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Proteins in The Exudation Sap from Birch Trees, *Betula platyphylla* Sukatchev var. *japonica* Hara and *Betula verrucosa* Her.

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Abstract

A complete analysis of the amino acids and proteins in the sap of *Betula platyphylla* Sukatchev var. *japonica* Hara and *Betula verrucosa* Her was conducted. N-terminal amino acid sequence analyses of the proteins revealed that a protein of 22 kDa in the sap of *B. platyphylla* var. *japonica* had high similarity with a protein of 25 kDa in the sap of *B. verrucosa* (97 % similarity). Moreover, these proteins had relatively high similarity with some proteins isolated from other plant sources such as flax, corn or tomato (65-74 % similarity), which are antifungal proteins. The amount of total amino acids and proteins increased as much as two-fold in the last stage of the season.

Key words: Birch (*Betula*), exudation sap, amino acids, proteins

Introduction

In Hokkaido, the northern island of Japan, six birch tree species grow mainly on flat land (Tabata 2000). More than one hundred birch tree species are distributed in the Northern Hemisphere, where they are pioneer species, appearing in the early stages after a fire or other forms of ground clearance (Sato 1975). Some of the energetic metabolites, which were synthesized in leaves during the growing season, are stored both in the roots and in the trunk over the winter season. The function of spring sap is to transfer nutrients that have been released from stored carbohydrates, lipids and proteins (Harms and Sauter 1992), as well as minerals absorbed from the soil by the roots, to the opening buds.

In early spring, sap exudes from birch tree trunks through a hole drilled to reach the xylem in each tree. The total amount of sap exuded from a birch tree is about 100-180 liters in one season. The chemical composition of the exuded sap changes throughout the season (Iguchi *et al.* 1985).

People living in forested areas of northern Europe have traditionally utilized birch sap as a beverage. According to a Chinese medical dictionary sap can be used to prevent a cough, fever and other illnesses (Jiang Su Medical College 1985). The functions of birch sap include the prevention of nervous asthenia, digestive derangement and joint diseases (Kurenkov 1990). In Finland birch sap has been used in the production of syrup (Kallio *et al.* 1987, 1988). In the Siberian area of Russia, birch sap has been used as a raw material for soft drinks, which contain sugar and citric acid and are pasteurized in glass bottles (Telishevskyj 1970).

Several papers have been published on the chemical composition (Kallio *et al.* 1989), exudation mechanism (Tyree 1995) and medical activity of birch sap (Drozdova *et al.* 1995, 2000; Shen *et al.* 2000). However, there are few studies dealing with the composition of birch sap proteins (Kallio *et al.* 1989). To better understand the utilization of sap as a beverage, basic nutritional aspects must be considered. Therefore, it is important to analyze the chemical constituents of the sap.

In this paper, we report the results of a complete analysis of the amino acids and the proteins in the sap of two birch tree species, *Betula platyphylla* Sukatchev var. *japonica* Hara and *B. verrucosa* Her. These two species are representative of the trees used for sap production in Japan and northern Europe.

Materials and Methods

1. Materials

Betula platyphylla Sukatchev var. *japonica* Hara and *Betula verrucosa* Her., which were planted in the Experimental Nursery of Hokkaido University Forests in Sapporo, Japan, were used for the collection of sap. The *B. platyphylla* var. *japonica* tree selected was 26 years old and about 14 m in height. The *B. verrucosa* tree selected was 27 years old and about 15 m in height. The sap was collected from a drilled hole (20 mm in depth, 18 mm in diameter) at a height of 80 cm. A silicon stopper with a glass and a polyethylene tube was inserted into the drilled hole. The sap collected in the tube was frozen at 25 ℃ immediately after collection of sap and stored until the biochemical analysis.

2. Assay of total amino acid concentration

The concentration of amino acids in the birch sap was determined by hydrolysis with hydrochloric acid and by performing an analysis with an amino acid analyzer (HITACHI 835-50, Japan).

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3. Separation and assay of proteins from the sap

The proteins in 10ml of the sap were precipitated in duplicate with 3.5 volumes of 99.9 % ethanol at 20 ℃ overnight. The precipitates were collected by centrifugation at 4 ℃ in 10,000 X g for 1 hr, then were suspended in 2 ml of distilled water and freeze-dried. The concentration of protein in the birch sap was determined using Bradford's method with bovine serum albumin as a standard protein (Bradford 1976).

4. SDS-PAGE analysis

The separated protein sample from 10 ml sap was dissolved in the sample buffer composed of 20 % (w/v) glycerol, 4 % (w/v) SDS, 0.1 M Tris-HCl (pH 6.8) and 0.1 M mercaptoethanol. SDS-PAGE was carried out by a gel with 4.5 % stacking and 10 % resolving regions. Myosin H-chain (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carboanhydrase (29 kDa), ß-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) were used as molecular mass standards (Kallio *et al.* 1995). After the electrophoresis, the gel was stained in 0.03 % Coomassie brilliant blue (CBB) for 2 hr and then was de-stained in 10 % acetic acid and 7 % methanol for 1 hr.

5. N-terminal amino acid sequence of the proteins

After separating proteins using SDS-PAGE, the gel was washed with distilled water for 5 mins and soaked in a transfer buffer, composed of 30 mM Tris, 17 mM Boric acid,20 % methanol and 0.055 % SDS, for 15 mins. The PVDF membrane (ABI, Trans Blot™,) for blotting protein was soaked in methanol for 5 seconds and further soaked in the transfer buffer for 15 mins. Filter papers were prepared and also soaked in the transfer buffer. Beginning from the negative pole, the filter papers, the gel, the PVDF membrane, and another filter papers were set up for the transfer of proteins from the gel to the membrane. The blotting was carried out under a voltage of 15 V for 15 mins, 20 V for 20 mins and 25 V for 30 mins. Then the PVDF membrane was washed with water for 5 mins, stained in CBB solution for 1 min and destained with 50 % methanol. The membrane regions with a band of protein were cut out and soaked first in 20, then 50 and finally in 100 % methanol. Then the membranes were washed with distilled water and used to analyze the N-terminal amino acid sequences of the protein, using an automatic protein sequencer (Applied Biosystem 477A)(Borgmeyer *et al.* 1992).

Results and Discussion

1. The flow rate

The amount of sap that had been exuded was measured every morning and recorded (Fig. 1). The sap exudation of *Betula platyphylla* var. *japonica* started earlier and reached a maximum flow rate earlier than *B. verrucosa*. The sap exudation stopped earlier in *B. platyphylla* var. *japonica* than in *B. verrucosa*. Since the sap exudation of birch trees is generally caused by root pressure (Kallio *et al.* 1995), the difference between when the sap exudation began in the two species may be caused by a difference in the sensibility of the roots to the change in temperature in early spring. The difference between when the sap exudation stopped in the two species may be due to the difference in the timing of physiological events such as the opening of flowers and leaves, which are specific to each species.

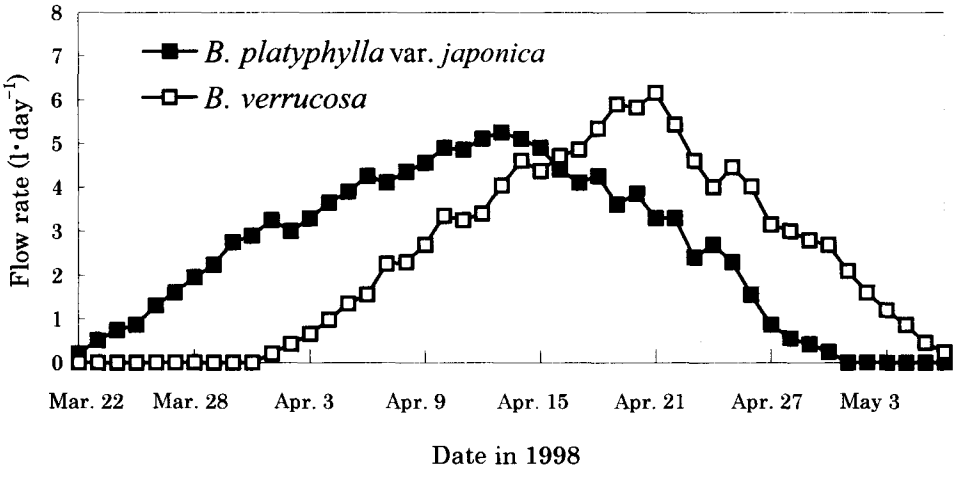

Fig. 1. The sap flow rates of *Betula platyphylla* var. *japonica* and *B. verrucosa* (March 22~May 6, 1998)

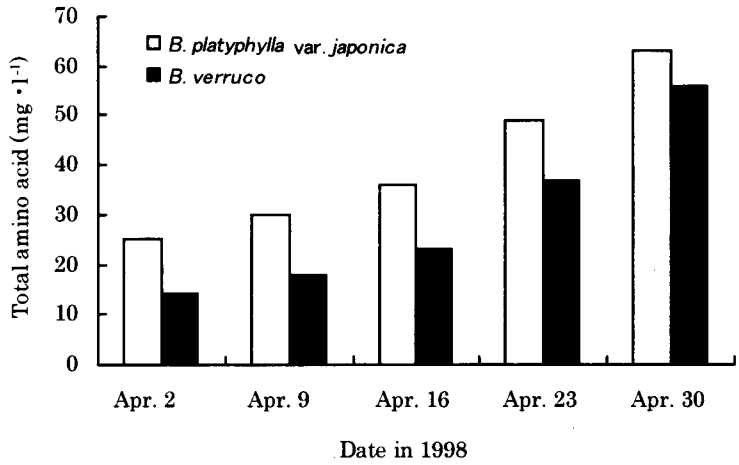

Fig. 2. The amino acid contents of the birch saps of *Betula platyphylla* var. *japonica* and *B. verrucosa* (April 2~30, 1998)

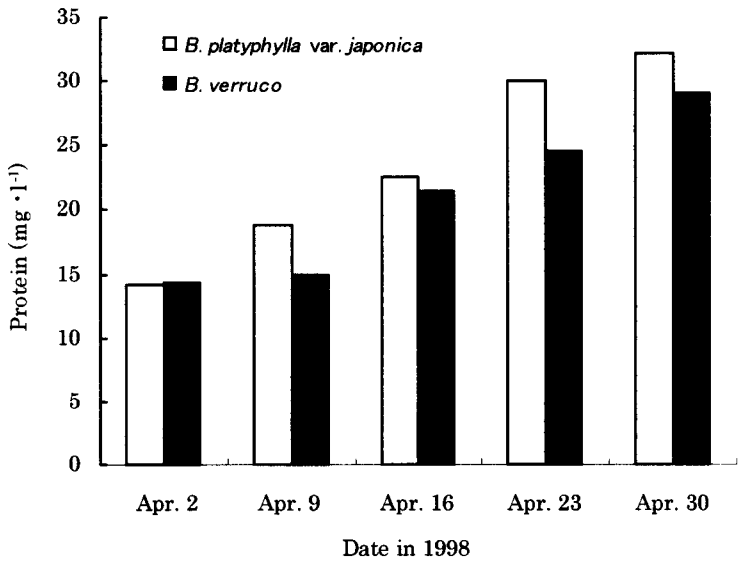

Fig. 3. The protein contents of the birch saps of *Betula platyphylla* var. *japonica* and *B. verrucosa* (April 2~30, 1998)

2. Total amino acids

The amino acid content of the *B. platyphylla* var. *japonica* sap increased from 25 to 63 mg · l^{-1} with exudation season proceeded accelerating the flow rate (Fig. 2). Although the pattern of the change of the amino acids content in the sap of *B. verrucosa* was similar to those of the sap of *B. platyphylla* var. *japonica*, their concentrations of the total amino acids in the sap were lower than those of *B. platyphylla* var. *japonica*.

3. Proteins

Analyzing the protein concentration of proteins of the two types of sap indicated that proteins continuously increased during the exudation period. The protein concentration increased as much as two-fold in the last stage of the season (Fig. 3). The concentration of proteins in the sap of *B. platyphylla* var. *japonica* was higher than that of *B. verrucosa* throughout the season.

4. SDS-PAGE

The proteins were applied to SDS-PAGE. About 10 protein bands were observed in both *B. platyphylla* var. *japonica* and *B. verrucosa* (Fig. 4). The molecular sizes of the major protein bands in the sap of *B. platyphylla* var. *japonica* and *B. verrucosa* were in the range of 20 to 30 kDa. Table 1 shows the seasonal changes of the protein content of the sap.

5. N-terminal amino acid sequences

The N-terminal amino acid sequences of the proteins in the sap of *B. platyphylla* var. *japonica* and *B. verrucosa* were analyzed (Table 2). A protein with a molecular mass of 22 kDa in the sap of *B. platyphylla* var. *japonica* was very similar to a protein with the molecular mass of 25 kDa in the sap of *B. verrucosa* (97% similarity). Moreover, these two proteins were found to be similar to several antifungal proteins found in other plants, such as flax, corn and tomato (Table 2). A protein with a molecular mass of 22 kDa found in flax seeds (Borgmeyer *et al.* 1992) had a 74 % similarity to the protein found in *B. platyphylla* var. *japonica* and a 71 % similarity to that found in *B. verrucosa*. A 22 kDa protein found in corn (Huynh *et al.* 1992) had a 71 % similarity to the protein of *B. platyphylla* var. *japonica* and a 68 % similarity to that of *B. verrucosa*. The similarity of the tomato proteins (Rodrigo *et al.* 1993; King *et al.* 1988) to the proteins in *B. platyphylla* var. *japonica* and *B. verrucosa* were 68 and 65%, respectively. The flax, corn and potato proteins were all membrane-permeable proteins and had anti-fungal properties.

The functions of the proteins in birch sap are not well known. In the future, it will be necessary to characterize them all in detail because drinking birch sap is becoming popular in Japan and the sap will be used to produce beverages. Also, it will be necessary to investigate the difference of the biological activities of the sap between different species of birch trees.

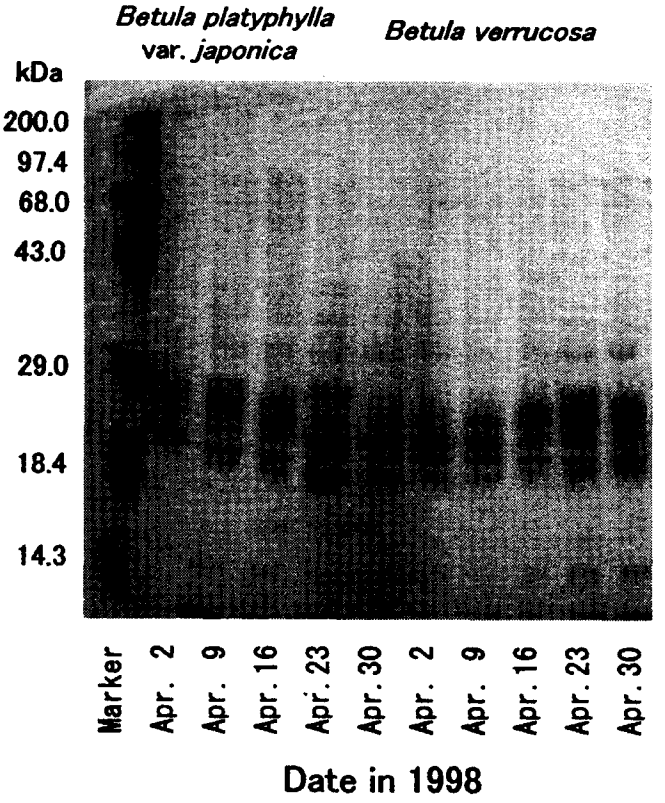

Fig. 4. Analysis of the proteins in the bircth saps of *Betula platyphylla* var. *japonica* and *B. verrucosa* with SDS-PAGE.
Lane 1: molecular mass standards {myosin H-chain (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carboanhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa)}; lanes 2~6: proteins in the saps of *B. platphylla* var. *japonica*; lanes 7~11: proteins in the saps of *B. verrucosa*. (April 2~30, 1998)

Table 1. The abundance of the proteins in the birch saps analyzed by SDS-PAGE

Molecular mass (kDa)	Abundance[a]									
	B. platyphylla var. *japonica*					*B. verrucosa*				
	Apr. 2	Apr. 9	Apr. 16	Apr. 23	Apr. 30	Apr. 2	Apr. 9	Apr. 16	Apr. 23	Apr. 30
14	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+		+	+	+	+	++
22		+	+	++	++	++	++	+	++	++
25	+	++	++	++	++	+++	+++	+++	+++	+++
27	+	+	+	+++	+++	++	++	++	++	++
30	+	++	+	++	++	++	++	++	+++	+++
35		+	+	++	+	+			+	+
68	+	+	+	+	+	+	+	+	+	+
70		+	+	+	+	+		+	+	+
75		+	+					+	+	+

[a]Abundance: +++ = major fractions; ++ = abundance fractions; + = visible fractions

Table 2. Comparison of N-terminal amino acids sequence (31 amino acids) of the proteins in the birch sap and of some anti-fungal proteins found in plants

Proteins	N-terminal amino acid sequence	Similarity (%) with *B. p.*	Similarity (%) with *B. v.*
B. p. (22 kDa)	MARFDVTNNCPFTVWAAAVPGGGRRQLNRGQT	---	97
B. v. (25 kDa)	MARFDVTNNCPFTVWAAAVPGGGRRQLNRRQT	97	---
Flax (25 kDa)	MARFDIQNKCPYTVWAASVPVGGGRQLNSGQT	74	71
Corn (22 kDa)	MAVFTVVNQCPFTVWAASVPVGGGRQLNRGES	71	68
Corn (A/TI)	MAVFTVVNQCPFTVWAASVPVGGGRQLNRGES	71	68
Tomato (PR23)	MATFEVRNNCPYTVWAASTPIGGGRRLDRGQT	68	65
Tomato (NP24)	MATIEVRNNCPYTVWAASTPIGGGRRLNRGQT	68	65

Abb: *B. p.*: *Betula platyphylla* var. *japonica*; *B. v.*: *Betula verrucosa*
Flax (25 kDa) (Borgmeyer *et al.* 1992) ; Corn (22 kDa) , Corn (A/TI) (Huynh *et al.* 1992) ;
Tomato (PR23) (Rodrigo *et al.* 1993) ; Tomato (NP24) (King *et al.* 1988).
Black squares : amino acids commonly found in the N-terminal amino acid sequences of the proteins.

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