Starch Breakdown during Banana Ripening: Sucrose Synthase and Sucrose Phosphate Synthase

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Starch contents, respiration rates, sucrose synthase (SS) activity, and sucrose phosphate synthase (SPS) activity in crude extract or in partially purified preparations were comparatively studied during fruit development and ripening in either attached or detached bananas. Data showed that while SS activity is almost abolished during ripening, SPS activity increased concomitantly to starch disappearance and sugar accumulation, confirming that it may be the enzyme involved in the process. The process is slower for attached fruits [final sucrose content 6%, SS activity 5 units (U), and SPS activity 33 U] when compared to the detached fruits (final sucrose content 12%, no SS activity, and SPS activity 50 U). During development of the fruit SPS was present but showed a very low activity, while SS activity was high and was kept constant during all of the starch synthesis phase falling during starch breakdown (climacteric) to a lower level (30 U) and then disappearing (postclimacteric).

Keywords: *Sucrose synthase; sucrose phosphate synthase; banana; ripening; starch; sucrose*

INTRODUCTION

The disappearance of starch reserve during banana ripening 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 hexoses (Arêas and Lajolo, 1981). Only 5% at the most is used for respiration (Biale and Young, 1981). In spite of the importance of this transformation for the fruit physiology, and it's eating and technological quality, not much is yet known about the mechanisms involved.

Starch–sucrose transformation during ripening of bananas involves several enzymes and more than one pathway. Amylases participate in starch hydrolysis but are probably not linked to sucrose synthesis. Garcia and Lajolo (1988) detected three α- and four β-amylase and α-1,4- and α-1,6-glucosidase activities in all stages of fruit ripening. They observed that their activities increased significantly but only at the climacteric peak when a large part of starch had already disappeared. Only β-amylase activity increased before onset of the respiration peak and parallel to starch decrease. Recently, Konishi et al. (1992) purified and partially characterized one acid α-glucosidase (AAG) and two neutral isoforms of α-glucosidase (NAG I and II) from preclimacteric banana pulp tissues. The AAG was recognized as a typical maltase, while the NAG forms were 50 times less active on maltase. The authors concluded the AAG is the enzyme responsible for the hydrolysis of maltose and also for the malto-oligosaccharides formed by the action of α- and β-amylases. Until recently, only trace amounts of maltose were found in ripening bananas.

In a previous paper (Chitarra and Lajolo, 1981), we demonstrated by electrophoresis the existence of four multiple forms of phosphorylases and that the overall phosphorylases activity increased slightly just before starch transformation began, followed by a slow reduction during climacteric. In both cases, their activities were low but still enough to account for the observed starch degradation, indicating the importance of this enzyme in the process. Infiltration of protein synthesis inhibitor in tissue slices did not reduce phosphorylase activity but slowed sucrose accumulation (Arêas and Lajolo, 1981). In a different variety, banana marmelo-(*Musa balbisiana* × *Musa accuminata*), the reduction of phosphorylase activity during the climacteric was sharp and concomitant with starch disappearance (Chitarra and Lajolo, 1981).

In a following work, Terra et al. (1983) reported that during ripening of bananas UDPG pyrophosphorylase activity remained constant and a small increment of sucrose synthase (SS) was detected; since they did not detect a significant sucrose phosphate synthase enzyme activity (SPS) during the respiratory rise, they concluded that SS was involved in sucrose synthesis in ripening bananas. These authors also observed that infiltration of [^{14}C-U]glucose-1-phosphate in thin banana slices produced incorporation of the label in sucrose and that it was 3 times faster in preclimacteric than in the climacteric period.

Beaudry et al. (1989) recently elucidated part of the systems involved in the carbon flux in both preclimacteric and climacteric bananas. In the preclimacteric fruits, glycolysis predominates over gluconeogenesis, but during the respiratory peak, the glycolytic carbon flux increases 4–5 times while the gluconeogenic flux increases 50–100 times. One day after the respiration peak, the gluconeogenic carbon flux falls while the glycolytic flux remains high. Fructose 2,6-biphosphatase does not seem to be involved in the control of observed effects; probably it is linked to the fine control of glycolysis, which is essential for sucrose production.

Recently, SPS was suggested to be the enzyme responsible for sucrose accumulation in muskmelon fruit (Hubbard et al., 1989) and bananas (Cordenunsi, 1989; Hubbard et al., 1990). The latter studies reported a decrease of SS and increase of SPS activity in crude extracts (measured in saturated conditions) during banana ripening after ethylene treatment and suggested that sucrose synthesis was mediated by SPS.

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The SPS, a key enzyme in sucrose biosynthesis in source tissues, seems to be also important in sink tissues. Besides bananas and muskmelon, other fruits are known to accumulate sucrose during ripening, i.e. mango, kiwi, and peach, and they also have shown an increment in SPS activity, linked to sucrose accumulation (Hubbard et al., 1991). In some cases, e.g. peach and strawberry fruits, the SS too has activity increased during the rise in sucrose levels, contrary to what happens in banana and other tissues that accumulate large quantities of starch. In potatoes for instance, the SS activity seems to be rather important in developing tubers, but only a negligible activity was reported in detached tubers (Geigenberger and Stitt, 1993). Detached tubers supplied with sucrose did not show this loss of activity, suggesting that sucrose might have a positive effect in SS gene expression in potatoes (Ross and Davies, 1992).

In this work, we are reporting new studies on the evolution of SS and SPS activities, during development and ripening of bananas, in crude extracts and also after partial purification of the enzymes. We are also reporting new investigations on these enzymes and on the carbohydrate changes in fruit that were left ripening on the tree as compared with fruit that were harvested green as is normally done.

MATERIALS AND METHODS

Plant Material. Unripe, fully developed (mature) bananas (*Musa cavendishii*) were obtained from the central warehouse before ethylene treatment. All of the fruits used had been stored from 1 to 2 days maximum after harvesting, which occurred when the central diameter of the fruit of the second bunch was 33−34 mm (full three-quarters, 110 days postanthesis). The hands in the bunch were detached, washed with a 1% sodium hypoclorite solution, and stored in respiration chambers at 18 °C as described previously (Terra et al., 1983). The respiration rate of the fruits, determined as CO_2 production, was measured by connecting the effluent air from each respiration jar to an infrared gas analyzer.

For the studies with developing (immature) fruits we used bunches, 30 days after flowering, which contained hands with ages varying from 3 to 30 days after anthesis and diameters of 3 and 16 cm, respectively.

For the study of ripening of the fruits attached on the tree, the whole bunch was covered by a protecting polyethylene bag. The fruits at the specific ripening stages were harvested and immediately peeled, sliced was stored in liquid nitrogen. The wound on the bunch due to harvesting of the samples was protected using a solution of copper chloride.

In each point samples for analysis were prepared by cutting the central portion of five fruits and running duplicate enzyme extraction.

Enzyme Extraction and Purification. Banana samples were homogenized for 2 min in a Polytron homogenizer, with the extraction buffer in a 1:4 tissue-to-buffer ratio. The extraction buffer contained 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% soluble PVP (MW 40 000), and recently neutralized 20 mM cysteine. The homogenate was centrifuged at 13200*g* for 15 min, and the supernatant was dialyzed against 20 mM Hepes (pH 6.5) containing 2 mM EDTA and 5 mM cysteine (buffer A) and used to measure SPS activity. Another part was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 2 mM EDTA and 5 mM cysteine (buffer B) and then used to assay for SS activity and for protein determination.

Frozen banana samples were ground in a chilled mortar and homogenized, using a 1:5 tissue-to-buffer extraction ratio, by mechanical agitation.

Experiments of mixing extracts from unripe (rich in phenolics) to ripe bananas confirmed that the same extraction buffer could be used for both stages. Moreover, addition of $MnCl_2$ or NaH_2SO_3 to the extraction media was not necessary.

The SS and SPS enzymes were partially purified with a saturated ammonium sulfate solution (30% final concentration) and left standing overnight at 4 °C to eliminate pectins. The supernatant was then precipitated with solid ammonium sulfate (50% final concentration). The precipitate obtained (13200*g*/20 min) was dissolved and dialyzed with buffer B and passed through a DEAE-cellulose column, equilibrated with the same buffer with pH 7.0. The enzymes were eluted with a 0−0.5 NaCl linear gradient in the same equilibrium buffer. Fractions containing SS and SPS activity were pooled and concentrated with a Centriprep 10 concentrator (Amicon).

Protein determination was done using either the method of Lowry et al. (1951) as modified by Petterson (1977) or the Bradford (1976) technique, using BSA as a standard.

Sucrose Synthase and Sucrose Phosphate Synthase Assays. Reaction mixtures (100 μL) to determine SS activity (measured in the sucrose synthesis direction) contained 10 μmol of Tris-HCl (pH 7.5), 1 μmol of NaF, 1 μmol of $MnCl_2$, 1 μmol of fructose, 0.5 μmol of UDPG, and 50 μL of dialyzed enzyme. Reaction mixtures were incubated at 30 °C and terminated at different times with 200 μL of 1.0 N NaOH. Remaining fructose was destroyed by placing tubes in boiling water for 10 min. Sucrose was then determined using the method of Roe (1934) as modified by Rufty et al. (1985).

In the sucrose hydrolysis direction, SS activity was measured by incubating at 30 °C the reaction mixtures (100 μL) containing 10 μmol of Hepes buffer (pH 6.5), 20 μmol of sucrose, 0.5 μmol of UDP, 10 μmol of NaF, 1.0 μmol of $MnCl_2$, and 30 μL of dialyzed enzyme for 10 min. Reaction was interrupted by heating in a boiling water bath for 1 min and the fructose content was determined enzymatically according to the method of Bernt and Bergmeyer (1974) with some modifications (Arêas and Lajolo, 1980).

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

One unit (U) of either SS or SPS activity was defined as the activity producing 1 μmol of sucrose or sucrose-6P h^{-1} (g of banana)$^{-1}$, respectively.

Carbohydrate Analysis. Samples (1 g) were extracted with 4 mL of 0.5 M NaOH in a Potter−Elvejhem homogenizer. The extract was neutralized with 4 mL of 0.5 M acetic acid and the volume completed with water. Starch, glucose, fructose, and sucrose were determined enzymatically as previously described (Arêas and Lajolo, 1980).

RESULTS AND DISCUSSION

Changes in Enzyme Activities during Ripening of Detached Bananas. In previous research Arêas and Lajolo (1981) reported the possible participation of phosphorylases in starch degradation and that transformation into sucrose occurred during ripening as demonstrated by incorporation of ^{14}C-labeled G-1-P into sucrose (Terra et al., 1983). The enzyme thought to be limiting on controlling sucrose synthesis was SS.

Recently Hubbard et al. (1990), using crude enzyme extract for measuring SS and SPS activity, suggested SPS, not SS, was the enzyme responsible. This is consistent with previous results obtained also with crude extracts of bananas (Cordenunsi, 1989). Our present data further test this suggestion in several ways. First, enzyme data obtained with crude extracts were compared with data obtained with partially purified preparations, and both showed that the activity of SS during ripening of the detached fruits fell rapidly (Figure 1A). Hydrolytic activity (not shown in the figure) was higher, starting at 380 U, and maintained a constant relation to synthetic activity (5:1). Both synthetic and hydrolytic activities dropped during ripening to very low but still detectable levels, before starch degradation started to occur, indicating a nonexistence of relation between the effects. At the same time SPS

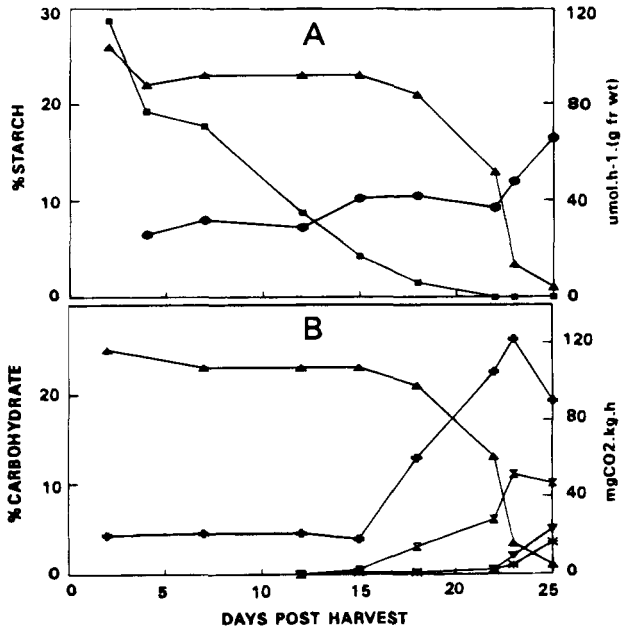

Figure 1. (A) Concentration of starch (▲), and SS (■) (sucrose synthesis direction), and SPS (●) activities in ripening detached banana fruit. Fruits were washed with 1% sodium hypoclorite solution and stored in respiration chambers at 18 °C. (B) Concentrations of starch (▲), sucrose (hour glass), glucose (▼), fructose (*), and CO_2 (+) of the same fruits. Data points are the mean of two replicates of samples prepared with the central portion of five fruits.

activity, which was very low in preclimacteric bananas (26 U) started increasing before the rapid phase of starch degradation began, rising 2 to 3 fold (66 U).

Figure 1B relates the starch disappearance and sugar synthesis to the respiration rate. It can be seen that sucrose accumulation is parallel to the increase of SPS activity as the starch content falls, after the 15th day. On the other hand, the SS activity fell sharply, and at the 15th day, when sucrose concentration started to increase, it was only one-sixth of the initial, showing that SPS is linked to sucrose synthesis during the banana ripening process.

Due to the complex composition and different contents of compounds such as organic acids, phenolics, and pectin and the presence of hydrolytic enzymes and proteinaceous inhibitors and because of the changes that occur during ripening and during enzyme extraction and activity measurement, data on changes of enzyme activity should be confirmed in different ways to avoid erroneous interpretation (Rhodes, 1977).

The results obtained after partial purification of the enzymes in the three stages studied confirmed what was shown for crude extracts. Figure 2 is self-explanatory, showing the existence of both enzymes in unripe bananas (21% starch), the different value of the SS/SPS ratio as starch drops to 16.3 (corresponding to the climacteric), and finally the disappearance of SS but a still high SPS activity in totally yellow, ripened, postclimacteric bananas. It is clear from the figure that while SPS is still being synthesized during ripening, SS is not.

Garcia and Lajolo (1988) reported that during ripening, starch disappearance started at the center portion of the fruit, radiating afterward toward the periphery. In this experiment we analyzed separately either the center or the external portion of the fruit for SS and SPS activity but, contrarily to our expectations, we could not detect any difference.

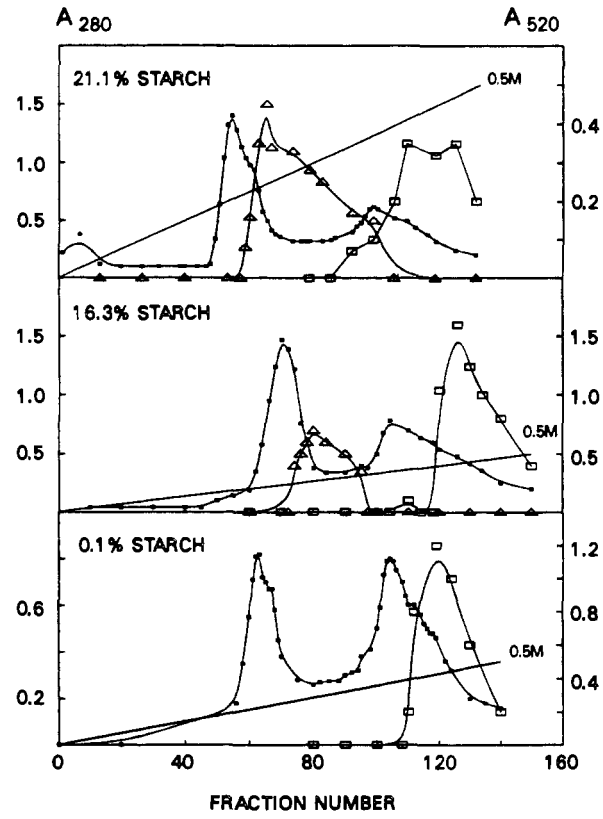

Figure 2. Elution profiles on DEAE-cellulose de extracts of preclimateric (21% starch), climacteric (16% starch), and ripened (0.1% starch) bananas. The fractions (30–40% ammonium sulfate) were applied to the columns and eluted with an NaCl gradient (0–0.5 M). SPS (□) and SS (△) activities were measured by the Roe method (A_{520}). Points (■) represent protein contents (A_{280}).

Effect of Time after Harvesting on SS and SPS Activity. It was observed in several experiments, conducted at different times, that while SPS activity in green bananas was practically constant and comparable to results obtained by other authors (Hubbard et al., 1990), the SS initial levels for unripe bananas varied considerably, even in fruits with similar starch contents and thus presumably in similar phases of the climacteric.

After accumulating results from several experiments, we realized that there was a variable that had not been considered before: time after harvest. Figure 3 illustrates the data obtained when time was considered.

Actually, there was not any correlation between SS activity and ripening stage as indicated by starch content, but between SS and time: the activity was reduced proportionally to the time after harvest. These data helped us to explain the different initial levels of SS observed in several experiments, as reported before and previously by Terra et al. (1983). A possible explanation is that SS synthesis in attached bananas is maintained by metabolites or hormones transported from the plant to the fruits, a process that is interrupted after harvesting. Since SS is known to participate in the process of starch synthesis from sucrose (Geingenberger and Stitt, 1993; Wang et al., 1993), it should be present and active during the starch formation and accumulation. As soon as the residual amounts of these compounds are metabolized, the synthesis of the enzyme stops or is greatly reduced. Vendrell et al. (1969) observed that when banana slices were infiltrated with a solution of growth regulators such as auxins, ripening

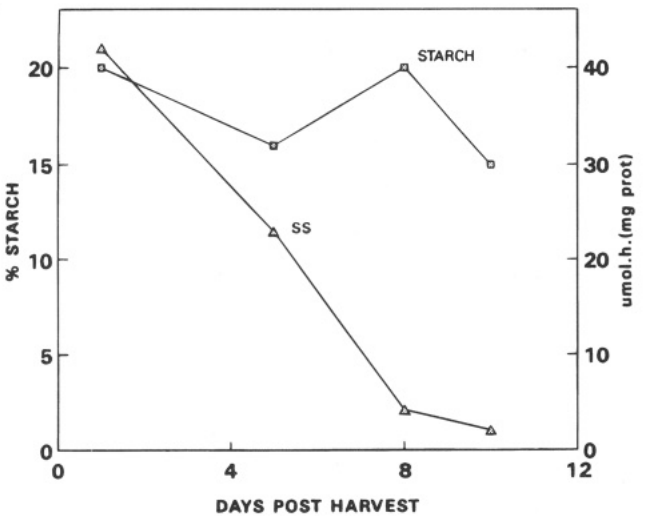

Figure 3. Specific SS activity (synthesis direction) (▲) of detached bananas of the same bunch but in different ripening stages (starch concentration) (■). Data points are the mean of two replicates of samples prepared with the central portion of five fruits.

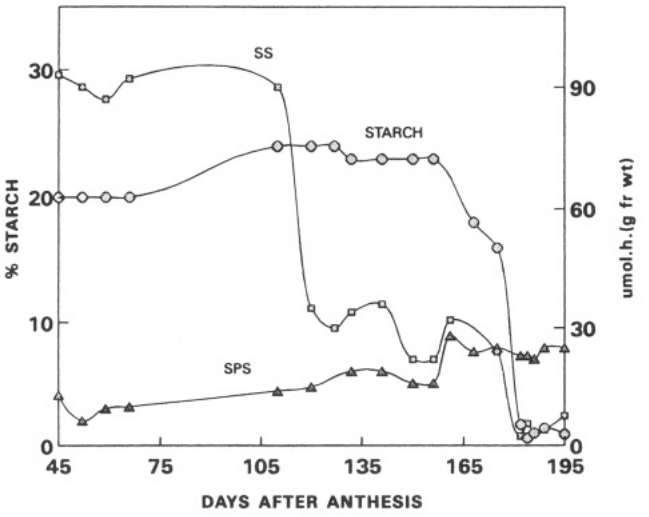

Figure 4. Starch concentration (●) and SS (■) and SPS (▲) activities in attached bananas as time function after anthesis. The whole bunch was covered by a polyethylene bag, and the fruits at specific ripening stages were harvested and immediately peeled, sliced, and stored in liquid nitrogen. Data points are the mean of two replicates of samples prepared with the central portion of five fruits.

was retarded. Sucrose is also known to participate in modulation of the SS gene in *Vicia faba* (Heim et al., 1993) and maize (Koch et al., 1992). In tomato tissue SS activity has a positive correlation with the increase of starch contents (Wang et al., 1993).

Changes of Enzyme Activities during Ripening of Attached Banana. To further explore these facts, we studied, in bananas left attached to the plant, the changes of SS and SPS activity and starch, sucrose, and hexose evolution, not only during ripening but also during fruit development when starch synthesis was still active. Figure 4 shows the data obtained from the 45th day after anthesis and on, but even before, in very small fingers (4 cm long, 3 days after anthesis, 0.5% starch), SS activity was already high (90 U). When the fruit reached its full length (16 cm, about 20 days after anthesis, 0.5% starch), it was about 120 U. SPS was also present in these initial stages, but with a very low activity. As shown in Figure 5, SS activity reached 120

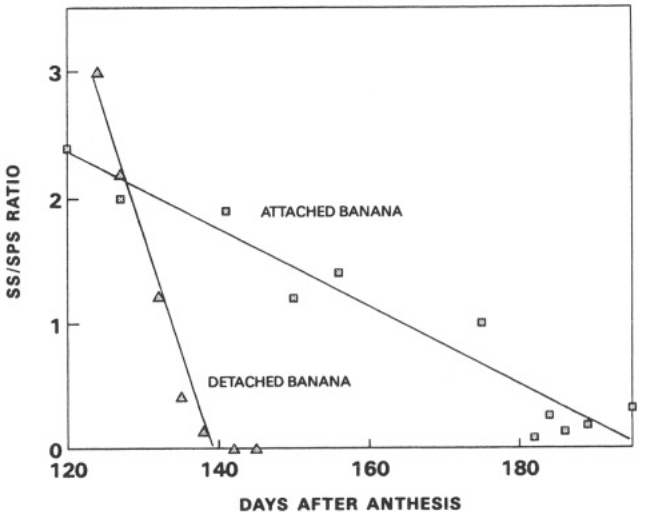

Figure 5. SS/SPS ratio in attached (■) and detached (▲) bananas.

U 45 days after anthesis, a value that remained constant until the 110th day, when starch accumulation stopped and the climacteric period, with starch degradation and sucrose accumulation, began. This is the time fruits are normally harvested. It is interesting to observe that the reduction of SS activity goes by steps: it is kept constant at a high level (120 U) during the entire starch synthesis phase and then drops to a lower but still significant level (30 U), which is maintained during the starch degradation period (climacteric phase); when finally starch has virtually disappeared and sucrose has accumulated, it is only 2 U. It is possible that this is related to the existence of different controls of enzyme synthesis and activity or to the presence of different forms of the enzyme. SPS behavior was different: its activity increased very slowly until the beginning of starch disappearance, when it went to a higher level at the same time that sucrose accumulated.

Comparing the data of Figure 1 with those of Figure 4, we can observe that when the fruits are left ripening on the tree, the overall behavior is similar to harvested fruits but proceeds at a different speed. In attached fruits the speeds of the changes of enzyme activity and carbohydrates content are slower, as is clearly shown in Figure 5, which illustrates the different changes of the ratio of SS/SPS in attached versus detached fruits. Also, SPS activity is lower, reaching only 50% of the value shown for detached fruits which may explain the reduced concentration of sucrose found (12% vs 6%) see table. These results seem to confirm the hypothesis we advanced before on the presence of metabolites or hormones being transported to the fruits, controlling the level of both enzymes, and to open new aspects for research on the composition of ripening bananas.

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LITERATURE CITED

Arêas, J. A. G.; Lajolo, F. M. Specific enzymic determination of starch, glucose, fructose, and sucrose in preclimacteric and climacteric bananas. *An. Farm. Quim. Sao Paulo* **1980**, *20* (1/2), 307–318.

Arêas, J. A. G.; Lajolo, F. M. Starch transformation during banana ripening: I. the phosphorylase and phosphatase behavior in *Musa acuminata*. *J. Food Biochem.* **1981**, *5*, 19–37.

Beaudry, R. M.; Severson, R. F.; Black, C. C.; Kays, S. J. Banana ripening: implications of changes in glycolytic intermediate concentrations, glycolytic and gluconeogenic carbon flux, and fructose 2,6-Biphosphate concentration. *Plant Physiol.* **1989**, *91*, 1436–1444.

Bernt, F. S.; Bergmeyer, H. U. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 3.

Biale, J. B.; Young, R. E. Respiration and ripening in fruit-retrospect and propect. In *Recent Advances in the Biochemistry of Fruits and Vegetables*; Friend, J., Rhodes, M. J. C., Eds.; Academic Press: New York, 1981.

Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Chitarra, A. B.; Lajolo, F. M. Phosphorylase, phosphatase, alpha amylase activity and starch breakdown during ripening of *marmelo* banana [(*musa acuminata colla*) x (*musa balbisiana colla*) ABB Group] whole fruit and thin slices. *J. Am. Soc. Hortic. Sci.* **1981**, *106* (5), 579–584.

Cordenunsi, B. R. Sucrose Synthesis During Banana Ripening: Sucrose Synthase and Sucrose Phosphate Synthase Involvement. Ph.D. Thesis, Universidade de São Paulo, 1989.

Garcia, E.; Lajolo, F. M. Starch transformation during banana ripening: the amylase and glucosidase behavior. *J. Food Sci.* **1988**, *53* (4), 1181–1186.

Geingenberger, P.; Stitt, M. Sucrose synthase catalyses a readly reversible reaction in vivo in developing potato tubers and other plant tissues. *Planta* **1993**, *189*, 329–339.

Heim, H.; Weber, H.; Baumlein, H.; Wobus, U. A sucrose-synthase gene of *Vicia faba* L.: Expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. *Planta* **1993**, *191*, 394–401.

Hubbard, N. L.; Huber, S. C.; Pharr, D. M. Sucrose phosphate synthase and acid invertase as determinants of sucrose concentration in developing muskmelon (*Cucumis Melo L.*) fruit. *Plant Physiol.* **1989**, *91*, 1527–1534.

Hubbard, N. L.; Pharr, D. M.; Huber, S. C. Role of sucrose phosphate synthsase in sucrose biosynthesis in ripening bananas and its relationship to the respiratory climacteric. *Plant Physiol.* **1990**, *94*, 201–208.

Hubbard, N. L.; Pharr, D. M.; Huber, S. C. Sucrose phosphate synthase and other sucrose metabolizing enzymes in fruits of various species. *Physiol. Plant.* **1991**, *82*, 191–196.

Koch, K. E.; Noite, K. D.; Duke, E. R.; McCarty, D. R.; Avigne, T. W. Sugar levels modulate differential expression of maize sucrose synthase genes. *Plant Cell* **1992**, *4*, 59–69.

Konishi, Y.; Kitazato, S.; Nakatani, N. Partial purification and characterization of acid and neutral α-glucosidases from preclimacteric banana pulp tissues. *Biosci., Biotechnol. Biochem.* **1992**, *56* (12), 2046–2051.

Lowry, O. H.; Rosenbrough, N. J.; Farr, A. C.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generaly applicable. *Anal. Biochem.* **1977**, *83*, 346–356.

Rhodes, M. J. C. The extraction and purification of enzymes from plant tissues. In *Regulation of enzyme synthesis and activity in higher plants*; Smith, H., Ed.; Academic: London, 1977; pp 245–267.

Roe, J. H. A colorimetric method for the determination of sucrose in blood and urine. *J. Biol. Chem.* **1934**, *107*, 15–22.

Ross, H. A.; Davies, H. V. Sucrose metabolism in tubers of potato (*Solanum tuberosum L.*). Effects of sink removal and sucrose flux on sucrose-degrading enzymes. *Plant Physiol.* **1992**, *98*, 287–293.

Rufty, T. W.; Kerr, P. S.; Huber, S. C. Caracterization of diurnal changes in activities of enzymes involved in sucrose biosynthesis. *Plant Physiol.* **1985**, *73*, 428–433.

Terra, N. N.; Garcia, E.; Lajolo, F. M. Starch sugar transformation during banana ripening: the behaviour of UDP-glucose pyrophosphorylase, sucrose synthetase and invertase. *J. Food Sci.* **1983**, *48*, 1097–1100.

Vendrell, M. Reversion of senescence: effects of 2,4-dichlorophenoxyacetic acid and indoleacetic acid on respiration, ethylene production, and ripening of banana fruit slices. *Aust. J. Biol. Sci.* **1969**, *22*, 601–10.

Wang, F.; Sanz, A.; Brenner, M. L.; Smith, A. Sucrose synthase, starch accumulation, and tomato sink strength. *Plant Physiol.* **1993**, *101*, 321–327.

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