When plasma membranes were prepared from tubers of Helianthus tuberosus L. (Jerusalem artichoke) frozen at a sublethal temperature (−10°C), the levels of some plasma membrane proteins, named frost-susceptible proteins (FSPs), decreased [Uemura, M., et al., Plant Physiol., 80, 187–195 (1986)]. The aim of this study was to characterize the response of FSP120, which is named FSP-3 in a previous report, to freezing treatment by immunoblotting. Levels of FSP120 in the plasma membranes of tubers decreased after sublethal freezing, whereas no degraded products were detected in the microsomes or the soluble fraction. The amount of FSP120 in the crude extract of frozen tubers remained at a comparable level to that of the unfrozen tubers. These results suggest that FSP120 might be released from plasma membranes during freezing treatment of the tubers of Jerusalem artichoke.

Key words: Helianthus tuberosus; tuber; freezing injury; plasma membrane protein

It is known that denaturation of freeze-labile proteins, such as lactate dehydrogenase and malate dehydrogenase, occurs during in vitro freezing and frozen storage.1) This might be caused by disorder of a tertiary or quaternary structure, such as aggregation, unfolding, or dissociation of the protein molecules. It has been reported that peripheral proteins of thylakoid membranes were released after in vitro freeze-thawing of the membranes in a salt solution due to the concentration of cryotoxic substances induced by freezing.2,3) Dysfunction of proteins during freeze-thawing of tissues, i.e., in vivo freeze-thawing, has also been reported. Plastocyanin, a soluble electron-carrier protein, was extruded from the thylakoid lumen by mechanical membrane rupture due to severe in vivo freeze-thawing of spinach leaves, and this resulted in the inactivation of photosynthesis after freeze-thaw cycles.4,5) It has also been reported that several metabolic enzymes, such as glucose-6-phosphate dehydrogenase, were inactivated in poplar twigs, resulting in injury during frozen storage for more than one year, although the mechanisms by which the enzymes were inactivated were not clarified.6)

It is generally accepted that freezing injury of plant cells probably results from dysfunction of the cellular membrane induced by structural changes during the freezing process.7–9) In the case of freezing-induced structural changes in the plasma membranes, differential hydration characteristics of membrane components first cause the exclusion of membrane proteins from the regions of the lipid bilayer that are in close proximity to plasma membranes and endomembranes during extracellular freezing. Upon further freezing, dehydration and deformation of the cells induced by severe extracellular freezing promote the demixing of membrane lipids due to lyotropic properties of the lipids, resulting in fusion of plasma membranes with endomembranes.10–14) These structural changes in plasma membranes may cause the freezing injury of plant cells. In recent years, many studies have focused on the effects of compositional changes in plasma membrane lipids on the freezing tolerance of plant cells.15) However, there have been few studies on protein components of plasma membranes that are related to freezing tolerance5,16,17) and freezing injury.18)

Uemura and Yoshida (1986) studied compositional changes in plasma membranes prepared from cold-acclimated tubers of Helianthus tuberosus L. (Jerusalem artichoke) that were subjected to in vivo freezing.18) They prepared plasma membranes from frozen tubers by homogenizing in a prechilled medium at 0°C as quickly as possible in order to eliminate the effects of thawing on tissues. Analyses of the chemical compositions of plasma membranes that had been prepared by aqueous two-phase partitioning from frozen tissues revealed a decrease in the ratio of sterols to phospholipids, a marked decrease in the content of phosphatidyethanolamine, and an increase in the contents of phosphatidylserine and phosphatidylinositol in comparison with those in unfrozen tubers. The relative fluidity of plasma membranes that had been prepared from frozen tubers was higher than that of plasma membranes prepared for more than one year, although the mechanisms by which the enzymes were inactivated were not clarified.6)
from unfrozen tubers, and this difference was thought to have been caused by the changes in lipid composition. Under the same conditions, the levels of plasma membrane proteins had also decreased after sublethal freezing treatment. These proteins, named frost-susceptible proteins (FSPs), were assumed to be degraded during freezing treatment since increases in levels of plasma membrane proteins with lower molecular masses were detected by protein staining after SDS-PAGE. However, it was not clear whether these proteins with lower molecular masses were degraded products of FSPs. The function of each FSP and the influence of the disappearance of FSPs from plasma membrane on cell physiology also remain unsolved.

In this study, we isolated FSP120, previously named FSP-3, from Jerusalem artichoke tubers, and we prepared an antibody to this protein. The properties of FSP120 were analyzed by immunoblotting to determine how FSP120 disappears from the plasma membrane during freezing treatment of tubers.

**Materials and Methods**

*Plant materials.* Tubers of Jerusalem artichoke grown in a field in the campus of Hokkaido University were harvested during the period from September 1996 to December 1998 and used as the plant materials for this study. Cold-acclimated tubers were collected from ground soil under snow cover from the middle of December to early March. Tubers were stored in wet vermiculite at 0 to 4°C in the dark until use.

*Freezing treatment.* Freezing treatment of tubers was done as described previously with some modification. Tubers were peeled and sliced into small pieces (ca. 5×5×1 mm). Sliced tissues were placed in a petri dish and were set in a programmed freezer at −3°C for about two hours. After ice nucleation, the tissues were kept overnight at −3°C to complete the equilibrium freezing and were then refrigerated at a cooling rate of 2.4°C per hour. When the desired freezing temperature had been reached, the frozen tissues in petri dishes were taken from the freezer and immediately used for preparation of plasma membranes or microsomes.

*Preparation of plasma membranes.* The whole procedure was done at 4°C. Tuber samples were prepared in the same way as that for freezing treatment described above. Unfrozen tissues and frozen tissues after freezing treatment were then homogenized in 40 ml per 30 g fresh weight tissues of a homogenizing medium [75 mM MOPS-KOH (pH 7.6), 0.5 M sorbitol, 10 mM EDTA, 5 mM potassium metabisulfite, 1 mM phenylmethylsulfonyl fluoride, 5% polyvinylpyrrolidone, 10 μg/ml 2, 6-di-t-butyl-p-cresol] prechilled at 0°C by polytron PT 10-20-350D (Kinematica, Luzern-Schweiz, Switzerland). After filtration of the homogenate through four layers of gauze, the filtrate was centrifuged at 10,000 g for 15 min and the supernatant was centrifuged at 156,000 g for 30 min. The precipitate was suspended in SKP buffer [10 mM potassium phosphate buffer (pH 7.8), 0.25 M sorbitol, 1 mM DTT] and recentrifuged at 156,000 g for 30 min. The resultant precipitate was suspended in SKP buffer and used for experiments as crude microsomes. The crude microsomes were subjected to aqueous two-phase partitioning to prepare the plasma membranes as described previously. The plasma membrane fraction was finally suspended in SH buffer [25 mM HEPES-BTP (pH 7.4), 0.25 M sorbitol, 1 mM DTT].

*Protein measurement.* The protein concentration was measured by the method of Bradford using gamma-globulin as a standard.

*Electrophoresis and protein staining.* SDS-PAGE was done essentially according to the method of Laemmli. The acrylamide concentration was usually 7% in a separating gel. Protein samples for SDS-PAGE were prepared by heating at 70°C for 10 min after the addition of the same volume of SDS-solute buffer [200 mM Tris-HCl (pH 6.8), 10% (v/v) 2-mercaptoethanol, 4% SDS, 10% (v/v) glycerol, 0.01% bromphenol blue]. Proteins in the gel after SDS-PAGE were made visible by silver staining. In two-dimensional electrophoresis, IEF for the first dimension and subsequent SDS-PAGE for the second dimension were done as described by O’Farrell et al. (1977). Protein samples for IEF were dissolved with 9 M urea, 5% (v/v) 2-mercaptoethanol, 2% (v/v) Triton X-100, and 2% (v/v) Pharmalyte (pH 8 to 10.5 and 5 to 8, Pharmacia, Sweden). The proteins in the gel after SDS-PAGE were made visible by silver staining. The apparent molecular masses of proteins were estimated by comparison with the mobility of molecular weight standard proteins (Daichi-kagaku, Japan) in the gel after SDS-PAGE.

*Isolation of FSP120 and preparation of anti-FSP120 antibody.* After ultracentrifugation of plasma membranes equivalent to 300 μg proteins, the precipitate was suspended in 300 μl of Triton X-114 solution [10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 2% Triton X-114] and agitated gently for 1 h at 2°C. After centrifugation of the sample at 189,000g for 30 min, the supernatant (solubilized protein fraction) was then put through to phase-partitioning of the Triton X-114 solution. The solubilized protein fraction was incubated at 30°C for 10 min and was then centrifuged at 7,000g for 10 min. The upper layer (aqueous phase), which contained a low content of the detergent, was collected and mixed with 50 μl of 6% Triton X-114 solution at 2°C. This was incubated at 30°C for 10 min and then centrifuged at 7,000g for 10 min. The resultant aqueous phase was put through to anion exchange column chromatography using a Mono Q HR5/5 column of the FPLC system (Pharmacia). The aqueous phase was diluted with four volumes of 10 mM Tris-HCl buffer and the filtrate was centrifuged at 10,000 g for 15 min and the supernatant was centrifuged at 156,000 g for 30 min. The precipitate was suspended in SKP buffer [10 mM potassium phosphate buffer (pH 7.8), 0.25 M sorbitol, 1 mM DTT] and recentrifuged at 156,000 g for 30 min. The resultant precipitate was suspended in SKP buffer and used for experiments as crude microsomes. The crude microsomes were subjected to aqueous two-phase partitioning to prepare the plasma membranes as described previously. The plasma membrane fraction was finally suspended in SH buffer [25 mM HEPES-BTP (pH 7.4), 0.25 M sorbitol, 1 mM DTT].
(pH 7.4) and was applied to a Mono Q column equilibrated with 10 mM Tris-HCl (pH 7.4). After washing with solvent-A [20 mM Bis-tris-HCl (pH 6.3), 30 mM NaCl], proteins were eluted with 30 to 350 mM of a NaCl linear gradient in solvent-A. Eluted fractions were analyzed by SDS-PAGE, and proteins in the gel were made visible by silver staining to detect the 120-kDa protein band of FSP120. FSP120-enriched fractions were collected and then concentrated. Proteins in this fraction were separated by SDS-PAGE and detected by using a copper-staining kit (Bio-Rad, Hercules, CA, USA). After the 120-kDa protein band of FSP120 had been excised and destained, proteins in the gel slices were eluted using an electroeluter Model 422 (Bio-Rad). Proteins in the eluant were precipitated by the addition of acetone to a final concentration of 80% (v/v), and the precipitate was dissolved in a small amount of PBS (as a FSP120 fraction).

The isolated FSP120 fraction was used as an antigen for preparation of anti-FSP120. The fraction was mixed with the same volume of TiterMax (CytRx Co., Atlanta, USA) and then used for immunization in a rabbit. The antiserum was partially purified by ammonium sulfate precipitation.23) The anti-FSP120 in PBS was stored at −80°C until use.

**Immunoblotting.** Electrotransfer of proteins from the gel to a PVDF membrane and detection of FSP120 by anti-FSP120 were done by the standard procedure.23) For immunodetection of FSP120 in this study, the anti-FSP120 and the secondary antibody of alkaline phosphatase-conjugated anti-rabbit IgG antibody from a goat (Bio-Rad) were diluted by 2 × 10^3-fold and 3 × 10^3-fold, respectively.

**Fractionation by centrifugation.** In order to study the distribution of FSP120 in the crude microsomes, the crude microsomes were separated by a linear sucrose-density gradient centrifugation as described by Ukaji et al. (1999).24) Activity of marker enzymes was measured in each fraction for vanadate-sensitive H^+-ATPase (P-ATPase: plasma membranes), vacuolar H^+-pyrophosphatase (Pase: tonoplasts), antimycin A-insensitive NADH cytochrome c reductase (NADH Cyt C reductase: endoplasmic reticulum), UDPase (Golgi membranes), and cytochrome c oxidase (Cyt C oxidase: mitochondria inner membranes).

To compare the distribution of FSP120 proteins before and after severe freezing, crude extracts from unfrozen and frozen tubers (15 g fresh weight each) were fractionated by differential centrifugation. After homogenization of tubers as described above, the extract was centrifuged at 330g for 10 min. The supernatant was further centrifuged at 8,000g for 10 min and then centrifuged again at 145,000g for 30 min. The resultant precipitates after centrifugation at 330g and 8,000g were washed with SH buffer three times and suspended in SH buffer. The precipitate after centrifugation at 145,000g was washed with SKP buffer once and suspended in SKP buffer.

**Results**

**Detection of FSP120 protein in the crude microsomes of Jerusalem artichoke tubers**

When the SDS-PAGE profile of plasma membrane proteins prepared from tubers of Jerusalem artichoke frozen at a sublethal temperature was compared with that from unfrozen ones, a decrease in the level of the major protein band with an apparent molecular mass of 120 kDa, FSP-3 in a previous study,18) was clearly revealed as shown in Fig. 1. In this study, FSP-3 was renamed FSP120 for our convenience.

Since the immunoblotting after two-dimensional electrophoresis of plasma membrane proteins showed a decrease in the level of 120-kDa protein, which was detected as a broad spot around pI 5.4, after sublethal freezing of the tubers (Fig. 2), it was suggested that the protein in this 120-kDa band was FSP120. Immunoblot analysis also showed a decrease in the level of FSP120 in the crude microsomes as well as in the plasma membranes (data not shown). Detection of FSP120 in the crude microsomes using anti-FSP120 without preparation of plasma membranes was convenient for semi-quantitative analysis of FSP120 levels during freezing treatment.

**Fig. 1.** SDS-PAGE Profiles of Plasma Membrane Proteins Prepared from Unfrozen Tubers of the Jerusalem Artichoke and Tubers Frozen at a Sublethal Temperature.

After plasma membranes were prepared from unfrozen tubers of Jerusalem artichoke (U, lane 1) and sublethally frozen tubers (F, lane 2), plasma membrane proteins were analyzed by SDS-PAGE. Each lane contains 10 µg of plasma membrane proteins. Proteins in an SDS-PAGE gel were made visible by silver staining. The arrow shows FSP120, which was renamed from FSP-3 (Uemura and Yoshida, 1986) in this study.
When the microsomes were separated by a linear sucrose-density gradient centrifugation, FSP120 bands were immunologically detected in a very similar manner to the distribution of activities of vanadate-sensitive H\(^{+}\)-ATPase, a marker enzyme of plasma membranes (Fig. 3). Distribution pattern of FSP120 protein was also similar to that of cytochrome c oxidase, a marker enzyme of mitochondria. But FSP120 protein was hardly detected in the 8,000 g-precipitate fraction prepared by differential centrifugation, indicating no accumulation of FSP120 in the mitochondria and plastids (Fig. 5B, lane 3). This result suggested that FSP120 is localized in the plasma membranes. Additionally, lower level of the 120-kDa protein was detected in low sucrose density fractions than in the peak fractions of activities of antimycin A-insensitive NADH cytochrome c reductase, a marker enzyme of endoplasmic reticulum. It is not certain whether the 120-kDa protein bands in the low sucrose density fractions are identical to FSP120 in the plasma membranes. However, changes in the levels of FSP120 in microsomes may be responsible for those in plasma membranes, since FSP120 was dominantly detected in plasma membrane fractions among the microsomes after sucrose-density gradient centrifugation.

No detection of degraded products of FSP120 in the tubers frozen at sublethal temperature
Whereas the levels of FSP120 decreased in the microsomes from the tubers frozen at \(-10^\circ\text{C}\) (data not shown), no polypeptide bands with lower molecular masses were immunologically detected in the microsomes or the soluble fraction of the frozen tubers (Fig. 4A). When the crude extracts of unfrozen tubers prepared in the absence of any protease inhibitors were frozen at \(-10^\circ\text{C}\), the levels of FSP120 in the microsomes from the extract did not decrease after freezing treatment and no degraded products were detected in either the microsomes or the soluble fraction from the crude extract by immunoblotting (Fig. 4B). These results indicated that the decrease in the level of FSP120 may not be due to proteolysis during the freezing process and the subsequent homogenization step. In addition, it is noteworthy that FSP120 was also detected in the soluble fraction from both unfrozen and frozen tubers (Fig. 4A).

Subcellular localization of FSP120 in unfrozen and frozen tuber tissues
To investigate the possibility that FSP120 is released from plasma membranes during freezing treatment at a...
Sublethal temperature, immunoblotting was done after subcellular fractionation of tubers by differential centrifugation. When the cell debris fractions obtained by filtration of the tissue homogenates were extracted with a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol and 2% SDS, almost no FSP120 proteins were detected in SDS-soluble fractions from the cell debris of unfrozen and frozen tubers (Fig. 5A). This meant that FSP120 is not tightly attached to the cell wall. The content of FSP120 in the crude extract, which was the filtrate prepared from unfrozen tubers (U) and tubers frozen at a sublethal temperature by programmed slow freezing (F), increased compared with that of unfrozen tubers (Fig. 5A).

In unfrozen tubers, FSP120 was dominantly detected not only in the polyethylene glycol (PEG) phase, which contained other membranes of the microsome fraction and a small amount of inside-out vesicles of the plasma membrane (Fig. 5B, column U). This indicated that FSP120 is present in both plasma membranes and the soluble fraction of unfrozen tubers. On the other hand, almost no FSP120 proteins were detected in the PEG phase in tubers frozen at a nonlethal temperature (Fig. 5B, column F).

Decrease in the levels of FSP120 in tubers during freezing treatment

To examine the stability of FSP120 in plasma membranes during the freezing process, immunoblotting was done using microsomes prepared from the tubers frozen at a nonlethal temperature (Fig. 5A). The level of FSP120 in the microsomes had already decreased after equilibrium freezing at −3°C overnight, and the level further decreased as the freezing temperature was lowered.
Discussion

It has previously been reported that levels of FSPs decreased markedly when plasma membranes were prepared from Jerusalem artichoke tubers frozen at a sublethal temperature. A decrease in the levels of FSPs is thought to be related to freezing injury of plant cells or freeze-induced dysfunction of plasma membranes. In this study, one of the most abundant FSPs, FSP120, which corresponds to FSP-3 in our previous study, was isolated and the responses of FSP120 to freezing treatments were characterized.

Most of the FSP120 proteins were not solubilized from plasma membranes with 1 M NaCl but were solubilized with Triton X-114 solution containing 0.15 M NaCl (data not shown). FSP120 was detected in the aqueous phase after phase partitioning of the Triton X-114-soluble fraction and was still soluble in the solution without any detergents in further purification steps (data not shown). FSP120 in plasma membranes may have a hydrophilic property in its structure. Several experimental results in the present study suggested that FSP120 is localized in plasma membranes. Furthermore, subcellular fractionation by differential centrifugation showed that FSP120 was present not only in the microsomes but also in the soluble fraction (Figs. 4A and 5B). FSP120 may exist endogenously as a plasma membrane-associated protein and a soluble isoform in unfrozen tubers. Alternatively, some portion of plasma membrane-associated FSP120 may be released to the soluble fraction during the freezing treatment and the subsequent homogenization process. It is possible that the 120-kDa protein bands in the low sucrose density fractions detected by anti-FSP120 (Fig. 3) may be a soluble FSP120. Further studies on FSP120, such as immunolocalization study and amino acid sequence analysis, are needed to identify and determine the physiological and biochemical properties of FSP120.

It was shown that there was almost no degradation of FSP120 during the freezing treatment of tubers and the subsequent homogenization process (Fig. 4). The level of FSP120 in crude extracts from tubers frozen at a sublethal temperature was almost the same as that in crude extracts from unfrozen tubers (Fig. 5). Treatment of the tubers with cycloheximide before freezing did not inhibit the increase in the level of FSP120 in the soluble fraction after freezing treatment of the tubers, suggesting that the increase in the level of FSP120 in the soluble fraction is not due to protein synthesis during freezing treatment (data not shown). As an explanation for these results, it is hypothesized that the decrease in levels of FSP120 in the membrane fraction is a consequence of the release from plasma membranes to a soluble fraction during freezing treatment or the subsequent homogenization process. The release of FSP120 from plasma membranes after sublethal freezing of tubers may be an irreversible process, since the levels of FSP120 in plasma membranes did not recover after thawing of the tubers frozen at a sublethal temperature, as shown in the SDS-PAGE profiles by protein staining.

Peripheral proteins were released from the thylakoid membranes by in vitro freeze-thawing of the membranes in a salt solution as shown in previous studies. This may be caused by the concentration of cryotoxic ions induced by freeze-dehydration of thylakoid vesicles. In another case, inactivation of vacuolar H^+-ATPase occurred after chilling treatment of the mung bean, a chilling-sensitive leguminous plant. The enzyme inactivation is thought to be caused by release of the peripheral domain from the tonoplast membrane domain of the enzyme. It has also been shown that inactivation of the enzyme is induced by chilling treatment with chaotropic anions in vitro in the presence of Mg-ATP. In both cases, dysfunction of the proteins was caused by the harmful effects of ions during freezing or chilling treatment. Most of the FSP120 was not released by treatment of plasma membranes with 1 M NaCl solution at 2°C (data not shown), although the effect of freezing treatment of plasma membranes in a solution with a high concentration of salts has not been examined. In addition, almost no release of FSP120 was detected after treatment of tissue extract by slow freezing to −10°C (Fig. 4), and most of the FSP120 was not released by freeze-thawing of plasma membranes with liquid nitrogen several times (data not shown). These results imply that in vivo freezing may promote the release of FSP120 from plasma membranes rather than in vitro freezing of the membrane vesicles or tissue extract.

In a previous study, it was thought that the decrease in the levels of lipid components of plasma membranes after freezing of Jerusalem artichoke tubers at a sublethal temperature might have resulted from the loss of membrane areas specifically enriched in sterols or phosphatidylethanolamine. According to the hypothesis of the mechanism underlying freezing injury of plant cells, it is possible that membrane fusion or structural changes in plasma membranes such as lyotropic-phase separation of membrane lipids during severe extracellular freezing may cause the loss of membrane lipids after preparation of plasma membranes from tubers frozen at a sublethal temperature. Therefore,
the mechanism of the disappearance of FSP120 from plasma membranes during extracellular freezing may be different from that of some plasma membrane lipids.

As shown in Fig. 6, a small portion of FSP120 had already been released from plasma membranes after nonlethal freezing treatment at $-3^\circ$C, and the decrease in levels of FSP120 in plasma membranes was increased in proportion to lowering of the freezing temperature. This suggested that FSP120 in plasma membranes may be susceptible to freezing treatment and/or subsequent homogenization. It is possible that the release of FSP120 may be one of the symptoms of structural changes in plasma membranes during extracellular freezing that results in freezing injury of tuber cells of the Jerusalem artichoke. In order to clarify the mechanism of the release of FSP120 from plasma membranes and its relationships with freezing injury, further characterization of FSP120 is in progress.

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