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Artigo/Article

Regulation of sucrose phosphate synthase in vascular bundles of Washington navel orange fruit (*Citrus Sinensis* L. Osbeck) by a protein kinase and a protein phosphatase

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SUMMARY

Sucrose phosphate synthase (SPS) is activated by a novel ATP-dependent, Ca-independent kinase and inactivated by a protein phosphatase in vascular bundles isolated from citrus fruit (*Citrus sinensis* L. Osbeck, cv. Washington navel orange). The SPS purified from dark-treated citrus leaves was used as the substrate for purification of the protein kinase and phosphatase. Sucrose phosphate synthase kinase (SPSK) purified from citrus fruit vascular bundles was resolved in a single band of 45 kDa by SDS-PAGE. The SPSK activated SPS and incorporated ^{32}P from [γ-^{32}P]ATP into the 110 kDa subunit of SPS. The sucrose phosphate synthase phosphatase (SPSP) was purified to a single band of 31 kDa determined by SDS-PAGE. The SPSP was able to dephosphorylate SPS previously phosphorylated by SPSK. The SPSP was shown to be a novel protein phosphatase 2C (PP2C) based on its insensitivity to okadaic acid, activation by Fe^{3+} and inhibition by Mg^{2+}, Fe^{2+} and Mn^{2+}. Activation of SPS by a Ca^{2+}-independent kinase and inactivation by a PP2C in citrus constitutes a novel mechanism regulating SPS activity in plants.

Index terms: protein phosphatase 2C, sucrose, sucrose phosphate synthase kinase, sucrose phosphate synthase phosphatase.

Regulação da sacarose fosfato sintase em feixes vasculares de fruta de laranja baía Washington Navel (*Citrus Sinensis* L. Osbeck) por uma proteína quinase e uma proteína fosfatase

RESUMO

Sacarose fosfato sintase (SFS) é ativada por uma nova quinase ATP-dependente, Ca-independente e inativada por uma proteína fosfatase em feixes vasculares isolados de frutos de citros (*Citrus sinensis* L. Osbeck, cv. Washington navel orange). A SFS purificada de folhas de citros incubadas no escuro foi usada como substrato para purificação da proteína quinase e fosfatase. Sacarose fosfato sintase quinase (SFSQ) purificada de feixes vasculares de frutos de citros foi resolvida em uma banda única de 45 kDa por SDS-PAGE. A SFSQ ativou SFS e incorporou ^{32}P de [γ-^{32}P]ATP na subunidade 110 kDa de SFS. A sacarose fosfato sintase fosfatase (SFSF) foi purificada em uma banda única de 31 kDa determinada por SDS-PAGE. A SFSF foi capaz de defosforilar SFS previmente fosforilada por SFSQ. Demonstrou-se que SFSF é uma nova proteína

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fosfatase 2C (PF2C) baseado em sua insensibilidade ao ácido ocadaico, ativação por Fe^{3+} e inibição por Mg^{2+}, Fe^{2+} and Mn^{2+}. Ativação de SFS por uma Ca^{2+}-independente quinase e inativação por uma PF2C em citros constituem um novo mecanismo regulando a atividade de SFS em plantas.

Termos de indexação: proteína fosfatase 2C, sacarose, sacarose fosfato sintase quinase, sacarose fosfato sintase fosfatase.

INTRODUCTION

Reversible phosphorylation of proteins mediated by protein kinases and protein phosphatases is a major mechanism regulating enzyme activity. Regulation of sucrose phosphate synthase (SPS; UDP glucose: D-fructose-6-phosphate-2-glucosyltransferase; EC 2.4.1.14) by reversible phosphorylation is perhaps the best characterized. The enzyme catalyzes the reversible reaction of UDP-glucose + fructose-6-phosphate ↔ sucrose-6-phosphate + UDP. Phosphorylation of SPS has been studied almost exclusively in photosynthetic tissues, where it is the basis of the light/dark activation/inactivation of the enzyme (Huber et al., 1989). For example, during the dark period in spinach (*Spinacia oleracea* L.) leaves, a sucrose-nonfermenting-1-related protein kinase (SnRK1) or a calmodulin-like domain protein kinase (CDPK) phosphorylates and inactivates SPS, causing sucrose synthesis to cease (Huang & Huber, 2001; Pagnussat et al., 2002; Sugden et al., 1999). Upon illumination, a light-regulated protein phosphatase 2A (PP2A) dephosphorylates and activates SPS, accelerating sucrose synthesis (Siegl et al., 1990).

There is significant evidence that SPS plays an important role in sucrose accumulation in nonphotosynthetic sucrose-storing sink tissues, such as fruit, roots and tubers (reviewed in Komatsu et al., 1999). The activity of SPS paralleled the increase in sucrose concentration in fruit of sucrose-storing tomato varieties (*Solanum lycopersicum* L.), muskmelon (*Cucumis melo* L.), Japanese pear (*Pyrus pyrifolia* [Burm.] Nak.), Satsuma mandarin (*Citrus unshiu* Marc.), roots of beet (*Beta vulgaris* L.) and tubers of potato (*Solanum tuberosum* L.) (reviewed in Komatsu et al., 1999). Despite, the strong relationship between SPS activity and the sucrose content of storage organs, regulation of SPS by reversible phosphorylation has been investigated only in potato tubers. Interestingly, in the tissues of this storage organ, SPS is regulated in a manner analogous to the light/dark modulation of the enzyme in spinach leaves (Reimholz et al., 1994). Additionally, SPS has also been documented to be located in the vascular bundles associated with sink tissues, including maize (*Zea mays* L.) (Cheng et al., 1996; Im, 2004) and grapefruit (*Citrus paradisi* Macf.) (Lowell et al., 1989; Nolte & Koch, 1993), suggesting a role for SPS in regulating phloem unloading. Thus, it is a striking omission that regulation of SPS by reversible phosphorylation has not been investigated in the vascular bundles of a sink organ.

To address this lack of information, vascular bundles isolated from Washington navel orange (WNO) fruit (*Citrus sinensis* L. Osbeck) were used as a model system to investigate the regulation of SPS by reversible phosphorylation. With the demonstration that partially purified SPS was activated in an ATP-dependent reaction and inactivated in the absence of phosphatase inhibitors, the putative SPS protein kinase and phosphatase were isolated, purified and characterized. The results provide strong evidence that the SPS from citrus fruit vascular bundles is regulated by a reversible phosphorylation mechanism uniquely different from that of SPS in the spinach leaf and potato tuber.

MATERIALS AND METHODS

Purification of sucrose phosphate synthase

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

The source of fully expanded, mature citrus leaves and fruit was 5-year-old WNO scions on Carrizo citrange (*C. sinensis* × *Poncirus trifoliata* L. Raf) rootstocks, maintained under standard nutrition and irrigation in a lath house. Dark-treated leaves, the source of SPS used as the substrate for the purification of SPS kinase and phosphatase, were collected from a subset of trees maintained in the dark for 16 h at 27 °C in a growth chamber. All tissue samples were immediately frozen in liquid nitrogen and stored at –80 °C until used. All purification steps were performed at 4 °C.

Tissue (50 g) was pulverized in liquid nitrogen, homogenized with three bursts of 10 sec each at full speed with a Polytron tissue homogenizer (Brinkman, Westbury, NY) in 200 mL of buffer A (100 mM Hepes-KOH [pH 7.5], 2% polyvinylpolypyrrolidone, 4 mM EDTA, 2 mM EGTA,

50 mM Na-ascorbate, 5 mM thiourea, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT], 5 mM ε-amino-n-caproic acid, and phosphatase inhibitors [50 mM NaF, 10 mM Na_2MoO_4, 1 mM activated orthovanadate, and 100 nM okadaic acid]). The homogenate was filtered through a 0.45-µm pore-size nylon membrane and centrifuged at 30,000*g* for 30 min. The supernatant was brought to 30% saturation with $(NH_4)_2SO_4$, stirred for 30 min, and then centrifuged at 30,000*g* for 30 min. The supernatant was further saturated to 50% with $(NH_4)_2SO_4$, stirred and centrifuged as above. The pellet was dissolved in a minimum volume of buffer B (20 mM Hepes-KOH [pH 7.5], 2 mM DTT and 10% glycerol) supplemented with phosphatase inhibitors and centrifuged at 30,000*g* for 30 min. The supernatant was desalted on a PD-10 column (Amersham Biosciences, Piscataway, NJ) equilibrated with buffer B supplemented with phosphatase inhibitors. The desalted 30% to 50% fraction was mixed with 20 mL of Q Sepharose fast flow (Amersham Biosciences) equilibrated in buffer B. After 20 min, the mixture was filtered through a 0.45-µm pore-size nylon membrane. The mixture was then extensively washed first with buffer B and then with 0.35 M KCl in buffer B. The enzyme was eluted with 40 mL of 0.6 M KCl in buffer B, concentrated with an Amicon YM30 ultra filtration membrane (MilliporeSigma, St Louis, MO), and desalted as described above. The desalted concentrated extract was applied to a 10-mL phosphocellulose-P11 column (Whatman, Florham Park, NJ), prepared according to manufacturer's recommendations. The SPS, which did not bind to phospocellulose-P11 column, was washed with 30 mL of buffer B at a flow rate of 0.5 mL per min and the eluate concentrated as described above. The concentrated enzyme was either used as partially purified SPS for the purification and characterization of SPSK and SPSP or applied to an 8-ml ω-aminohexyl agarose column (Amersham Biosciences) equilibrated with buffer B for further purification. The column was washed with 30 mL of buffer B and the enzyme was eluted using a linear 80 mL gradient of 0 to 0.5 M KCl in buffer B at a flow rate of 1 mL per min. Pooled active fractions were concentrated with an Amicon Ultra-4 100K concentrator and applied to a 5-mL Blue Sepharose CL-B (Sigma-Aldrich) column equilibrated with buffer B. The column was washed with 15 mL of buffer B and the enzyme was eluted with 15 mL of 0.8 M KCl in buffer B. The 0.8 M active fraction was desalted, concentrated using an Amicon Ultra-4 100K concentrator, frozen in liquid nitrogen, and stored at –80 °C.

Purification of sucrose phosphate synthase kinase

All purification steps were performed at 4 °C. Dissected fruit vascular bundles (50 g) were pulverized in liquid nitrogen, homogenized in 200 mL of buffer A (minus phosphatase inhibitors) and centrifuged as described above. The supernatant was treated with $(NH_4)_2SO_4$ to obtain the 30% to 70% fractions. The pellet was desalted as described above and applied to a 10-mL phosphocellulose-P11 equilibrated with buffer B. The column was washed with 30 mL of buffer B at a flow rate of 1 mL per min and the bound proteins were eluted at the same flow rate with a linear gradient (90 mL) of 0 to 0.3 M KCl in buffer B. Active fractions were pooled, concentrated with an Amicon Ultra-4 30K concentrator and desalted on a PD-10 column. The desalted extract was applied to a 2-ml ATP-agarose column (Amersham Biosciences) equilibrated with buffer B. The column was washed with 20 mL of buffer B at a flow rate of 0.5 mL per min and the bound proteins were eluted with 5 mM ATP in buffer B at the same flow rate. Pooled active fractions were concentrated with an Amicon Ultra-4 30K concentrator to a final volume of 500 µL. The enzyme was applied to a MQ column (Amersham Biosciences), equilibrated with buffer B and active fractions were eluted with a linear 10 mL gradient of 0 to 0.5 M KCl in buffer B at a flow rate of 0.5 mL per min. Active fractions were concentrated and desalted as previously described. $MgCl_2$ and dithiothreitol (DTT) were added to the enzyme at a final concentration of 10 mM each and frozen in liquid nitrogen and stored at –80 °C.

Purification of sucrose phosphate synthase phosphatase

Fruit vascular bundle extracts were applied to a 10-mL phosphocellulose-P11 column as previously described for SPSK. Active fractions were pooled, concentrated with an Amicon Ultra-4 10K concentrator and desalted on a PD-10 column. The desalted extract was concentrated with an Amicon Ultra-4 10K concentrator to a final volume of 500 µL, applied to a MQ column and eluted as described above. Active fractions were concentrated, desalted, and frozen in liquid nitrogen and stored at –80 °C.

Enzyme assays

The activity of SPS in forming sucrose-6-phosphate was determined under saturating substrate (*V*max) concentrations in the absence or presence of phosphatase inhibitors. The *V*max assay contained 20 mM Hepes-KOH (pH 7.5), 10 mM UDP-glucose, 10 mM fructose-6-phosphate, 40 mM glucose-6-phosphate, 5 mM $MgCl_2$, 5 mM DTT, 10% glycerol plus or minus phosphatase inhibitors (20 mM NaF, 5 mM Na_2MoO_4, and 1 mM activated orthovanadate) in a total volume of 90 µL. All assays were initiated with the addition of 10 µL of SPS and incubated for 20 min at 28 °C. The reaction was stopped with 50 µL 30% KOH and boiled for 10 min, followed by centrifugation at 3,000*g* for 5 min. The supernatant was mixed with 1 mL of 0.14% anthrone in 13.8 N H_2SO_4, incubated at 40 °C for 20 min, at which time optical density (OD) at 620 nm was determined (Huber et al., 1989; Van Handel, 1968). One unit of activity is defined as 1.0 µmol of sucrose-6-phosphate produced per min per mg protein. Protein concentration was determined as OD at 595 nm, using γ-globulin as the standard (Bradford, 1976).

Activity of SPSK was determined as the activation of SPS under limiting substrate concentrations in comparison with the activity under saturating substrate (*V*max) concentrations (Huber et al., 1989). A typical reaction contained 20 mM Hepes-KOH (pH 7.5), 10 µL of partially purified leaf SPS (phosphocelullose-P11 fraction), 2 mM ATP, 5 mM $MgCl_2$, 10 mM DTT, 10% glycerol and phosphatase inhibitors (10 mM NaF, 5 mM Na_2MoO_4, 1 mM activated orthovanadate, and 100 nM okadaic acid) in a final volume of 50 µL. All assays were initiated with the addition of 20 µL of SPSK and incubated for 20 min at 28 ºC. For comparison, SPS was assayed either under limiting substrate concentrations (20 mM Hepes-KOH (pH 7.5), 10 mM UDP-glucose, 3 mM fructose-6-phosphate, 12 mM glucose-6-phosphate, 10 mM Pi, 5 mM $MgCl_2$, 5 mM DTT, 10% glycerol and phosphatase inhibitors (20 mM NaF, 5 mM Na_2MoO_4, 1 mM activated orthovanadate) or under saturating substrate (*V*max) concentrations described above. One unit of activity is defined as the increase by 1.0 µmol of sucrose-6-phosphate produced per min per mg protein. Since ATP gives a positive reaction with anthrone, the reaction mixture without added enzymes was used as the reagent blank.

The activity of SPSP was determined as dephosphorylation of *p*-nitrophenylphosphate (*p*NPP) to *p*-nitrophenol (*p*NP) (Garen & Levinthal, 1960). Reaction mixtures contained 50 mM Bis-Tris Propane (pH 7.0), 20 mM *p*NPP, and 5 mM DTT in a total volume of 90 µL. All assays were initiated with the addition of 10 µL of SPSP. The reaction was monitored at 405 nm using a Bio-Rad 3550-UV microplate reader. One unit of activity is defined as 1.0 µmol of *p*NP produced per min per mg protein. In addition, SPSP activity was also assayed as the inactivation of SPS under limiting substrate concentrations. A typical reaction contained 50 mM Bis-Tris Propane (pH 7.0), 10 µL of partially purified vascular bundle SPS (phosphocellulose-P11 fraction), 5 mM DTT, and 10% glycerol in a final volume of 50 µL. All assays were initiated with the addition of 20 µL of SPSP and incubated for 20 min at 28 °C.

Gel electrophoresis

Proteins were separated by SDS-PAGE (Laemmli, 1970), nondenaturing PAGE (Davis, 1964) and isoelectric focusing gel (Robertson et al., 1987) using a Bio-Rad Mini Protean II under standard conditions (Bio-Rad, Hercules, CA). Nondenaturing PAGE and isoelectric focusing gel at 4 ºC were used to assay for SPS activity as follows: each lane was cut into segments of 0.25 cm and was assayed under saturating substrate concentrations in a final volume of 100 µL. In addition, the 0.25-cm segments were incubated in SDS sample buffer at 60 ºC for 30 min and separated in a second dimension by 10% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue G (Neuhoff et al., 1988).

Reversible phosphorylation of sucrose phosphate synthase using [γ-^{32}P] ATP

A typical phosphorylation assay contained 20 mM Hepes-KOH (pH 7.5), 30 µL of SPS, 20 µL of SPSK, 2 mM [γ-^{32}P] ATP (220 TBq/mmol; Amersham Biosciences), 5 mM $MgCl_2$, 10 mM DTT, 10% glycerol and phosphatase inhibitors (20 mM NaF, 5 mM Na_2MoO_4, 1 mM activated orthovanadate, and 100 nM okadaic acid) in a final volume of 60 µL incubated for 1 h at 28 °C. Subsequently, the assay mixture was subjected to electrophoresis on 6% nondenaturing PAGE and in a second dimension by 10% SDS-PAGE. Alternatively, the phosphatase inhibitors and $MgCl_2$ were removed from the reaction mixture by desalting with an Amicon Microcon 100K concentrator. The resulting phosphorylated SPS was added to a reaction containing 20 µL of SPSP, 50 mM Bis-Tris Propane (pH 7.0), 5 mM Fe^{3+} and 10% glycerol

in a final volume of 50 µL incubated for 30 min at 28 °C. The reaction mixture was boiled in SDS sample buffer and subjected to 10% SDS-PAGE. Proteins labeled with ^{32}P were detected by exposing the dry gels to X-ray film (Kodak BioMax).

Reversible phosphorylation of a synthetic SPS peptide

The phosphorylation assay contained 20 mM Hepes-KOH (pH 7.5), 100 µg of the synthetic peptide (RIKRNV**S**CYGK) (Syn-Pep, Dublin, CA), which was based on the sequence of the citrus SPS gene homologous to the region flanking the Serine424 that is phosphorylated during the osmotic activation of spinach leaf SPS (Komatsu et al., 1996), 10 µL of SPSK, 5 mM ATP, 5 mM $MgCl_2$, 5 mM DTT and 10% glycerol in a final volume of 50 µL incubated for 30 min at 28 °C, and then applied to a column (0.5 × 0.5 cm) of phosphocellulose-P11 packed in a Gelman Z-spin filter (0.45-µm pore size) (Sigma-Aldrich). After 10 min, the phosphocellulose-P11 column was washed with 400 µL of water. The eluted peptide solution was dehydrated in a SpeedVac concentrator (ThermoFisher Scientific, Waltham, MA) and the phosphorylated peptide was suspended in 10 µL of 50 mM Bis-Tris Propane (pH 7.0). The dephosphorylation assay contained 50 mM Bis-Tris Propane (pH 7.0), the phosphorylated peptide, 10 µL SPSP, and 10 mM Fe^{3+} in a final volume of 50 µL incubated for 30 min at 28 ºC and then applied to a mini-phosphocellulose-P11 column as described. Assays without the addition of the synthetic peptide were used as the control. Aliquots (2 µL) of each peptide solution (4 µg peptide) were spotted on a polyvinylidene fluoride (PVDF) membrane developed using anti-phosphorserine antibodies and an ECL Western blotting analysis system (Amersham Biosciences).

RESULTS

Regulation of WNO sucrose phosphate synthases

The addition of ATP increased the activity of the partially purified SPS from WNO fruit vascular bundles in a time and Ca^{2+}-independent manner when assayed under limiting substrate concentrations (Figure 1a and b). The ATP-dependent activation of SPS also did not require the presence of other ions in the reaction volume (Figure 1b). Analysis of the partially purified fruit vascular bundles also revealed the activity of a SPS phosphatase capable of time-dependent inactivation of SPS (Figure 1a).

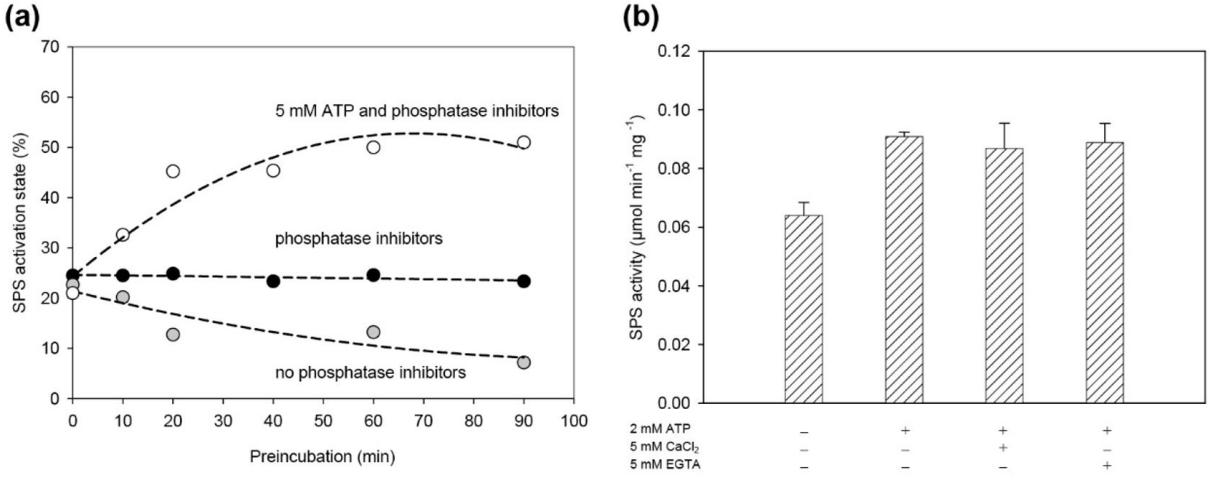

Figure 1. Regulation of SPS in the vascular bundles of navel orange fruit. (a) Time-dependent, ATP-dependent activation of SPS and inactivation of SPS in the absence of phosphatase inhibitors (20 mM NaF, 5 mM Na_2MoO_4, 1 mM activated orthovanadate, and 100 nM okadaic acid) and 5 mM ATP; (b) ATP-dependent, calcium-independent activation of SPS from fruit vascular bundles assayed with phosphatase inhibitors (20 mM NaF, 5 mM Na_2MoO_4, 1 mM activated orthovanadate, and 100 nM okadaic acid) and with or without 2 mM ATP, 5 mM $CaCl_2$, or 5 mM EGTA, to sequester all ions in the reaction medium.

The results suggested that regulation of SPS in WNO fruit vascular bundles was distinctly different from the classic mechanism reported for the spinach leaf (Huber et al., 1989) and potato tuber (Reimholz et al., 1994). It is noteworthy that the activity of SPS in photosynthetic tissues of WNO, including leaves (data not shown) and the exocarp (flavedo) of young developing fruit was inactivated in an ATP-dependent reaction (Figure 2a and b), consistent with inactivation by a SPS kinase exactly as described for spinach leaves (Huber et al., 1989).

Sucrose phosphate synthase kinase from WNO fruit vascular bundles

The SPSK purified from vascular bundles of WNO fruit had an optimum pH of 7.0 to 7.5 and a K_m for ATP of 0.15 mM based on the activation of SPS. The 42% activation of SPS by SPSK was Ca^{2+}-independent (Figure 1b). The activation SPS by SPSK resulted in a lower K_m for UDP-glucose and a negligible change in the K_m for fructose-6-phosphate. To confirm that SPSK phosphorylates SPS, the purified citrus leaf SPS was used as a substrate for incorporation of ^{32}P from [γ-^{32}P]ATP by SPSK. After 1 h, the reaction was subjected to nondenaturing PAGE, resolving in only one protein band containing radiolabeled phosphate. Each lane of the nondenaturing gel was divided into equal 0.25-cm segments and run in a second dimension on a SDS-PAGE. A single band corresponding to the 110-kDa band of SPS and containing the radioactivity was obtained confirming the phosphorylation of SPS (Figure 3a). The SPSK from WNO fruit vascular bundles also was able to phosphorylate the synthetic peptide (RIKRNV**S**CYGK), which was based on the citrus SPS gene sequence (Komatsu et al., 1999) analogous to the

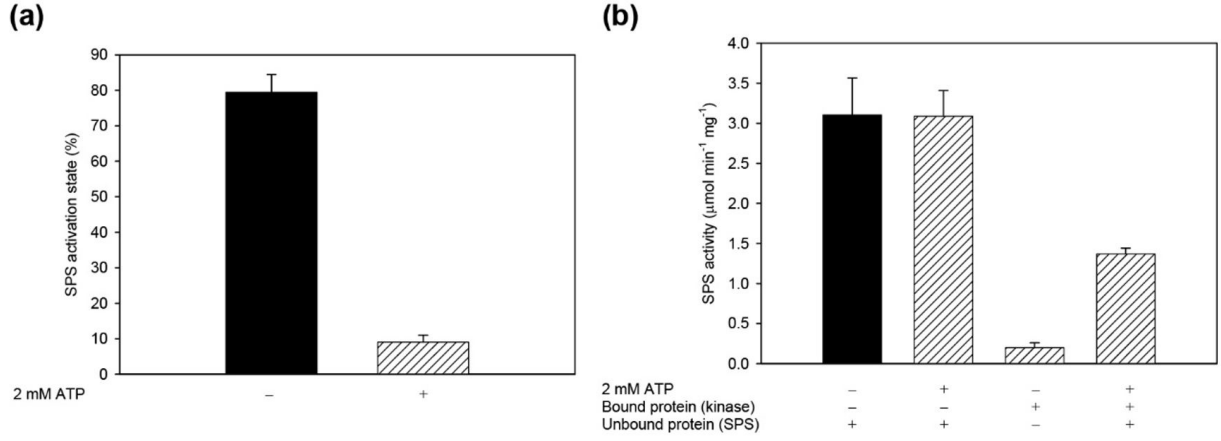

Figure 2. (a) ATP-dependent inactivation of SPS in the exocarp (flavedo) of WNO fruit; (b) ATP-dependent inactivation of vascular bundles derived SPS (unbound protein fraction) by a protein bound kinase from dark-treated citrus leaves.

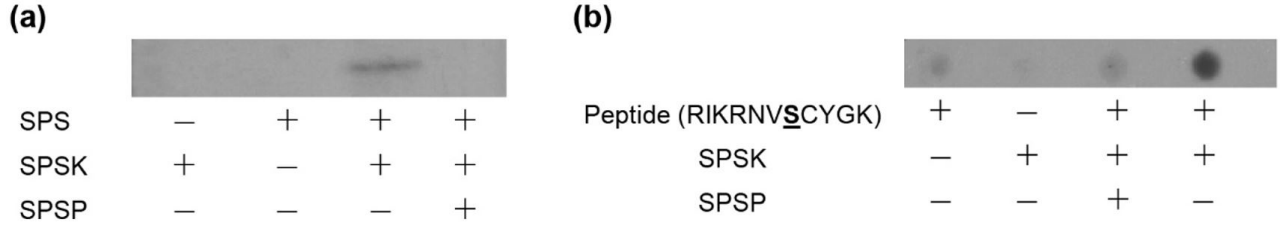

Figure 3. (a) Phosphorylation and dephosphorylation of citrus leaf SPS by fruit vascular bundle SPSK and SPSP. Purified SPS was phosphorylated by SPSK and subsequently dephosphorylated by SPSP. (b) Phosphorylation and dephosphorylation of the synthetic peptide RIKRNVSCYGK by SPSK and SPSP. In (a) and (b), [γ-^{32}P]ATP was the source of ^{32}P in the phosphate transfer reactions. Four micrograms peptide were spotted on a PVDF membrane developed using phosphorserine antibodies.

spinach leaf SPS osmotic stress phosphorylation site (Huber & Huber, 1992) (Figure 3b).

Sucrose phosphate synthase phosphatase from WNO fruit vascular bundles

The SPSP purified from vascular bundles of WNO fruit was characterized as PP2C, based on its insensitivity to okadaic acid, activation by Fe^{3+} and inhibition by Mg^{2+}, Fe^{2+} and Mn^{2+} (Figure 4a and b). As expected, SPSP was strongly inhibited by 20 mM NaF and 2 mM activated orthovanadate (data not shown). Further, the WNO fruit vascular bundle SPSP dephosphorylated leaf inactivated SPS and the synthetic peptide RIKRNV**S**CYGK, both of which had been previously phosphorylated by SPSK (Figure 3a and b). Additional experiments were undertaken to confirm that the reduced activity of SPS from WNO fruit vascular bundles was not due to dephosphorylation of its substrate fructose-6-phosphate by SPSP added to the assay. The results demonstrated that fructose-6-phosphate was not dephosphorylated under assay conditions that inactivated SPS.

DISCUSSION

Since the initial discovery of the light/dark modulation of SPS in spinach leaves by reversible phosphorylation (Huber et al., 1989), subsequent attention has predominantly been given to regulation of the enzyme in photosynthetic tissues (Winter & Huber, 2000). In each case, the enzyme was inactivated by an ATP-dependent, Ca^{2+}-dependent protein kinase and activated by a protein phosphatase (PP2A). Surprisingly, SPS in nonphotosynthetic potato tuber tissue was regulated by the same mechanism (Reimholz et al., 1994). In contrast, it was demonstrated subsequently that SPS was activated in an ATP-dependent manner when spinach leaves were under osmotic stress (Toroser & Huber, 1997). This putative mechanism was also suggested for SPS in bundle sheath cells of C4 plants (Lunn et al., 1997). However, the activation/inactivation mechanism was not characterized in either tissue.

The current research is the first to report activation of SPS by a protein kinase and inactivation by a protein phosphatase in vascular bundles of fruit. The SPSK purified from WNO fruit vascular bundles activates SPS in an ATP-dependent manner, but unlike spinach leaves under

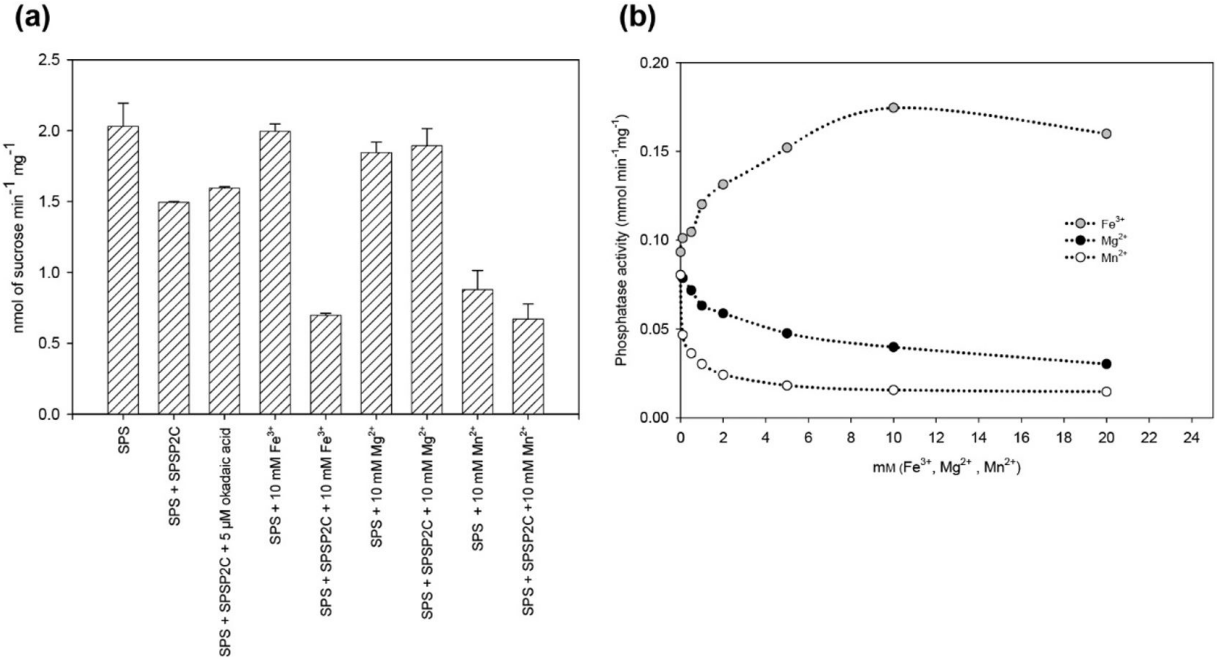

Figure 4. SPSP from vascular bundles of WNO fruit. (a) The effect of cations on SPSP activity assayed as the inactivation of partially purified vascular bundle SPS. (b) The effect of cations on SPSP2C activity (µmol min^{-1} μg^{-1}) was measured as the production of *p*NP at 405 nm in the presence of different concentrations of metal anions. The SPSP from vascular bundles of WNO fruit was characterized as a PP2C (SPSP2C) based on insensitivity to okadaic acid, activation by Fe^{3+} and inhibition by Mg^{2+} and Mn^{2+}.

osmotic stress, the activation of SPS in WNO fruit vascular bundles is Ca^{2+}-independent. Activation by ATP resulted in SPS having a lower K_m for its substrate UDP-glucose, but not fructose-6-phosphate, as was observed for SPS in the bundle sheath cells of C4 grasses (Lunn et al., 1997). The kinase activating the spinach leaf SPS under osmotic stress phosphorylates Serine424 (RMRRGV**S**CHGR) in the regulatory sequence of the SPS protein (Toroser & Huber, 1997). Similarly, the SPS from WNO fruit vascular bundles phosphorylated the serine in the synthetic peptide RIKRNV**S**CYGK, which was based on the sequence of the citrus SPS gene homologous to the region flanking the Serine424 of the osmotic activation site of spinach leaf SPS (Komatsu et al., 1996). Thus, the two protein kinases target a common phosphorylation site, but have distinctly different biochemical properties. This is not unique. The spinach leaf SNF1-related kinases and CDPKs both phosphorylate a relatively conserved sequence of SPS to inactivate the spinach leaf SPS (Huang & Huber, 2001).

The research presented here is also the first to purify and characterize the phosphatase that inactivates SPS in the vascular bundles of WNO fruit. The purified phosphatase dephosphorylated both SPS and the synthetic peptide RIKRNV**S**CYGK, which both had been previously phosphorylated by the WNO fruit vascular bundle SPSK. Further, additional assays confirmed that desphophorylation of fructose-6-phosphate was not a factor in the decrease in SPS activity in the presence of SPSP. The SPSP in WNO fruit vascular bundles was characterized as PP2C based on its insensitivity to okadaic acid, inhibition by Mg^{2+}, Fe^{2+} and Mn^{2+}, and activation by Fe^{3+}, which previously has been reported to activate PP2C in mammals (Fjeld & Denu, 1999). To the authors' knowledge, this is the first report of a plant PP2C inhibited by Mg^{2+}, Fe^{2+} and Mn^{2+} and activated by Fe^{3+}. Furthermore, PP2C is a novel protein phosphatase in the regulation of SPS. The spinach leaf SPSP in the light/dark modulation of SPS belongs to the PP2A family (Huber & Huber, 1990; Siegl et al., 1990).

The uniqueness of the mechanism regulating SPS activity in vascular bundles of citrus fruit appears to be related to the fact that vascular bundles are nonphotosynthetic. The activity of SPS in citrus leaves and in the exocarp of young developing fruit, both photosynthetic tissues, was regulated by a mechanism analogous to the one described for spinach leaves (Huber et al., 1989). Localization of SPS, SPSK and SPSP in the vascular bundles of the citrus fruit, combined with the unique mechanism regulating SPS activity in this tissue, suggests SPS might play a key role in sucrose unloading and sink strength that is carefully regulated by SPSK and SPSP during citrus fruit development.

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