

## Phosphorylation of sucrose synthase from maize seedlings\*

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**Two isoforms of sucrose synthase (SS1 and SS2) from maize (*Zea mays*, var. *Mona*) seedlings co-purified with a calcium and phospholipid dependent protein kinase. The enzymatic preparation obtained gave a positive reaction with the antibody against mammalian protein kinase C. Maize sucrose synthase was phosphorylated by the endogenous protein kinase. Also, mammalian protein kinases (protein kinase C and protein kinase A) were able to phosphorylate the 86 kDa subunit of sucrose synthase. When excised seedlings were fed [<sup>32</sup>P]orthophosphate, sucrose synthase was also phosphorylated. Microsequencing of *in vivo* labelled enzyme has shown phosphorylation of Ser-15 in SS2. The present work provides evidence that maize sucrose synthase is the physiological substrate of the endogenous calcium and phospholipid dependent protein kinase(s).**

Sucrose synthase (UDP-glucose: D-fructose 2-glucosyl transferase EC 2.4.1.13) which catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose, seems to play an important role in sucrose degradation [1-3]. Two sucrose synthase isoforms, named SS1 and SS2, encoded by genes designated *Sh1* and *Sus1* have been found in

maize [2, 4]. Differential expression of the two genes was observed in different tissues in response to environmental stimuli. The maize *Sus1* and its homolog *Ss2* gene appear to be constitutively expressed, whereas *Sh1* is expressed at high levels only in response to specific developmental and environmental stimuli [4, 5]. The two genes show markedly

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**Abbreviations:** SS, sucrose synthase; SPS, sucrose-phosphate synthase; PKA, protein kinase A; PKC, protein kinase C; CDPK, calcium dependent protein kinase/calmodulin like domain protein kinase; PMSF, phenylmethylsulphonyl fluoride.

different responses to changes in tissue carbohydrate status. The *Sh1* mRNA was expressed under conditions of limited carbohydrate content, whereas *Sus1* transcript levels were nearly undetectable under sugar-depleted conditions [3]. The maize *Sh1* gene shows an increased expression under anaerobic conditions and its transcript level is light dependent [2, 4].

Cloning of these genes has demonstrated that their products are composed of 802 and 763 amino acids, respectively. Both isoforms show a high degree of sequence homology and more than 80% of amino acids is situated in identical positions [6, 7]. Products of the two genes have been purified and characterized. These proteins, consisting of about 90 kDa subunits are biochemically similar and immunologically crossreactive [1, 8, 9]. Because of high degree of homology of SS1 and SS2 isoenzymes, encoded by *Sh1* and *Sus1* genes, the common name SuSy is frequently used for these maize proteins.

Recent data indicate that SuSy is a phosphorylated protein [8, 10]. Sucrose metabolism in plant tissues seems to be regulated by reversible phosphorylation. It has been recognized that phosphorylation of sucrose-phosphate synthase (SPS) can play a role in regulation of sucrose synthesis [11] leading to starch accumulation. From the physiological point of view, starch in plants corresponds to glycogen in mammalian organisms. Therefore, the metabolism of these compounds might be regulated in a similar way. The finding that the enzymes involved in starch metabolism undergo phosphorylation seems to be in accordance with the known regulation pathway of metabolism of storage material in eukaryotes. These data prompted us to study the phosphorylation of sucrose synthase to elucidate the role of this modification on the regulation of sucrose metabolism in plants.

## MATERIALS AND METHODS

**Chemicals.** [ $^{32}$ P]orthophosphate and [ $\gamma$ - $^{32}$ P]ATP were purchased from Amersham International. Histone H1 (type III S), pepstatin A, 1,3-diolein, phosphatidylserine,

UDP and phenylmethylsulphonyl flouride (PMSF) were products of Sigma. DEAE-cellulose and 3MM paper were from Whatman. PD-10 column was from Pharmacia. Polyacrylamide, low molecular mass markers and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad. The antibody raised in rabbit against a peptide from  $\beta$  subunit of protein kinase C was from Boehringer Mannheim. All other chemicals were of the highest purity commercially available.

**Biological material.** Maize seeds (*Zea mays* var. *Mona*) were soaked for 12 h in distilled water, then grown in the dark for 72 h at 26°C. The apical parts of the seedlings, which reached a length of about 2.5 cm, were harvested, immediately frozen in liquid nitrogen and stored at -70°C.

**Purification of sucrose synthase from maize seedlings.** Frozen seedlings, 2.5 g, were ground in a mortar to a fine powder in the presence of a small volume of liquid nitrogen, and extracted with 7.5 ml of homogenization buffer (20 mM Tris/HCl, pH 7.5, 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 0.2 mM PMSF, 0.7  $\mu$ g/ml pepstatin). The homogenate was centrifuged at 20000  $\times g$  for 15 min at 4°C. Ammonium sulphate was added to 0.36 saturation. The mixture was stirred for 30 min at 4°C and centrifuged at 12000  $\times g$  for 15 min; then another portion of ammonium sulphate was added to the supernatant to 0.48 saturation. The mixture was stirred for 30 min and then centrifuged as above. The pellet was suspended in 900  $\mu$ l of buffer A (20 mM Tris/HCl buffer, pH 7.0, 10 mM magnesium chloride, 1 mM dithiothreitol) and desalted by chromatography on a PD-10 column equilibrated with buffer A. The eluted protein fractions were pooled and diluted with an equal volume of buffer A, then applied to a 0.5 ml DEAE-cellulose column equilibrated with buffer A. The column was washed with buffer A until the absorbance at 280 nm of the eluate was below 0.2. The bound proteins were eluted using a 3 ml linear gradient of 0–0.3 M sodium chloride in buffer A at a flow rate of 12 ml/h. All steps of purification were performed at 4°C. The fractions were assayed for protein kinase activity, sucrose synthase activity, protein content and ionic strength. Protein concentration

was determined either by measuring the absorbance at 280 nm or by the method of Bradford [12] using bovine serum albumin as a standard.

**Labelling of maize seedlings with [ $^{32}\text{P}$ ]orthophosphate and purification of  $^{32}\text{P}$ -sucrose synthase.** The seedlings (2.5 g) grown as described above were packed into a beaker and incubated for 16 h in 1 ml of 10 mM morpholinoethanesulphonic acid buffer, pH 6.0, containing 100 mCi [ $^{32}\text{P}$ ]orthophosphate. In some experiments the seedlings were labelled with [ $^{32}\text{P}$ ]orthophosphate in the presence of 1% of sucrose. The seedlings were then washed with deionized water and dried on tissue paper. Sucrose synthase was purified as described above by ammonium sulphate fractionation and chromatography on DEAE-cellulose.

**Determination of sucrose synthase activity.** The activity of the enzyme was measured by determination of fructose cleaved from sucrose in the presence of UDP according to the spectrophotometric method of Avigad & Milner [13].

**Determination of activity of the phospholipid and calcium dependent protein kinase.** The reaction mixture (total volume 70  $\mu\text{l}$ ) contained histone H1 (1 mg/ml), Tris/HCl buffer, pH 7.5 (50 mM), calcium acetate (0.7 mM), magnesium chloride (7.5 mM), phosphatidylserine (60  $\mu\text{g}/\text{ml}$ ), diolein (5  $\mu\text{g}/\text{ml}$ ), [ $\gamma$ - $^{32}\text{P}$ ]ATP (0.1 mM, 50 c.p.m./pmol) and 20  $\mu\text{l}$  of protein fraction after DEAE-cellulose chromatography as the source of protein kinase. After incubation for 5 min the reaction was stopped by spotting 50  $\mu\text{l}$  of the reaction mixture onto phosphocellulose filter papers, followed by immediate washing of the filters 4 times with 75 mM phosphoric acid and drying. The radioactivity was measured as Čerenkov radiation [14].

**Purification and definition of activity of mammalian protein kinases.** PKC was isolated from pig spleen according to Parker *et al.* [15] as modified by Ferrari *et al.* [16]. One unit of PKC activity was defined as the amount of enzyme that catalyses the incorporation of 1 pmol of [ $^{32}\text{P}$ ]phosphate per minute into histone H1 under conditions previously described by Humble *et al.* [17]. PKA was purified from pig heart essentially as

described by Zoller *et al.* [18]. One unit of protein kinase A activity was defined as the amount of kinase required for incorporation of 1 pmol of [ $^{32}\text{P}$ ]phosphate per minute into mixed histone under the specified conditions [19].

**Phosphorylation of maize sucrose synthase by mammalian and endogenous protein kinases.** The phosphorylation of sucrose synthase by PKC was performed in a total volume of 70  $\mu\text{l}$  at 30°C. The mixture contained 20  $\mu\text{l}$  of chromatographic fraction of sucrose synthase (about 10  $\mu\text{g}$  of protein), 2–30 units of protein kinase from spleen, Tris/HCl buffer, pH 7.5 (25 mM), calcium acetate (0.7 mM), magnesium chloride (5.3 mM), phosphatidylserine (64  $\mu\text{g}/\text{ml}$ ), diolein (5  $\mu\text{g}/\text{ml}$ ). The reaction was initiated by addition of [ $\gamma$ - $^{32}\text{P}$ ]ATP (0.1 mM, 100 c.p.m./pmol).

The phosphorylation of plant sucrose synthase by PKA was performed in a total volume of 40  $\mu\text{l}$  at 30°C. The mixture contained 10  $\mu\text{l}$  of chromatographic fraction of sucrose synthase (about 5  $\mu\text{g}$  of protein), 20 units of PKA from pig heart, morpholinoethanesulphonic acid buffer, pH 6.9 (25 mM), EGTA (0.25 mM), potassium phosphate, buffer pH 6.5 (1.25 mM), and mercaptoethanol (1.25 mM). The reaction was initiated by addition of [ $\gamma$ - $^{32}\text{P}$ ]ATP (0.1 mM, 100 c.p.m./pmol).

The phosphorylation of endogenous proteins was performed as described for the activity of maize protein kinase, except that the substrate (histone H1) was not added to the incubation mixture.

All phosphorylation reactions were terminated after 5 min of incubation by adding SDS-sampling buffer. The proteins were electrophoretically separated on polyacrylamide gels.

**Polyacrylamide gel electrophoresis, autoradiography and immunoblotting.** After termination of the reaction the samples were heated for 15 min at 95°C under reducing conditions. The proteins were separated on a 10% polyacrylamide gel according to Laemmli [20] as modified by O'Farrell [21]. The resulting gels were stained with Coomassie Brilliant Blue R-250, dried and autoradiographed. In some cases transfer to nitrocellulose filter was performed. Western

blots of plant proteins were developed using a monoclonal antibody raised against  $\beta$  subunit of mammalian PKC.

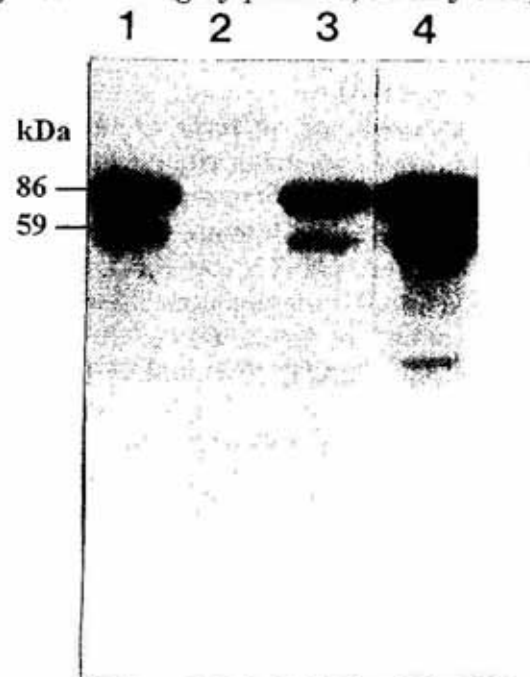
**Preparative separation of proteins by electrophoresis.** About 100  $\mu$ g of maize proteins from fractions after DEAE-cellulose chromatography were applied on 10% SDS/polyacrylamide gel 16 cm in length. Electrophoresis was carried out overnight at room temperature, then the gel was stained for 20 min with 0.2% (w/v) Coomassie Brilliant Blue containing 20% methanol and 0.5% acetic acid. The gel was destained with 30% methanol. The segment of the gel containing the band of about 86 kDa was cut out using a sharp scalpel.

**Procedure for sequencing of peptides.** Proteins from the band of interest were digested in the gel as described by Rosenfeld *et al.* [22] either with trypsin or with LysC (WAKO). In the latter case the recommended buffer, Tris/HCl, pH 8.5 (100 mM) was used. The extracted peptides were separated on a SMART (Pharmacia) chromatography system equipped with a  $\mu$ RPC C2/C18 SC 2.1/10 column. Peptides were eluted using a gradient of 0–60% acetonitrile in water/1% trifluoroacetic acid over 60 min at a flow rate of 100  $\mu$ l/min. Peptides were sequenced either on an Applied Biosystem model 470A gas phase sequencer with on line PTH analyzer 120A, or on a model 476A according to the instructions of the manufacturers.

## RESULTS

Two proteins from maize seedlings preparation were endogenously phosphorylated. The electrophoretically estimated molecular masses of those proteins were about 59 kDa and 86 kDa (Fig. 1, lane 1). The phosphorylation was totally inhibited in the presence of 0.5 mM EGTA, a potent chelating agent of calcium ions (Fig. 1, lane 2), whereas addition of an equimolar concentration of calcium acetate abolished the effect of EGTA (Fig. 1, lane 3). The phosphorylation of both proteins was strongly stimulated by phospholipids (phosphatidylserine and diolein) (Fig. 1, lane 4). In the present work, the attempts at identification of higher molecular mass pro-

tein the phosphorylation of which is stimulated by phospholipids were undertaken. The phosphorylated 86 kDa protein was separated by preparative electrophoresis, and subsequently digested by lysine specific proteases. After separation by chromatography (see Materials and Methods, and Fig. 2), ten predominant peptides were sequenced. The sequenced peptides showed complete homology to the sucrose synthase isoenzymes SS1 and SS2 from maize (Table 1). The results indicated that the protein band of about 86 kDa contained two isoforms of sucrose synthase (SS1 and SS2). For identification of the phosphorylation site of maize sucrose synthase, the enzyme was purified using a combination of ammonium sulphate precipitation and anion exchange chromatography (see Materials and Methods). SS1 and SS2 flow out together from DEAE-cellulose column at 75–150 mM NaCl (Fig. 3). The isoenzymes were highly purified, as only one pro-



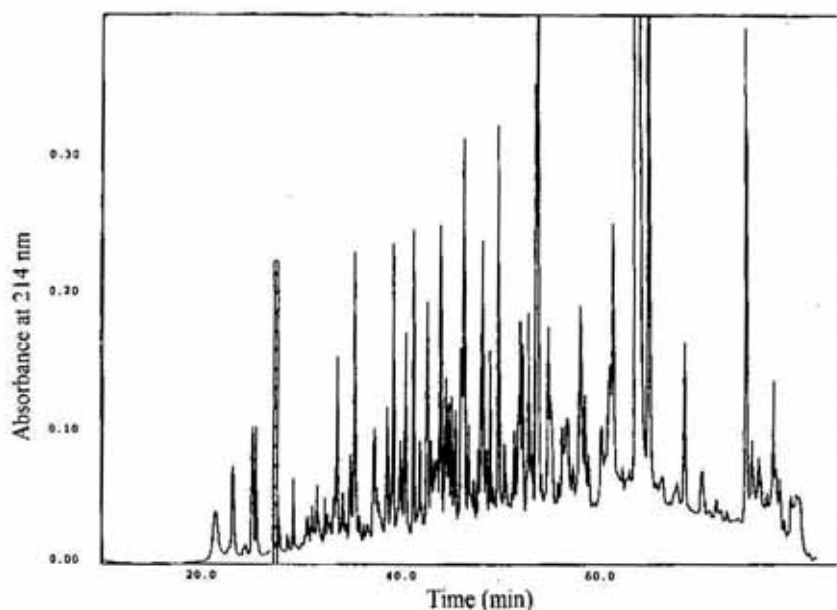
**Figure 1. Autoradiography of maize seedling proteins separated by SDS/PAGE.**

The proteins were phosphorylated alone (lane 1); and in the presence of: 0.5 mM EGTA (lane 2); 0.5 mM EGTA and 0.5 mM calcium acetate (lane 3); and 0.12 mM calcium acetate, phosphatidylserine (75  $\mu$ g/ml) and diolein (6.7  $\mu$ g/ml), (lane 4). Before electrophoresis the proteins were purified by chromatography on DEAE-cellulose and Octyl-Sepharose as described previously [23]. Molecular masses were estimated using appropriate markers.

**Table 1. Comparison of the sequence of the peptides isolated from 86 kDa maize protein, with the sequences of the peptides from SS1 and SS2 isoenzymes**

Protein	Sequence	Homologous to	Protein	Sequence	Homologous to
SS1	<sup>116</sup> KEQLVDGQS <sup>124</sup>		SS1	<sup>515</sup> KFNIVSPGADMS <sup>526</sup>	
Peptide 1	K-QLV - - - S	SS1	Peptide 6	KFNIVSPGADLS	SS2
SS2	<sup>124</sup> KEQLVEEGP <sup>132</sup>		SS2	<sup>523</sup> KFNIVSPGADLS <sup>534</sup>	
SS1	<sup>169</sup> KESLYPL <sup>175</sup>		SS1	<sup>638</sup> RWISAQMNR <sup>646</sup>	
Peptide 2	K - - LYPL	SS1	Peptide 7	RWISAQMNR	SS1, SS2
SS2	<sup>177</sup> KESMYPL <sup>183</sup>		SS2	<sup>646</sup> RWISAQMNR <sup>654</sup>	
SS1	<sup>169</sup> KESLYPLLN <sup>177</sup>		SS1	<sup>641</sup> SAQMNR <sup>646</sup>	
Peptide 3	K - -MYPLLN	SS2	Peptide 8	SAQ - NR	SS1, SS2
SS2	<sup>177</sup> KESMYPLLN <sup>185</sup>		SS2	<sup>649</sup> SAQMNR <sup>654</sup>	
SS1	<sup>308</sup> RALENEM <sup>314</sup>		SS1	<sup>736</sup> ISQGGLQR <sup>743</sup>	
Peptide 4	RALEN - M	SS1	Peptide 9	ISQGGLQR	SS1, SS2
SS2	<sup>316</sup> RAMENEM <sup>322</sup>		SS2	<sup>744</sup> ISQGGLQR <sup>751</sup>	
SS1	<sup>504</sup> RVVHGID <sup>510</sup>		SS1	<sup>748</sup> YTWK <sup>751</sup>	
Peptide 5	RV - HGID	SS1, SS2	Peptide 10	YTWK	SS1, SS2
SS2	<sup>512</sup> RVVHGID <sup>518</sup>		SS2	<sup>756</sup> YTWK <sup>759</sup>	

- Amino acids not detectable. The sequence of peptides isolated from the maize protein of molecular mass of 86 kDa was compared with sequences of peptides derived from isoenzymes of sucrose synthase. SS1 and SS2 were sequenced by Werr *et al.* [6] and Huang *et al.* [7], respectively.



**Figure 2. Separation on the Reverse-Phase HPLC of tryptic fragments from <sup>32</sup>P-phosphorylated protein of 86 kDa.**

Separated by SDS/PAGE the radioactive protein was cut out from the gel, digested by trypsin and subjected to chromatography on a C18 column according to [22]. The chromatogram shows the peptide pattern. For details see Materials and Methods.

tein band of about 86 kDa was predominant (Fig. 4, lane 2). The sucrose synthases flow out together with the calcium and phospholipid dependent protein kinase (Fig. 3).

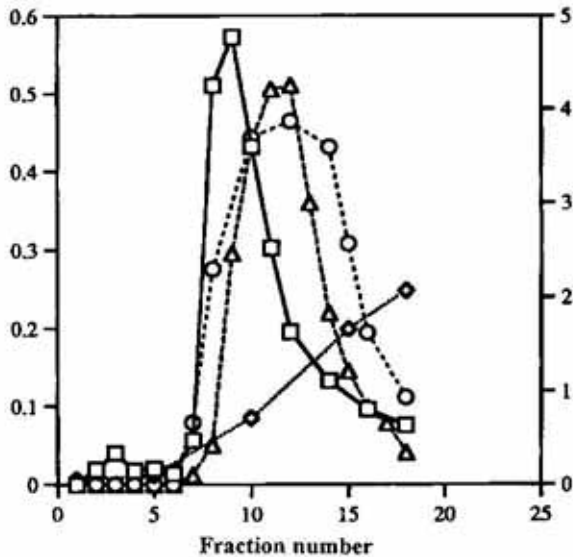


Figure 3. Separation of sucrose synthase on DEAE-cellulose.

□, Sucrose synthase activity ( $A_{660}$ ); ◇, NaCl (M); △, protein ( $\mu\text{g}/\text{ml}$ ) (left axis for both); ○, protein kinase activity (pmol/min per  $\mu\text{l}$ ) (right axis for both). The separation was performed as described under Materials and Methods.

The presence of protein kinase made possible endogenous phosphorylation of sucrose synthase. It is worth to point out that a protein of about 90 kDa present in sucrose synthase preparation, reacted very strongly with the antibody against  $\beta$  type mammalian PKC. A faint positive immunoreaction with the antibody against PKC was also noticeable with maize protein of about 59 kDa (Fig. 4, lane 1). The maize sucrose synthase(s) was phosphorylated not only by endogenously present protein kinase, but also by PKC from pig spleen and PKA from pig heart (Fig. 4, lanes 6, 7, 8, respectively). Autoradiography of the analyzed proteins showed that the endogenously present protein kinase phosphorylated sucrose synthase both *in vitro* and *in vivo* (Fig. 4, lanes 6 and 9, respectively), whereas phosphorylation by mammalian protein kinases was less specific (Fig. 4, lanes 7 and 8, respectively). For identification of the site of sucrose synthase phosphorylation, the phosphorylated enzyme was isolated from [ $^{32}\text{P}$ ]phosphate labelled maize seedlings (Fig. 4, lane 9). When the seedlings were radioactively labelled in the presence of 1% sucrose, the degree of sucrose synthase phos-

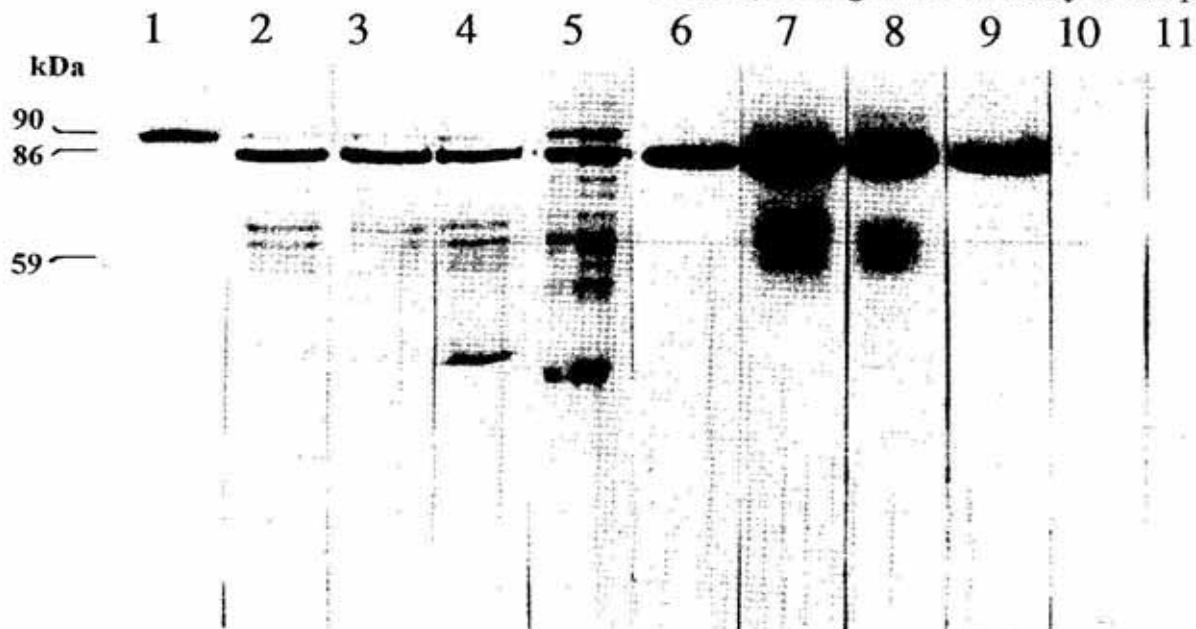


Figure 4. SDS/PAGE of proteins from maize seedlings after chromatography on DEAE-cellulose columns.

Coomassie-stained gel (lanes 2–5) and its corresponding autoradiogram (lanes 6–9). The fraction of highest sucrose synthase activity was phosphorylated with endogenously present protein kinase (lanes 2 and 6); with pig spleen PKC (lanes 3 and 7); PKA from pig heart (lanes 4 and 8). Sucrose synthase(s) was isolated from maize seedlings  $^{32}\text{P}$ -labelled (lanes 5 and 9) *in vivo*. Western blot analysis was performed using antibodies raised against the  $\beta$  subtype of PKC (lane 1). Lanes 10 and 11, autoradiogram of PKC and PKA, respectively, phosphorylated in the absence of sucrose synthase. The experiments were performed as described in Materials and Methods.

phorylation was about 2-fold decreased (not shown). The  $^{32}\text{P}$ -labelled sucrose synthase was subjected to trypsin digestion, followed by separation of peptides on C18 column. One peak, eluted from the column after 27.5 min, marked in Fig. 2 as a bar, contained the radioactive material. The determined sequence of the peptide from this peak evidenced phosphorylation of Ser-15 from SS2 isoenzyme (Table 2). To confirm this result a synthetic peptide with the sequence corresponding the residues surrounding Ser-15 in SS2 was constructed. The serine in this peptide was effectively phosphorylated by the maize calcium and phospholipid dependent protein kinase (not shown).

**Table 2. Comparison of the sequence of the phosphorylated peptide from sucrose synthase  $^{32}\text{P}$ -labelled *in vivo***

Peptides	Sequence	Homologous to
SS1	<sup>7</sup> RLHSLR <sup>12</sup>	
Peptide A	RLHSVR	SS2
SS2	<sup>12</sup> RLHSVR <sup>17</sup>	

Peptide A was obtained from sucrose synthase isolated from  $^{32}\text{P}$ -labelled maize seedlings.

## DISCUSSION

Phosphorylation by calcium dependent serine-threonine protein kinases regulates many cellular events in a plant cell. Widely characterized plant enzymes form a family of calcium dependent protein kinases (CDPK) also known as calmodulin like domain protein kinases [24]. CDPK has been characterized, purified and cloned from higher plants [25, 26]. At present only several biological functions of CDPK are known, but the ubiquitous distribution of this enzyme in the plant kingdom argues for a role in a variety of phenomena which implicate free calcium as the second messenger of the plant cells signal transduction system.

Evidences accumulating over the past few years strongly suggest that, in addition to the predominant CDPK, the plants contain another protein kinase which is calcium and phospholipid dependent [23, 27, 28]. Such a dependence of the plant enzyme resembles the properties of the well known PKC, the

enzyme crucial in signal transduction in mammalian cells [29]. Recently it has been reported that some of CDPK activities can be stimulated by phospholipids, however clear evidence for the existence of plant PKC is still lacking. Now we have found that phosphorylation of two proteins of 59 kDa and 86 kDa is inhibited by EGTA and stimulated by phospholipids. The phosphorylated protein of 86 kDa was identified as SuSy, the mixture of two isoforms of sucrose synthase (SS1 and SS2). Because of differences in mobility of the phosphorylated and non-phosphorylated protein, the electrophoretic estimation of molecular mass of sucrose synthase as 86 kDa was not very accurate. The calcium and phospholipid dependent protein kinase, which is able to phosphorylate SuSy, reacts with a mammalian anti-PKC antibody. This may point the existence of common structural elements in the two protein kinases. It seems to be an important observation that only one sucrose synthase, namely SS2, is phosphorylated and only one residue, Ser-15, was found to be phosphorylated *in vivo*. The *in vitro* experiments and the phosphorylation of the synthetic peptide based on the sequence surrounding Ser 15, confirmed the observation that serine located at the N-terminal sequence of SS2 was phosphorylated. Also, Huber *et al.* [10] have recently reported that serine located at N-terminal of SS2 can be phosphorylated in a calcium dependent manner. It is worth to point out that the present results provide evidences that phosphorylation of SS2 is catalyzed by an endogenous calcium and phospholipid dependent protein kinase. It is interesting that under both *in vivo* and *in vitro* conditions, serine at the N-terminal part of sucrose synthase was phosphorylated. The terminal modifications might have an influence on conformation, recognition in protein-protein interaction, and thereby on the activity of the target protein. It is possible that in plants, the biological complex involving the enzyme(s) of starch metabolism and protein kinase(s) does exist. Such a possibility is supported by the observations (i) that the protein coeluted (or associated) with sucrose synthase reacts with antibody against protein kinase; and (ii) that the sucrose influences the phosphoryla-

tion of sucrose synthase. Also, the fact that, in the developing rice seeds, a gene encoding protein kinase is located in the region immediately upstream of the gene encoding the branching starch enzyme [30] seems to indicate the important role of protein phosphorylation in the regulation of the metabolism of carbohydrates and their storage in plants.

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