Partial Purification and Characterization of Sucrose Phosphate Synthase from Preclimacteric and Climacteric Bananas

João R. O. Nascimento, Beatriz R. Cordenunsi, and Franco M. Lajolo*

Departamento de Alimentos e Nutrição Experimental, FCF Universidade de São Paulo, Caixa Postal 66083, CEP 05389-970, São Paulo, Brazil

Sucrose phosphate synthase (SPS) (EC 2.4.1.14) was purified from bananas (*Musa acuminata* cv. Nanicão) at different developmental and ripening stages (70, 110, and 130 days after anthesis), corresponding to tissue with different structure and composition. Banana SPS exists in three polymeric forms as observed by electrophoresis and immunoblotting, similar in all stages studied, with M_w respectively of 180, 254, and 686 kDa. The three forms have the same 116 kDa subunit, and their activity increased during ripening associated with increased protein synthesis as shown by Western blotting.

Keywords: *Musa acuminata; banana; sucrose phosphate synthase; ripening; sucrose*

INTRODUCTION

Recent reports have shown that sucrose phosphate synthase (SPS) has a central role in sucrose biosynthesis from triose phosphate in the cytoplasm of photosynthetic (source) tissues and probably also in sucrose accumulation in others tissues, as seeds and fruits (sinks), linked or not to starch transformation.

The role of SPS in sucrose metabolism in leaves has recently been discussed by Bruneau et al. (1991). SPS together with fructose 1,6-biphosphatase is important to control carbon partition between starch and sucrose (Stitt et al., 1987). It was also shown recently that transgenic tomato leaves expressing a cloned SPS gene, had increased amount of sucrose and reduced content of starch (Worrell et al., 1991). The enzyme in leaves of higher plants is regulated by two mechanisms: (1) a fine control by allosteric effectors, such as glucose-6-phosphate (G-6P, activator) and P_i (inhibitor), which affect the kinetic behavior, and (2) a coarse control, involving covalent protein modification by phosphorylation (inactivation) by an SPS-kinase and dephosphorylation (activation) mediated by a phosphatase (Weiner et al., 1992). Recently Reinholtz et al. (1994) postulated that potato SPS is regulated via metabolites and phosphorylation of the enzyme similarly to what happens with leaves.

In seeds such as wheat, apparently there is not an allosteric regulation by these metabolites and the SPS is not inhibited in the same way by P_i, indicating at least two types of the enzyme (Salerno et al., 1991) that may differ, as suggested by Nielsen and Huber (1989), in their primary or secondary structure.

In fruits not much is known about the properties and regulation of this enzyme, but there is increasing evidence of participation of SPS in the sucrose synthesis linked to starch degradation, to sorbitol, sucrose, and raffinose in saccharide-translocating species (Hubbard et al., 1989), and regulation by similar mechanism. In tomatoes, SPS activity during development was positively correlated to sucrose contents (Dali et al., 1992). Tomato varieties having higher SPS activity (*Lycopersicum hirsutum*) had also higher sucrose contents when compared to low SPS and sucrose varieties (Miron and Shaffer, 1991).

In muskmelon it was recently shown an increase of SPS activity during ripening (Hubbard et al., 1989). The authors suggested that sucrose accumulation was determined by the balance between sucrose synthesis (SPS activity) and degradation by SS (sucrose synthase) or invertase action. In Asian pears similarly the balance of SPS with SS (but not with invertase) seem important in determining sugar accumulation (Moriguchi and Yamaki, 1988). Only in peaches did Moriguchi et al. (1992) find a relation of sucrose synthesis with SS and not SPS.

During bananas ripening the disappearance of starch reserve is very fast. The average starch content drops from 25% in the preclimacteric phase to less than 1% during the climacteric period. Concomitantly, sucrose content increases 12 times and precedes the increase of glucose and fructose (Arêas and Lajolo, 1981).

In a previous paper (Cordenunsi, 1989; Cordenunsi and Lajolo, 1995) we reported that, synchronously to starch disappearance and to sucrose accumulation during ripening of green bananas, there was a complete reduction of SS activity and a 3−5-fold increase of SPS activity, detected in crude extracts, which are results also obtained by Hubbard et al. (1990). Similarly to bananas or potatoes, kiwi fruit SPS activity, when measured with saturating substrate concentrations, increased during ripening but before starch−sugar conversion and was not associated to the climacteric phase. Only when measured in limited substrate conditions was SPS activity coincident to starch−sugar conversions (MacRae et al., 1992).

While SPS from leaves and seeds has been purified and cloned and several of its properties have been established (Salerno et al., 1991; Walker and Huber, 1989; Klein et al., 1993), in fruits it has received less attention. Fundamental information about the properties of SPS remain to be determined because the enzyme has not been yet purified to homogeneity and characterized. The objective of this research was to establish a procedure for SPS purification from both preclimacteric and climacteric bananas and to study some of its properties.

* Author to whom correspondence should be addressed [fax 55 (011) 815 4410; e-mail fmlajolo@spider.usp.br].

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Table 1. Partial Purification of SPS from Bananas 70 d.a.a. (20% Starch)

purification step	volume (mL)	total activity	protein (mg)	specific activity	yield (%)	purification factor
crude extract	1760	6880	1378	5.0	100	−
30−60% ammonium sulfate (1)	370	13800	735	18.7	200	3.7
DEAE-cellulose	265	5308	128	41.4	77	8.3
30−60% ammonium sulfate (2)	50	4128	70	58.4	60	11.7

MATERIALS AND METHODS

Plant Material. Unripe bananas (*Musa acuminata* cv. Nanicão) either immature (70 days after anthesis) or mature (110 and 130 days after anthesis), were obtained from the experimental orchard of Universidade Federal de Minas Gerais. The preclimacteric fruits were harvested 110 days after anthesis when the central diameter of the fruits of the second bunch was 33−34 mm (full three quarters). Some bunches were used immediately (preclimacteric fruits), and others were stored under controlled temperature (18 °C) until ripe (climacteric), which took about 20 days.

Enzyme Extraction and Purification. Samples were peeled, sliced, and ground in a chilled mortar. The materials (100−250 g) were homogenized using a 1:10 tissue-to-buffer extraction ratio by mechanical shaking. The extraction buffer contained 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% polyvinylpyrrolidone (M_w 40 000) and recently neutralized 20 mM cysteine. The homogenate was centrifuged (13200*g*, 20 min), and the supernatant was collected. Ammonium sulfate solution was added (0.43 vol) to give 30% saturation and centrifuged (13200*g*, 20 min). The pellet was discarded, and ammonium sulfate was added to give a 60% saturation. The resulting precipitate (30−60% fraction) was dissolved and dialyzed in cellulose dialysis tubing (12000−14000 M_w cutoff) against Tris buffer (pH 7.0), containing 20 mM cysteine and 20 mM EDTA (buffer A).

The dialysate was mixed with DEAE-cellulose equilibrated with buffer A to bind SPS, followed by centrifugation (1400*g*, 10 min). The pellet was washed 3 times with buffer A; a column (60 cm × 1.6 cm) was packed with the slurry and a linear gradient of 0−0.6 M NaCl in buffer A was used at a flow rate of 0.5 mL/min. Fractions with SPS activity were combined and dialyzed against buffer A. From this pool, another ammonium sulfate fraction (30−60%) was obtained by the same procedure and stored in glycerol at −15 °C.

Carbohydrate and Protein Determination. Samples (1 g) were extracted with 0.5 N NaOH in a Potter-Elvehjem homogenizer. The extract was neutralized with 0.5 N acetic acid. Starch, glucose, fructose, and sucrose were determined enzymatically as described by Areas and Lajolo (1981). Protein determination was done either using the method of Lowry et al. (1951) as modified by Peterson (1977) or by the Bradford (1976) technique using BSA as a standard.

Sucrose Synthase (SS) and Sucrose Phosphate Synthase (SPS) Assays. Reaction mixtures to determine SS activity (measured in the sucrose synthesis direction) contained 10 mmol of Tris-HCl (pH 7.5), 1 mmol of NaF, 1 mmol of $MnCl_2$, 1 mmol of fructose, 0.5 mmol of uridine diphosphoglucose (UDPG), and 50 μL of dialyzed enzyme (100 μL final volume). Reaction mixtures (in triplicate) were incubated at 30 °C, and the reactions were terminated at different times (usually between 5 and 15 min) with 200 μL of 1.0 N NaOH. Remaining fructose was destroyed by immersing the solution in a boiling water bath for 10 min. Sucrose was then determined using the thiobarbituric method (Percheron, 1962).

The procedure for the SPS assay was performed as described for the SS assay by substituting fructose by fructose-6P and Tris-HCl by Hepes buffer (pH 6.5).

Identification of SPS Bands in the Gels. Nondenaturating polyacrylamide slab gels (6% monomer concentration) were prepared according to Davis (1964). Samples (10−20 μg of protein) were applied in duplicate, and runs were conducted in a cold room at 100 volts. One of the runs was stained for protein with Coomassie Blue R-250; the other was cut into 2 mm strips that were incubated overnight in 100 μL of the reaction mixture (see SPS assay above) to show the protein bands having SPS activity.

Molecular Weight and Isoelectric Point. M_w was determined by Sepharose CL-6B column (62 cm × 1.8 cm) in 50 mM Tris-HCl, pH 7.0. The column was previously calibrated with standards ranging from 29 to 660 kDa. SDS−PAGE (7.5%) was used to obtain the subunits M_w (Laemmli, 1970), using markers ranging from 29 000 to 205 000.

For the determination of the M_w of SPS forms in nondenaturating PAGE protein standards ranging from 14 to 545 kDa were used as described in the Sigma Technical Bulletin. The proteins were separated in a set of slab gels of different polyacrylamide concentrations (ranging from 4.5% to 10%), and the electrophoretic mobility (R_f) of each protein in each one of these gels was determined. The molecular weight were calculated from the mobility data obtained according to Ferguson (1964) and Hedrick and Smith (1968).

Total Protein Extraction. Because bananas have several interfering compounds, we used the method described by Dominguez-Puigjaner et al. (1992) to extract total protein for SDS−PAGE, omitting the acetone:hexane extraction mixture. The homogenate was extracted with phenol, and the proteins in the phenolic phase were precipitated with 4 vol of methanol/0.1 M ammonium acetate. The pellet was dried and recovered in SDS−PAGE sample buffer.

Preparation of Rabbit Polyclonal SPS Antibodies against SPS. The purified enzyme was run on preparative native gel electrophoresis to separate the different SPS forms. The band corresponding to SPS_3 (approximately 150 mg of protein) was cut out, homogenized in complete Freund's adjuvant, and injected subcutaneously into the dorsal region of rabbits. The procedure was repeated twice at 20 day intervals. One week after the last injection, blood was collected.

Western Immunoblots. Proteins were separated in DAVIS 6% and SDS−PAGE at 7.5% acrylamide concentration and electroblotted overnight on polyvinylidene fluoride (PVDF) membrane (Millipore), using a 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine for transference. The membranes were stained with starch black or tested with anti-SPS_3 antibodies. After the membrane was treated with 5% defatted milk in TBS (50 mM Tris, pH 7.6, containing 150 mM NaCl) the first antibody (anti-SPS_3 serum) was added at a 1:1000 dilution and the system was incubated at room temperature for 2 h. The membrane was then washed with TBS, treated with a second antibody goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in TBS-milk medium (1:1000 dilution), and incubated at room temperature for 2 h.

RESULTS

Purification of SPS from Banana Pulp Tissues. Soluble pectin substances, especially in ripened tissues, increase the viscosity of the crude extracts, making it very difficult the passage through columns or fractionation by ammonium sulfate. The difficulty was overcome by using a preliminary fractionation (0−30%) with a saturated ammonium sulfate solution followed by storage overnight at 4 °C to remove some of the soluble pectin. For the same reason we used a batch method to bind the 30−60% fraction to DEAE-cellulose and packed the column afterward for elution.

Concentration of the column fractions by ultrafiltration usually caused a great loss of activity. That is the reason why we normally used ammonium sulfate, which resulted in a very good recovery of activity in addition to increasing the specific activity due to separation of other non-precipitating proteins.

The results of Table 1 illustrate a typical purification obtained for pre-climacteric (70 days) bananas. The protein elution peak profiles (not shown here) were similar for the fruits at different physiological stages.

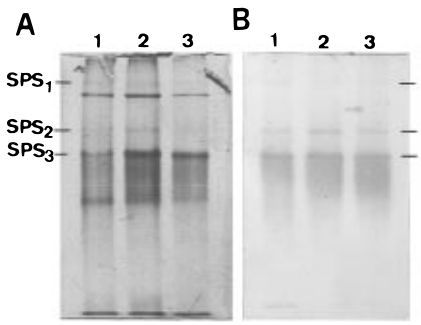

Figure 1. (A) Partially purified banana SPS−native PAGE. Lane 1, 70 days after anthesis (a.a.); lane 2, 110 days a.a.; and lane 3, 130 days a.a. (Coomassie staining). (B) Western blot probed with Ab SPS_3: lane 1, 70 days a.a.; lane 2, 110 days a.a.; and lane 3, 130 days a.a.

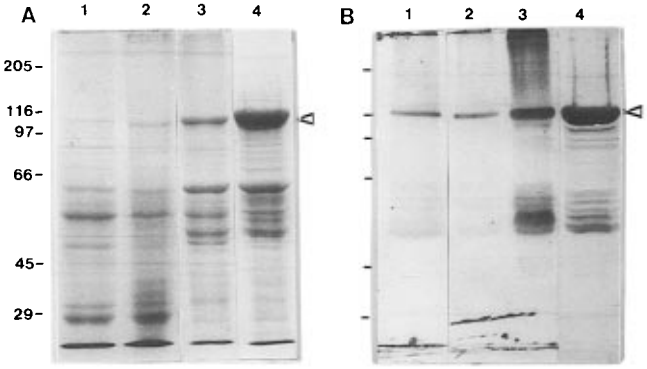

Figure 2. SPS purification steps SDS−PAGE of bananas 110 days after anthesis: Lane 1, crude extract; lane 2, 30−60% ammonuim sulfate fraction; lane 3, DEAE pool; lane 4, 30−60% ammonium sulfate fraction from DEAE pool (Coomassie staining). (B) Western blot probed with Ab SPS_3: lane 1, crude extract; lane 2, 30−60% ammonuim sulfate fraction; lane 3, DEAE pool; lane 4, 30−60% ammonium sulfate fraction from DEAE pool. Molecular weight markers: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

Using the technique we established, it was possible to purify SPS from bananas at 70, 110, and 130 days maturity with yields of 60, 20, and 34% and purification factors of 12-, 3-, and 4-fold, respectively.

General Properties of the Purified Enzyme. Chromatography on Sepharose CL-6B resulted in an SPS activity peak having an estimated M_w around 440 kDa which was similar in all the physiological stages studied. The stability of the preparation changed according to the physiological stage of the fruits, following the yield of the purification.

The SPS preparations obtained from bananas in the three development stages studied, revealed three bands by the enzymatic activity of slices from non-dissociating gels (named SPS_1, SPS_2, and SPS_3) with SPS activity (data not shown). Antibodies produced specifically with SPS_3, which was the main protein band, cross-reacted with SPS_1 and SPS_2 (Figure 1B). The other bands observed with Coomassie staining neither reacted with antibodies nor revealed SPS activity. The M_w values of each one of the three bands were respectively 686 kDa for SPS_1, 254 kDa for SPS_2, and 180 kDa for SPS_3 and did not change with the age of the fruit.

SDS−PAGE at the different purification steps showed clearly the enrichment of the 116 kDa polypeptide and appearance of others from 50 to 70 kDa (Figure 2A). Western blots showed that the 116 kDa band was recognized by the SPS_3 antibody (Figure 2B). The antibody also reacted with those smaller (50−70 kDa) bands but not with the 71 kDa band. The reaction was more or less intense depending on the physiological stage of the fruit and on the preparation. Further tests showed that liquid nitrogen grinding with the extraction buffer and the use of protease inhibitors greatly reduced the appearance of the smaller bands. When the extracts were incubated at room temperature we observed that the appearance of the bands between 50 and 70 kDa was related to the disappearance of the 116 kDa band. This could be reduced by the protease inhibitors. Among the inhibitors tested, benzamidine (1 mM) and antipain (10 μg/mL) were effective while PMSF (1 mM) was not. The band with 71 kDa was a co-purified polypeptide since it did not react with SPS_3 antibody, and its appearance was not related to proteolysis of the 116 kDa band.

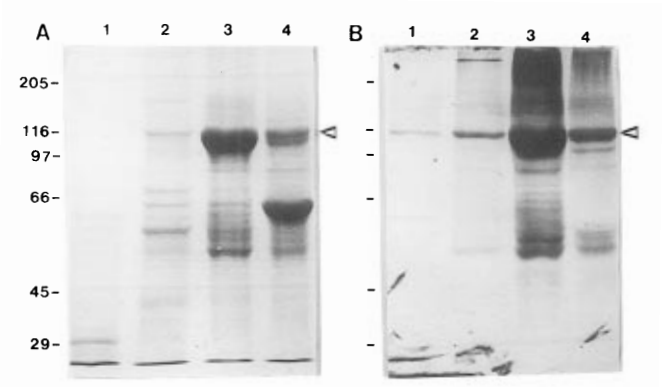

Figure 3. (A) SDS−PAGE of electroeluted native bands: lane 1, SPS_1; lane 2, SPS_2; lane 3, SPS_3; lane 4, band devoid of SPS activity (Coomassie staining). (B) Western blot probed with Ab SPS_3: lane 1, SPS_1; lane 2, SPS_2; lane 3, SPS_3; lane 4, band devoid of activity showing the contamination with SPS_3. Molecular weight markers: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

Figure 3 shows the profile formed when the three SPS bands obtained by DAVIS−PAGE were electroeluted and individually run on SDS−PAGE. Western blot showed that the 116 kDa polypeptide was present in all SPS forms (Figure 3B). Lane 4 corresponded to the fifth band eluted from native gel. The presence of the 116 kDa polypeptide in this lane was caused by contamination of the fifth band with SPS_3 due to spreading of the bands in the moment the bands were excised from the gel.

When crude extracts from bananas in the three physiological stages studied were separated on SDS−PAGE an increase of the amount of the SPS 116 kDa polypeptide was observed (Figure 4). The densitometry of lanes 1−3 showed that the SPS band represented 2.3%, 3.2%, and 4.6% of the total protein, respectively, in the 70, 110, and 130 days, corresponding to a 2-fold increase in SPS antibody-reacting protein. The SPS activity assayed in crude extracts also incresed throughout the development and ripening; the activity obtained from fruits with 70, 110, and 130 days were respectively 22, 47, and 66 μmol/h/g fresh weight, corresponding to a 3-fold increase.

DISCUSSION

Purification of SPS. Purification of oligomeric high M_w enzymes from vegetable tissues is difficult as it usually requires several steps and passages through different types of column chromatography. Only recently was purification to homogeneity of SPS from leaves and seeds reported (Salerno et al., 1991; Son-

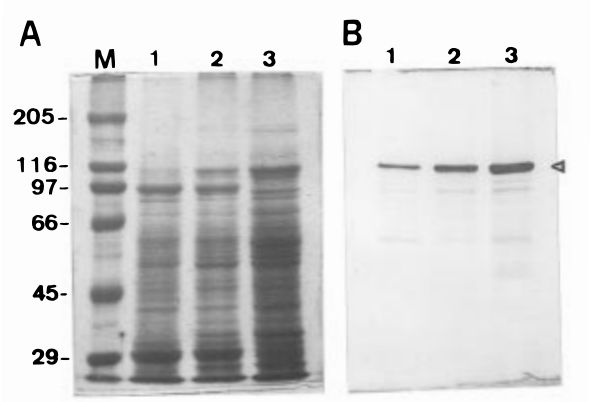

Figure 4. (A) SDS−PAGE crude extracts of bananas: lane 1, 70 days a.a.); lane 2, 110 days a.a.; and lane 3, 130 days a.a. (Coomassie staining). (B) Western blot probed with Ab SPS_3: lane 1, bananas 70 days a.a.; lane 2, 110 days a.a.; and lane 3, 130 days a.a. Molecular weight markers: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

newald et al., 1993). In fruits this is the first report of purification of SPS, which was accomplished with a reasonable yield with a reduced number of steps. The DEAE-cellulose fractionation step was critical to our procedure since it decreased the yield but, at the same time, it was an efficient way for retaining pectins and for separating SS from SPS in only one run. The SS activity in crude extracts and in 30−60% fractions was respectively 8 and 38.2 μmol/h/mL and was eliminated after DEAE step. The absolute value for yield and purification factors must be interpreted cautiously considering the dilution effect on the activity. For instance, we have observed a 5-fold increase of the SPS specific activity when either crude extracts or purified preparations of the enzymes were diluted 10 times (0.28 to 0.028 mg of protein/mL). This effect was consistently observed, and it may be due the presence of an inhibitor or to the association of the molecules in solution.

The SPS yield difference obtained for 70 day bananas (60%) as compared to 110 and 130 day bananas (around 20%) was probably due to the different composition of the tissues mostly to the state of the pectin which greatly affect protein extraction (Cordenunsi, 1989). Also, the action of the protease, mostly in the ripe tissues, cannot be ruled out since its activity is much increased in this stage, an effect that was reduced when we used protease inhibitors such as benzamidine and antipain.

General Properties of SPS. The preparation after elution from a Sepharose CL-6B column yielded a main protein peak with enzymatic activity, corresponding to a mean M_w of 440 kDa. This value is within the range 380−540 kDa found for the M_w of SPS from seeds (Salerno et al., 1991) and leaves (Kalt-Torres, 1987) but M_w values as low as 380 kDa were also found (Bruneau et al., 1991). The M_w did not change at the different physiological stages studied, suggesting that the molecular form of SPS synthesized are probably the same during the period considered. The three active bands found in native PAGE could correspond to different associations of the enzyme since all of them have the 116 kDa polypeptide, but they were not separated by the column. Different techniques may give different M_w values for SPS due to the interaction to the column matrix as already observed by Salvucci et al. (1990). In our case, the effect of dilution on activity, as discussed previously, associated with the presence of three active bands with different M_w in PAGE is an indication of the existence of different polymeric forms similarly to was also observed by Sonnenwald et al. (1993) for purified spinach SPS.

As was shown for other vegetable organs, banana SPS seems to be a tetramer of identical subunits. This was shown by the enrichment of only the 116 kDa band during purification, which occurred in the three forms of the enzyme separated by electrophoresis. Using the SPS_3 antibody it was possible to see that the 116 kDa polypeptide was present in all the bands: SPS_1, SPS_2, as well as in SPS_3 (Figure 3). The presence of the 116 kDa polypeptide increased about 2-fold as the age of the fruits advanced. This is clear in Figure 4 which shows Western blotting of the crude extracts of fruits 70, 110, and 130 days after anthesis and submitted to SDS−PAGE. These increase the amount of SPS subunit was parallel to enzymatic activity, which increased about 3-fold.

The bands observed between 50 and 60 kDa which cross-reacted with the antibody of SPS_3 are hydrolysis products of the main 116 kDa band. This was demonstrated by the fact that they did not appear clearly in the initial stages of the preparation but appeared mostly after the preparation was stored for some time and were hardly present when the tissue was ground in liquid nitrogen and rapidly mixed with the SDS buffer, before electrophoresis. Also, the appearance of these bands were greatly reduced by benzamidine and antipain, protease inhibitors.

The 71 kDa polypeptide is a co-purified protein not related to SPS since it did not react with the SPS_3 antibodies; as can be seen in Figure 3 (lane 4) that polypeptide corresponds to the band in the native gel that it devoid of SPS activity. The presence of the 116 kDa band in the lane 4, as shown by the reaction with the anti-SPS_3 serum, was caused by diffusion of the SPS_3 in the gel (Figure 1). The 71 kDa polypeptide with seems to be the subunit of the fifth band observed in the non-dissociating gel, which, as we detected later, has phosphoglucose isomerase activity (enzyme currently under study).

In previous experiments (Cordenunsi, 1989) we observed that during ripening of bananas there was an increase of SPS activity and a virtual disappearance of SS as measured in partially purified extracts (Hubbard et al., 1990). The increase of activity could be due to increased protein synthesis, "de novo" synthesis of a pre-existing enzyme, or to activation or reversal of inhibition. The present results indicate that the observed effect was probably not due to the synthesis of new enzyme forms but may be related to the increase of the synthesis of one or all of the pre-existing ones. This suggestion is supported by the fact that PAGE showed (Figure 1) in the three stages studied, the same SPS_1 to SPS_3 forms and that the SPS subunit became more evident after 110 days and could be responsible for the enhancement of total SPS activity previously to and during ripening. Any way, if not de novo at least protein synthesis seems to be partially involved in the process as shown by the increase of the 116 kDa polypeptide (Figure 4) and of the SPS_3 band in the gel (Figure 1). Thus in bananas, synthesis of SPS−protein during ripening may to be also part of a coarse control mechanism of SPS activity and sucrose formation. We also observed in a previous paper that infiltration of protein synthesis or nucleic acid synthesis inhibitors reduced

the incorporation of [^{14}C]glucose-1-phosphate into sucrose during ripening of banana slices "in vitro" (Terra et al., 1983). Studies on the effect of protein synthesis inhibition, mRNA isolation, and SPS cloning are under way.

The physiological stages of the bananas studied covered periods of starch synthesis [bananas 70 days after anthesis (a.a) had 20% starch], a pre-climacteric period (fruit 110 days a.a. when the starch content reached 25%), and a climacteric period when starch degradation was almost complete (130 days a.a., 6% starch). The difference in activity of total SPS or of the main fraction SPS_3 was more pronounced when comparing the 70 day with the 110/130 day fruits than when we compared the 110 with the 130 day bananas. This indicates that the observed changes of SPS activity and sucrose accumulation are related and that both are initiated at the end of the starch accumulation phase rather than being associated to the beginning of the climacteric (respiration) period.

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