et al. 1999) in the phloem sap suggest that sieve tubes can act as a communication network that enables to coordinate processes throughout the plant body.

Several attempts have been made to clarify the physiological functions that take place inside sieve tubes. To investigate physiological events in the sieve tubes, it is necessary to collect phloem sap or phloem exudate for analyses. The three methods described below have most commonly been used to collect phloem sap or exudate from plant materials. First, phloem sap can be collected using an insect stylet that has been severed by laser. For example, phloem sap has been collected from rice plants through the stylet of a brown planthopper (Kawabe et al. 1980) and from wheats using the stylet of an aphid (Fisher and Frame 1984). In this method, the purity of the collected phloem sap is very high, but the amount is very small. Second, treating a vertically cut stem surface with ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) facilitates phloem sap exudation (Girousse et al. 1991). However, the amount of sap collected using this method is insufficient for the analysis which is difficult because of the presence of EDTA. Third is a simple incision method, which allows sap to be collected by cutting the plant stem. However, this method is only possible for a few types of plants (cucurbits: Sabnis and Hart 1978;

To whom correspondence should be addressed.
E-mail: sinnaka@akita-pu.ac.jp
Present addresses: [2]Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Takasaki, Gunma, 370–1207 Japan; [3]694 Futago, Aki, Higashi-kunisaki-gun, Oita, 873–0356 Japan.

legumes: Pate et al. 1974). So far, therefore, the very small amounts of phloem sap available for analysis have hindered the thorough investigation of the function of the sieve tubes. To overcome this problem, we attempted to collect large quantities of phloem sap from coconut palm trees.

Palm trees, which are mainly cultivated in tropical regions, are utilized in a variety of purposes, such as food and materials. People in these regions frequently cut the top of the plant's fruit-bearing stems with a sharp knife and collect a large amount of exudate from the severed surface. This exudate is used as a beverage and as a basic material for the production of sugars. The high sugar concentration of the exudate from the palm tree, *Arenga saccharifera*, approximately 400 mM (Van Die and Tammes 1975), suggests that the palm tree exudate has a potential for the study of phloem sap.

Exudate can be collected from coconut palm trees in a similar way to that of the incision method. MacRobbie (1971) demonstrated in his review that the chemical composition of phloem exudates reflected the chemical composition of phloem sap from the source organs. Although the purity of phloem exudate is lower than that of the phloem sap, large quantities of phloem exudate are available for the study of sieve tubes. Many researchers have used the exudates from sieve tubes for their experiments. If the exudate collected from the cut end of a flower-bearing stem is derived from sieve tubes, its chemical composition is likely to be similar to that of the phloem sap or phloem exudate, analyzed previously. The objective of the current study was to demonstrate that the exudate from coconut palm trees is a phloem exudate by analyzing the chemical composition in detail. Studying the molecules in the exudates and their circulation in the sieve tube network should enable to determine the physiological functions of the phloem system to be determined.

## MATERIALS AND METHODS

**Plant material and collection of phloem sap from palm trees.** Coconut palm trees (*Cocos nucifera* L. cv. Namhom) were grown at the Petchaburi Horticultural Experiment Station Center (the Petchaburi district in Thailand) for 6 years on sandy loam soils, under natural climatic conditions (Fig. 1A). Exudate from these trees was taken from the top of their fruit-bearing stems. Stems were severed transversely with a sharp knife and the exudate from the cut surfaces was collected in polyethylene bottles (Fig. 1B). These samples were stored at −20°C until the subsequent biochemical analysis.

**Determination of sugar, amino acid, and inorganic cation concentrations.** Sugar concentrations in the exudate were analyzed by HPLC (Nippon Dionex K.K., Osaka, Japan). Exudates (2 μL) were diluted with 5,998 μL of distilled water, applied to a column (CarboPac™ PA1, Nippon Dionex K.K.; 4 × 250 mm) and eluted with 100 mM NaOH (1 mL min⁻¹). Eluted sugars were detected with a pulse amperometric detector (Nippon Dionex K.K.).

Concentrations of amino acids in the exudate were determined by using an amino acid analyzer (L-8000, Hitachi Ltd., Tokyo, Japan). Two μL of exudate was diluted with 198 μL of 0.02 M HCl. After centrifugation (at 11,000 × *g* and 4°C for 10 min), the supernatant was collected and applied to the analyzer.

To analyze the concentrations of inorganic cations, 15 μL of exudate was diluted with 5,985 μL of 0.1 M $HNO_3$. The concentrations of inorganic cations in the solution were determined by inductively coupled plasma

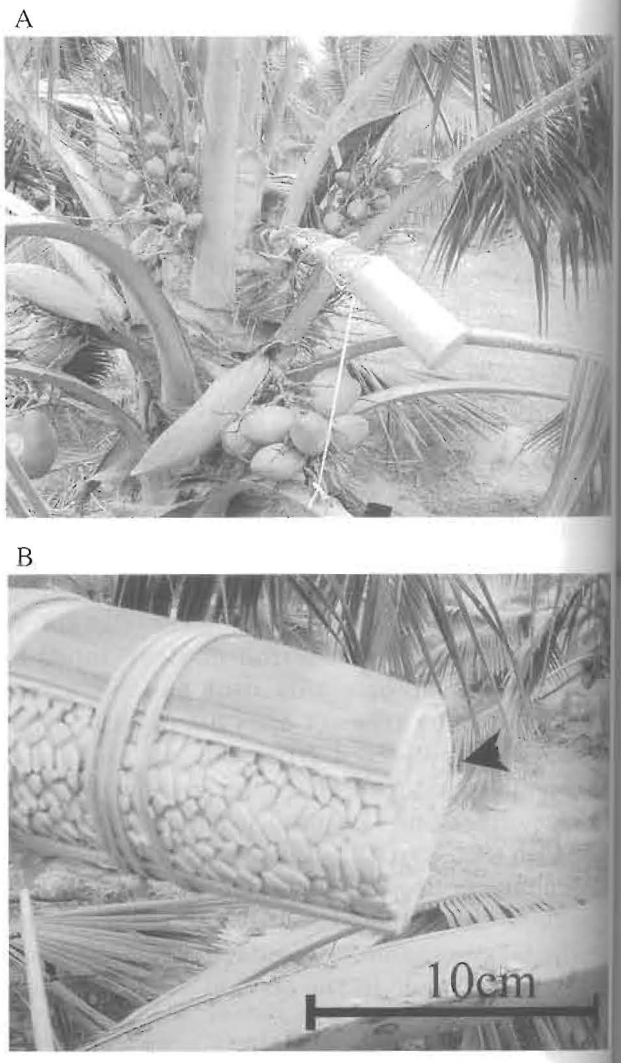


**Fig. 1.** Collection of exudate from coconut palm trees. A: Coconut palm tree. B: Exudate obtained from the cut end of the fruit-bearing stem (arrowhead).

spectrometry (IRIS Duo, Nippon Jarrell-Ash Co., Ltd., Kyoto, Japan).

**Analysis of the proteins in the exudate and those extracted from leaves and fruit-bearing stems.** Soluble proteins were also extracted from both fruit-bearing stems and leaves of coconut palm trees. These samples were frozen with liquid nitrogen in a cold mortar and crushed to a powder, followed by the addition of an extraction buffer containing 100 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride. Subsequently, these extracts were homogenized with a pestle and centrifuged at 18,300 × *g* at 4°C for 10 min. After centrifugation, the supernatants were collected as soluble protein fractions.

The concentrations of the proteins in the exudate and in the leaf and stem extracts were determined by the Bradford method (Bradford 1976). The protein profiles were examined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O'Farrell 1975). The proteins were separated on the gels and detected by the silver staining method (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

**Western blotting analysis of the protein that cross-reacted with anti-thioredoxin *h* antibody.** First, the proteins in the exudate and in the leaf and the stem extracts were separated on a 15% polyacrylamide gel and transferred electrically to a polyvinylidene difluoride membrane (Towbin et al. 1979). After transfer, immunoblotting was performed following the method of Towbin et al. (1979), with an antiserum raised against rice thioredoxin *h*. Immune complexes were detected by using *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt.

## RESULTS AND DISCUSSION

### Collection of exudate from coconut palm trees

After vertically cutting the top of the fruit-bearing stems, most of the coconut palm trees produced an exudate for at least 2 d, allowing us to collect more than 500 mL of exudate on the average from a single cut surface. We assumed that the large sieve tube size of the coconut palm enabled the flow of such a large volume of exudate. The average exudation rate from the cut surface was 133 μL $min^{-1}$ (mean value of three trees): this rate was calculated by measuring the amount of exudate collected and dividing by the collection time. The large standard deviation (± 117.3) emphasized the variation between trees and / or the repeatability of the incision method. The exudation rate of these coconut palm trees was more than 1,000 times higher than that of castor bean plants (0.14 μL $min^{-1}$; Geigenberger et al. 1993) and more than 10,000 times higher than that of rice plants using the insect stylet technique (0.011 μL $min^{-1}$; Hayashi and Chino 1985). The pH of the palm tree exudate was estimated (using pH test paper) to be approximately 7.5, a value similar to that reported for the phloem sap elsewhere (Hall and Baker 1972; Hocking 1980; Fukumorita and Chino 1982).

### Sucrose is the major sugar form in coconut palm exudate

As in the case of the phloem sap from other plant species, sucrose was the major sugar form in the exudate from coconut palm trees. The concentration of sucrose sugar in the exudate amounted to 339 mM (Table 1), whereas for the phloem sap of rice and castor bean plants, the sucrose concentration had been reported to be approximately 600 mM (Fukumorita and Chino 1982) and 175 mM (Geigenberber et al. 1993), respectively. The sucrose concentration in the palm tree exudate was therefore comparable to that in rice and castor bean plants. We also detected small amounts of glucose (5.45 mM) and fructose (9.60 mM) in the coconut palm tree exudate (Table 1). In the *Ajuga* plants, although raffinose family oligosaccharide(s) are the main carbohydrates transported via the sieve tubes (Bachmann et al. 1994), we observed that they were only minor sugars in the palm tree exudate (Table 1).

### Glutamine is the predominant amino acid in the exudate from coconut palm trees

The results of the free amino acid analysis are shown in Fig. 2. The concentration of the total amino acids in the exudate was 17.1 mM, averaged across three independent samples. The amounts of free amino acids in the exudate were approximately ten times lower than those found in rice (Fukumorita and Chino 1982) and *Brassica napus* (Lohaus and Mollers 2000) and similar to that found in castor bean plants (Hall and Baker 1972). Our results indicated that glutamine was the predominant amino acid in the coconut palm tree exudate

**Table 1.** Measurement of the concentrations of sugars in the exudate collected from the fruit-bearing stems of coconut palm trees.

| Sugar | Concentration (mM) |
|---|---|
| Sucrose | 339.0 ± 47.2 |
| Fructose | 9.6 ± 7.8 |
| Glucose | 5.5 ± 1.2 |
| Raffinose | 0.74 ± 0.33 |
| Sorbitol | 0.65 ± 0.11 |
| Stachyose | 0.29 ± 0.08 |

All the values are given as means ± SD ($n = 3$).

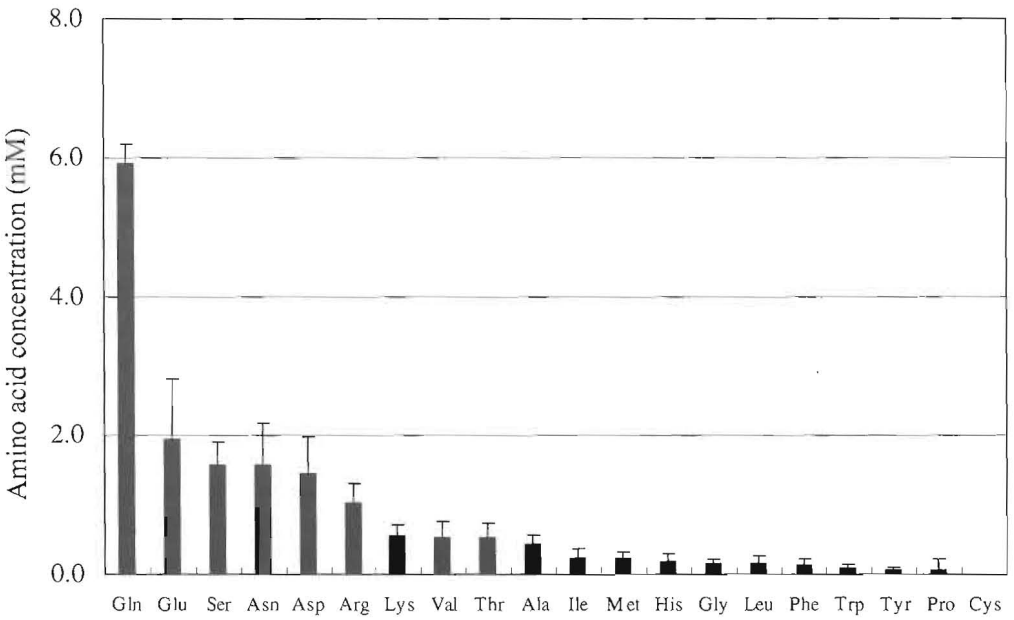


**Fig. 2.** Concentrations of amino acids in the exudate of coconut palm trees. Values are means of three independent samples.

(Fig. 2), occurring at a concentration of 5.8 mM. Glutamine accounted for approximately one-third of the total amino acids present: it was also the predominant amino acid in the phloem sap of the plants mentioned above. These results further support the assumption that the exudate obtained from coconut palm trees was mostly derived from sieve tubes.

The ratio of the sucrose concentration to the total amino acid concentration, calculated as C / N ratio, was 19.8 in the coconut palm tree exudate, a value much higher than that in the phloem sap of rice (3.0) (Fukumorita and Chino 1982), castor bean plants (7.1) (Hall and Baker 1972) and *Brassica napus* (4.8) (Lohaus and Moellers 2000). It has been reported that the C / N ratio of the phloem sap from *Sinapis alba* increased markedly during floral treatment (Corbesier et al. 2002). Because the exudate here was collected during the trees' reproductive stage, it was assumed that the C / N ratio had been modified by the process of floral induction.

## Inorganic cation concentrations in the exudate from coconut palm trees

The concentrations of inorganic cations in the palm tree exudate are presented in Table 2. Potassium, sodium, magnesium, and calcium predominated. The concentrations of $K^+$, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$ were 48.33, 3.36, 2.33, and 0.10 mM, respectively. MacRobbie (1971) demonstrated that high proportions of potassium level (20–85 mM) to sodium level (0.06–0.3 mM) and magnesium level (2.3–23 mM) to calcium level (0.25–0.5 mM) are characteristic features of the phloem sap of many plants. In our results, the sodium level was slightly higher than that reported in the review of MacRobbie (1971) and the calcium level was slightly lower than that reported in the review of MacRobbie (1971). Based on his theoretical concept, we calculated the ratios of potassium to sodium and magnesium to calcium in coconut palm exudates. These ratios were 14.4 and 23.3, respectively. These values indicated that there was a molecular imbalance in the ratios of the potassium level to sodium level and magnesium level to calcium level. These results also supported the assumption that the exudate from coconut palm trees was derived from sieve tubes.

**Table 2.** Concentrations of inorganic cations in the exudate from coconut palm trees.

| Cation | Concentration |
|---|---|
| Potassium | 48.33 ± 3.29 mM |
| Sodium | 3.36 ± 2.54 mM |
| Magnesium | 2.33 ± 0.16 mM |
| Calcium | 0.10 ± 0.04 mM |
| Copper | 18.78 ± 10.66 μM |
| Zinc | 17.54 ± 4.62 μM |
| Iron | 17.31 ± 5.55 μM |
| Manganese | 4.97 ± 1.87 μM |

Values are mean values ± SD ($n$ = 3).

## Protein analysis

The characterized profiles demonstrated that the exudate we collected was indeed derived from sieve tubes. The large amount collected can potentially provide a rich sample of molecules circulating in the plant's sieve tubes, in which many kinds of proteins with some physiological functions have been reviewed (Hayashi et al. 2000). Protein assay analysis revealed that the protein concentration in the coconut palm exudate was approx-

mately 0.1 μg μL$^{-1}$ (across three plants) on the average. This concentration was comparable to that in the phloem sap from rice (Nakamura et al. 1993) and wheat (Fisher et al. 1992).

The SDS-PAGE analysis depicted in Fig. 3 demonstrates that the protein composition in the exudate was distinctly different from that in the extracts from leaves and fruit-bearing stems. A detailed profile of the proteins in the phloem exudate is shown in Fig. 4: 2D-PAGE separation indicated the presence of more than 150 proteins. The major protein was approximately 14 kDa in size with an isoelectric point in the region of 5.5 (arrowhead in Fig. 4).

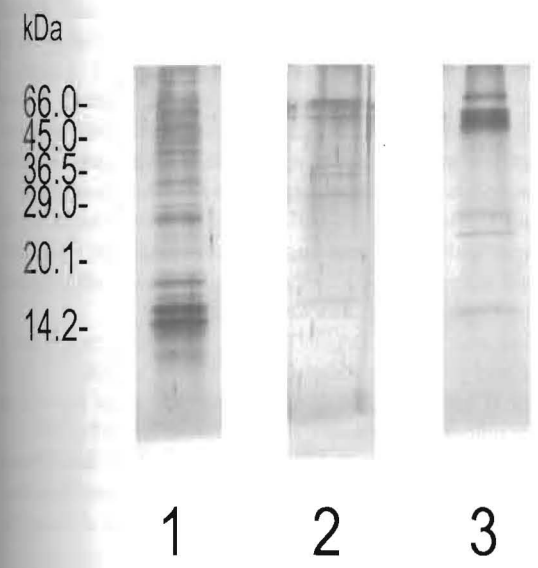


Fig. 3. Analysis by SDS-PAGE of proteins in coconut palm tree phloem exudate, leaves, and fruit-bearing stems. Lane 1, phloem exudate; lane 2, leaves; lane 3, fruit-bearing stems. One μg of protein was subjected to SDS-PAGE analysis and detected by silver-staining in each lane.

Phloem sap from rice plants contains a major 13 kDa protein with an isoelectric point of about 4.8, which has been identified as a thioredoxin *h* (Ishiwatari et al. 1995). It was confirmed that the gene of this protein was mainly expressed in the companion cells of the rice phloem (Ishiwatari et al. 1998). A laser capture microdissection technique also revealed that transcripts of this protein were located in the rice plant phloem cells (Asano et al. 2002). Because the molecular weight and isoelectric point of the major protein in the phloem exudates from coconut palm trees were similar to those of thioredoxin *h*, we carried out a western blotting analysis to determine whether the protein was recognized by the anti-thioredoxin *h* antibody. Figure 5 shows the results of the western blotting analysis. The 14 kDa protein was recognized by the anti-thioredoxin antibody. In an electrophoretic test, the protein was only detected in the lane where phloem exudate was loaded, suggesting that it occurs in higher proportions in the phloem exudate than in the leaves or fruit-bearing stems (Fig. 5). The high proportion of thioredoxin *h* is considered to be characteristic of the phloem sap (Ishiwatari et al. 1995). Thioredoxin *h* has also been detected in the phloem sap of *Triticum aestivum* L., *Yucca filamentosa* L., and other plant species (Schobert et al. 1998). Future studies using a large amount of phloem exudate from coconut palm trees might enable to elucidate the structure and function of this 14 kDa protein within the sieve tubes.

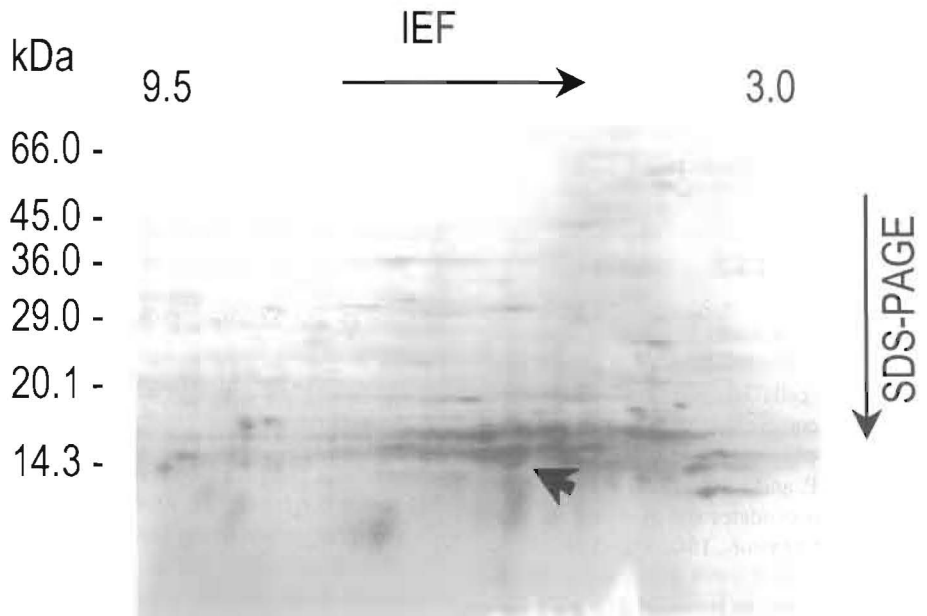


Fig. 4. Two-dimensional PAGE analysis of proteins in the phloem exudate from coconut palm trees. A 30 μg aliquot of protein in the phloem exudate was subjected to 2D-PAGE analysis. After electrophoresis, the gel was silver-stained and proteins on it were detected.

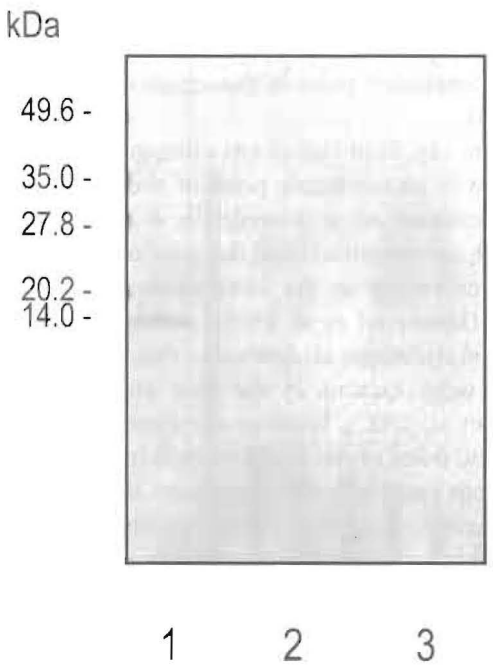


**Fig. 5.** Detection of protein, which was recognized by anti-thioredoxin *h* antibody, in samples from coconut palm trees. Lane 1, protein from phloem exudate (1 μg); lane 2, protein from leaves (1 μg); lane 3, protein from fruit-bearing stems (1 μg). The procedure used to detect the protein recognized by antibody is described in the MATERIALS AND METHODS section.

The results of our analysis indicated that the exudate from coconut palm trees contains various compounds including sugars, amino acids, inorganic cations, organic acids, proteins, etc. and that the composition of these compounds is similar to that of the compounds in the phloem sap from many kinds of plants. Our studies demonstrated that the exudate from the cut surface of the fruit-bearing stems of coconut palm trees is in fact derived from the sieve tubes. This exudate, available in large quantities, could allow to gain further information about the role of the sieve tubes in the mediation of physiological signals within the whole plant.

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