Carbohydrate Content and Metabolism As Related to Maturity and Chilling Sensitivity of Cv. Fortune Mandarins

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Fruits of cv. Fortune mandarin were periodically harvested throughout the ripening period to evaluate changes in carbohydrate content and metabolism in flavedo tissue and to determine the potential role of carbohydrates in the tolerance of citrus fruit to chilling injury (CI). Sucrose showed little change in the flavedo during the season, but fructose and glucose increased, in nearly equal amounts, throughout the fall and winter, reaching a maximum in January. Starch levels were less abundant than soluble carbohydrates and rose continuously until March. Sucrose phosphate synthase (SPS; EC 4.1.14) activity decreased from December throughout ripening. Changes in sucrose synthase (SS; EC 2.4.1.13) and acid and alkaline invertase (Inv; EC 3.2.1.26) activities correlated with changes in the reducing sugars, but acid invertase was less active than the other sucrose-metabolizing enzymes. Carbohydrate changes in the flavedo of Fortune mandarins with fruit maturity appear not to be related to the chilling tolerance of fruits during cold storage.

Keywords: *Acid and alkaline invertase; carbohydrates; citrus; chilling injury; maturation; starch; sucrose phosphate synthase; sucrose synthase*

INTRODUCTION

Fortune mandarin fruit (*Citrus reticulata* Blanco), a hybrid of Clementine mandarin × Dancy tangerine, is a late ripening cultivar highly appreciated for its good quality. It can be marketed when other mandarins are no longer available, but fruit marketing is difficult because of its accute sensitivity to chilling, which induces pitting and necrosis in the flavedo (the pigmented portion of the peel) along the fruit. The susceptibility of this cultivar to chilling injury (CI) changes during the season, but the mechanisms involved in its acclimation to cold stress are still unclear. Numerous studies provide evidence linking carbohydrate content and metabolism of plants to environmental stresses, including chilling (King et al., 1988; Tognetti et al., 1990).

Changes in sucrose, glucose, and fructose levels during maturation and ripening of citrus fruit have been described in different cultivars. Tadeo et al. (1987) found an accumulation of sugars during ripening in different Clementine mandarins and orange cultivars, which was mainly due to an increase of sucrose in the juice and of reducing sugars in the peel (albedo plus flavedo). Purvis and Grierson (1982) observed an increase in reducing sugar content in mid-season in grapefruit flavedo, when the mean field temperatures dropped to 10 °C. Changes in the starch content in the flavedo of citrus fruits in relation to the soluble carbohydrates content and metabolism have not previously been studied. However, it is known that the starch content in the peel (flavedo plus albedo) of Satsuma mandarin was very low as compared to the soluble carbohydrate levels (Kawase and Hira, 1983). In other fruits, such as tomato, it has been suggested that starch accumulation may function as a carbohydrate reservoir in the developing fruit and may contribute to the soluble hexoses level in the mature fruit (Dinar and Stevens, 1981).

Little is known about changes in carbohydrate metabolism in citrus flavedo during growth and development. Purvis and Rice (1983) reported that reducing sugar levels in grapefruit flavedo paralleled acid invertase activity (EC 3.2.1.96), whereas sucrose contents were inversely related to the activity of this enzyme. To our knowledge, no other published information relates carbohydrate changes in the flavedo of citrus during fruit ripening with the mechanism underlying carbohydrate accumulation. Besides invertase, sucrose synthase (SS; EC 2.4.1.13) and sucrose phosphatase synthase (SPS; EC 2.4.1.14) are two key enzymes in sucrose metabolism. Hubbard et al. (1989) reported that sucrose accumulation is determined by the balance between sucrose synthesis (SPS activity) and degradation (invertase and SS activities). SPS is involved in sucrose synthesis, whereas SS is thought to function primarily in a sucrose-degrading direction (Cano-Medrano and Darnell, 1997). Extensive data support the view that the involvement of SS in sink activity is predominantly through sucrose metabolism in sink cells, for the production of either respiratory substrates or UDPG for synthesis of complex carbohydrates (Sung et al., 1988). Considerable work has been done on the role of SPS in regulation of sucrose synthesis in source plant tissues. However, SPS may also be important in sucrose synthesis in many sucrose-storing sink tissues. The SPS activity remained relatively constant during tomato fruit ripening (Islam et al., 1996), but SPS was associ-

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ated with the synthesis of sucrose during maturation and ripening in muskmelon fruit (Lingle and Dunlap, 1987) and buttercup squash (Irving et al., 1997). Recently, three partial cDNA clones encoding SPS isoforms from a citrus fruit have been isolated. The levels of expression of these cDNAs were compared in Satsuma mandarin fruits harvested at two different maturity stages, and it was shown that the accumulation of one of those cDNAs (CitSPS1) was higher in the more mature fruits (Komatsu et al., 1996). However, Lowell et al. (1989) reported that the activity of SPS in the albedo, phloem-free juice sacs and transport tissues of the fruit was lower in the mature stage of grapefruit. Those authors also found that SS was particularly active in extracts of sink tissue samples of stage I fruit, when cell division and respiration rates were maximal.

Several studies indicate that carbohydrate metabolism is involved in protecting plants against cold stress, although controversial results have been found. Sucrose was more effective than glucose or fructose in reducing chilling susceptibility in tomato seedlings (King et al., 1988). In grapefruit flavedo, however, reducing sugars, not sucrose, correlated with decreased chilling sensitivity of the fruits during the season (Purvis and Grierson, 1982). Higher levels of sucrose as well as of levels of enzyme activity of SS and SPS were found as the hardiness of different wheat cultivars increased (Tognetti et al., 1990). In spinach, sucrose content and SPS activity were also significantly increased by low-temperature treatment, whereas SS and invertases were not (Guy et al., 1992). In other plants, SPS was reduced under cool conditions (Rufty et al., 1985; Khayat and Zieslin, 1987).

The aim of this study was to investigate changes in starch and soluble carbohydrates contents in the flavedo of Fortune mandarin fruits at various maturity stages in relation to SS, acid and alkaline invertase, and SPS activities and to examine if seasonal changes in the susceptibility to CI of this cultivar could be attributed to differences in carbohydrate accumulation and metabolism.

MATERIALS AND METHODS

Plant Material. Fruits of Fortune mandarin (*Citrus reticulata* Blanco) were harvested at random from adult trees growing in a commercial orchard at Sagunto (Valencia, Spain) during the seasons 1992/1993, 1993/1994, and 1996/1997. Fruits were periodically hand-harvested from November to May and immediately delivered to the laboratory. For each harvest time, three replicates of 10 fruits were selected to evaluate changes in fruit color and size during the season. Thereafter, the flavedo tissue was separated from the whole fruit, cut into small pieces, and frozen in liquid nitrogen for later sugar and starch content analysis and for enzyme assays. The internal maturity index was determined in the same fruits. For CI damage estimation, three additional replicates of 20 fruits were used.

CI Index. The CI index was estimated after 21 days of holding the fruits at 2 °C. Brown pitlike depressions of the fruit are the main CI symptoms in Fortune mandarins. CI was rated visually on a scale from 0 to 3, and a CI index was determined by summing the product of the number of fruits in each category multiplied by the score of each category and then dividing the total by the number of fruits examined (Lafuente et al., 1997).

Fruit Color and Surface Area. Fruit color and surface area were determined immediately after the fruits were harvested. Peel color was measured using a Hunter Lab Meter at four locations around the equatorial plane of the fruit and the hue angle ($h°$) determined. Hue angle: 0° = red-purple, 90° = yellow, 180° = bluish green, and 270° = blue.

Fruit diameter and height were measured and fruit surface area was determined according to Turrell (1946).

Chemicals and Reagents. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except amyloglucosidase, which was obtained from Boehringer Mannheim Co. (Indianapolis, IN).

Maturity Index. Fruit juice was extracted using an electric rotary juicer and filtered through cheesecloth. Soluble solids (°Brix) content was determined with an Atago/X-1000 refractometer. Acids content was titrated with 0.1 N NaOH using phenolphthalein as indicator and expressed as grams of anhydrous citric acid in 100 mL of juice. The maturity index was calculated by dividing the °Brix of the extracted juice by its acids content.

Sugar Analysis. Flavedo sugars were extracted according to the method of Purvis et al. (1979). One gram of frozen flavedo was extracted three times with 10 mL of 80% boiling ethanol using a Polytron. The ethanol extracts were combined and filtered through a glass fiber filter pad. Two milliliters of 2.1% raffinose was added as an internal standard, the alcoholic solution was centrifuged, and the resulting supernatant vacuum evaporated. The residue was dissolved in water (5 mL) and 1 mL of the resulting aqueous solution purified by passing through a C-18 Elud Bond cartridge from Varian (Harbor City, CA). The purified solution was filtered through an HV-4 filter (pore size = 0.45 μm) from Millipore Ibérica, S.A. (Barcelona, Spain) for HPLC analysis. Sugars were eluted through a 300 × 7.8 mm Phenomenex (Phenomenex, Torrance, CA) column packed with a Rezex sulfonated polystyrene resin using water as the mobile phase at a flow rate of 0.6 mL min^{-1} in 20 min at 85 °C. Sugars were detected with a Waters 410 refractive index detector (Waters, Franklin, MA) and quantified by peak area comparison using raffinose as the internal standard and standard curves for sucrose, glucose and fructose.

Starch Analysis. Starch was determined from the insoluble residue obtained after ethanol extraction according to the method previously described by Lafta and Lorenzen (1995). About 60 mg of dry residue was obtained from 1 g of freshly weighed flavedo. The dried residue (20 mg) was rehydrated in 1 mL of water and heated for 1 h at 90 °C. The gelatinized starch sample was incubated at 40 °C for 48 h with 1 mL of amyloglucosidase solution (10 units mL^{-1}, 20 mM NaF, 100 mM acetate buffer, pH 4.5), and the glucose released was determined colorimetrically at 490 nm in a glucose oxidase-coupled reaction. The reagent contained 7 units mL^{-1} glucose oxidase, 0.5 unit mL^{-1} peroxidase, and 44 mM *p*-hydroxybenzoic acid in 100 mM phosphate buffer (pH 7.0).

Enzyme Extraction and Assays. Enzymes were extracted from 1 g of fresh weight of frozen flavedo by grinding the tissue in a chilled mortar with 5 mL of 50 mM MOPS/NaOH (pH 7.5) buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 15000*g* for 30 min and the supernatant desalted in a Sephadex G-25 (1 × 5 cm) column from Amersham Pharmacia Biotech AB (Uppsala, Sweden) equilibrated with extraction buffer minus Triton X-100 (Guy et al., 1992). The activities of SPS, SS, and acid and alkaline invertase were determined from the same extract. SPS was assayed with limiting substrates plus P_i (limiting assay) or with saturating substrates (V_{max} assay). The activities of SPS were determined according to the method of Guy et al. (1992) except that quantification of the sugars formation was measured using the phenol−sulfuric method (Dubois et al., 1956) instead of that of anthrone. Acid invertase was assayed for 5−20 min according to the procedure of Purvis and Rice (1983). The reaction mixture contained 100 mM sucrose in 80 mM acetate buffer (pH 4.7). SS, in the degradative direction, and alkaline invertase were also assayed for 5−20 min. For alkaline invertase, the reaction mixture contained 50 mM sucrose in 50 mM MOPS/NaOH (pH 7.5) buffer. The same reaction mixture was used for SS but contained 5 mM UDP. The glucose and fructose produced were determined according to the Nelson assay (Nelson, 1944).

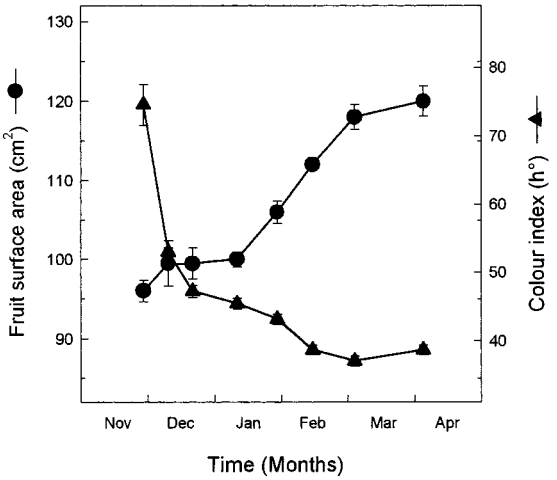

Figure 1. Seasonal changes in fruit size (●) and color (▲) of Fortune mandarin fruits. The data are from fruit harvested during the season 1996/1997. Values are the mean of three replicate samples containing 10 fruits ± SE.

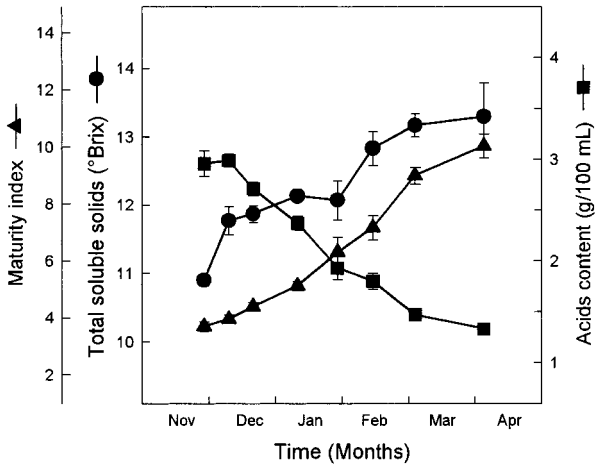

Figure 2. Seasonal changes in soluble solids (●), acids content (■), and maturity index (▲) of Fortune mandarin fruits. The data are from fruit harvested during the season 1996/1997. Values are the mean of three replicate samples containing 10 fruits ± SE.

The soluble protein concentration in the extracts was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as the standard.

RESULTS AND DISCUSSION

Fruit Growth, Color, and Maturity Index. Figure 1 shows the changes in fruit color and size, and Figure 2 shows the changes in in total soluble solids, acids content of the juice, and maturity index of Fortune mandarins as references of the external and internal degree of fruit development and ripening, respectively. Fruit surface area increased continuously with fruit ripening from November till March. The pattern of changes in fruit color during the 1996/1997 season agree with that found in previous seasons (Lafuente et al., 1997). Fruit color, expressed as *h*°, changed very slowly from the beginning of December until April (Figure 1). The most adequate color index for commercialization was reached in January (Figure 1), whereas the commercial maturity index was reached in mid-February, when the fruit presented a maturity index of nearly 8 (Figure 2). The total soluble solids content increased

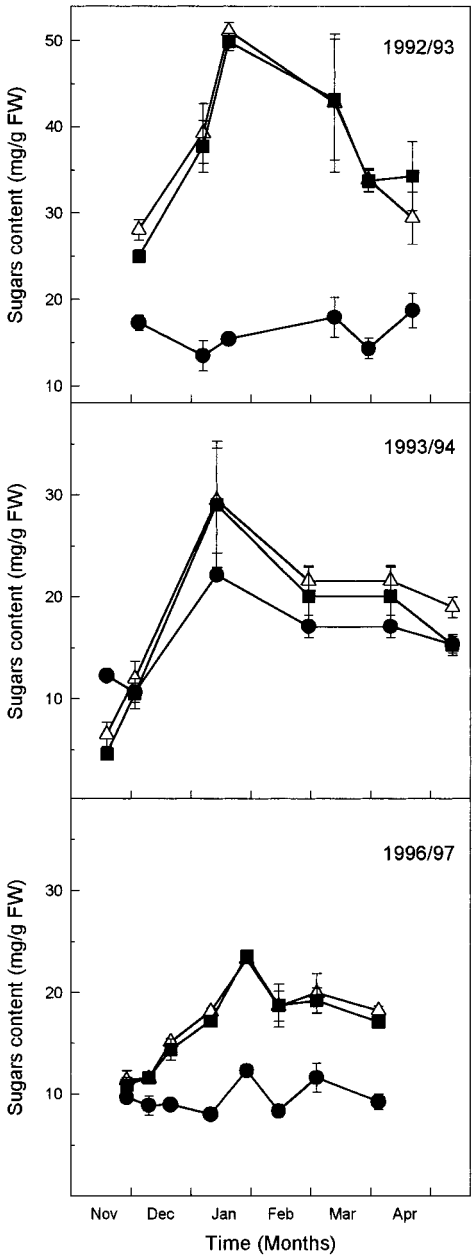

Figure 3. Seasonal changes in sucrose (●), glucose (△), and fructose (■) contents in the flavedo of Fortune mandarins. The data are from fruit harvested during the seasons 1992/1993, 1993/1994, and 1996/1997. Values are the mean of three replicate samples containing 10 fruits ± SE.

during maturation, whereas the acidity decreased, probably due to the use of the acids as respiratory substrates and the dilution of the remaining acids due to the increase in size and water content of the fruits.

Changes in Chilling Sensitivity and Carbohydrate Content in the Flavedo. Glucose and fructose levels increased in the flavedo of Fortune mandarins throughout the fall and winter. The maximum content corresponded to the mid-season fruit harvested in January, which was ~4−5 times that of the less mature fruits harvested at the end of November (Figure 3). This pattern of changes was consistent throughout the three seasons studied. Sucrose only increased with maturity during the season 1993/1994, but its increase was lower than that of reducing sugars. Thus, the sucrose content of this cultivar was, in general, lower than that of

Table 1. Seasonal Changes in Chilling Susceptibility of Fortune Mandarin

season 1992/1993		season 1993/1994		season 1996/1997	
harvest date	CI index[a]	harvest date	CI index[a]	harvest date	CI index[a]
Dec 4	1.21 ± 0.21	Dec 2	1.07± 0.15	Dec 9	0.53 ± 0.11
Jan 5	2.05 ± 0.23	Jan 13	1.67 ± 0.17	Jan 27	0.40 ± 0.09
Feb 20	1.73 ± 0.09	Feb 28	1.42 ± 0.24	March 3	0.43 ± 0.05
March 29	0.71 ± 0.02	April 9	0.52 ± 0.06	April 2	0.27 ± 0.12

[a] CI index was determined in fruits stored for 21 days at 2 °C. Values are the mean of three replicate samples containing 20 fruits ± SE.

glucose and fructose as occurred in the peel of other citrus cultivars (Purvis and Grierson, 1982; Tadeo et al., 1987).

Purvis and Grierson (1982) reported that the reducing sugars increased in the peel of grapefruits when the mean low weekly temperatures dropped to 10 °C and suggested that the reducing sugar content in the flavedo correlated with decreased chilling sensitivity of the fruits. Reducing sugars also reached a maximum in the flavedo of Fortune mandarins at mid-season, probably as a consequence of the decline in field temperatures occurring during the coolest months of the growing period in the citrus area of Valencia. The influence of other environmental or growth factors on carbohydrate levels in Fortune mandarins cannot be ruled out because those levels were different among the three seasons studied. Our results differ, however, from those obtained in grapefruit, because sugar accumulation during the maturity of Fortune mandarins did not precede resistance to CI. A careful comparison of the carbohydrate changes (Figure 3) and the CI susceptibility determined periodically during the 1992/1993 and 1993/1994 seasons (Table 1) indicates that fruits containing higher glucose and fructose contents were, in general, the most susceptible to cold stress during storage. In these seasons, fruits harvested in the coolest months (January and February) were the most susceptible to CI. Those fruits presented a CI index >1.4 when they were stored for 21 days at 2 °C, whereas the CI index of the fruits harvested earlier or later in the season was lower. The chilling sensitivity of fruits from the 1996/1997 season was considerably lower than that of fruits harvested during the previous seasons, and the differences in the chilling susceptibility in the fruits harvested during the season were not so noticeable. The pattern of changes in the reducing sugars was, however, similar to that found in the other seasons (Figure 3). It is also important to point out that the levels of reducing sugars found during the 1996/1997 season were similar to those of the 1993/1994 season and even lower than those of the 1992/1993 season. King et al. (1988) showed that tomato seedlings in which chilling started at different times during the light/dark cycle were most chilling sensitive at the end of the dark period and found that the increase in chilling sensitivity thoughout the course of the dark period could be due to carbohydrate depletion and that light acts by restoring the levels of carbohydrate and starch in the tissue. Our results, as well as those of Purvis and Grierson (1982), may suggest that the increase in reducing sugars content in the peel of the fruits during the winter could be related to an osmotic hardiness process of citrus fruits still attached to the trees, to cope with environmental stress. The increase in reducing sugars in Fortune mandarins was not sufficient, however, to protect the fruits against CI after storage. Carbohydrate changes in Fortune mandarins could also be related to the osmotic adjustment occurring during development and maturation of the fruit. By cleaving sucrose into two hexoses moieties, the osmotic potential would be doubled, thus assisting in cell expansion. During the period studied, the size of the fruit still increased (Figure 1). Sala et al. (1992) showed that accelerating peel color changes during maturity of Navelina orange fruits using different nutritive solutions also accelerated the sugar increase during fruit ripening, and all of the fruits were from cuttings grown in a greenhouse under the same temperature conditions.

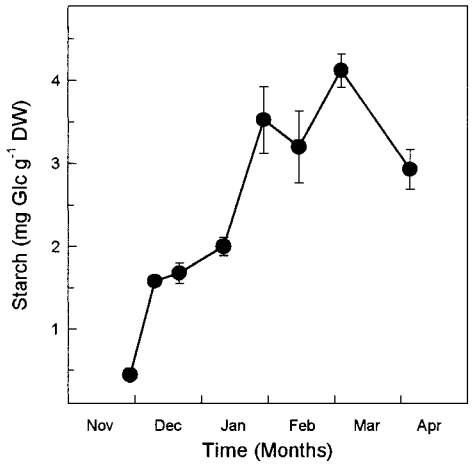

Figure 4. Seasonal changes in starch content in the flavedo of Fortune mandarins. The data are from fruit harvested during the season 1996/1997. Values are the mean of three replicate samples containing 10 fruits ± SE.

In many fruits the breakdown of starch to glucose, fructose, or sucrose is a characteristic ripening event. In the present work, we have shown that starch content in the flavedo of Fortune mandarins is less abundant than that of soluble carbohydrates and that it increases with fruit maturity. The starch content in the flavedo may differ from one season to the other (data not shown), but it always increased during the season (Figure 4). Its pattern of changes was similar to that of the reducing sugars, but starch did not decline after mid-January. Yelenosky and Guy (1977) found that in the leaves and stems of Valencia oranges, the reducing sugars increased with cold temperatures but that there was little starch hydrolysis above 0 °C. Their results suggested that relatively low starch−sugar conversion restricts maximum cold hardening in citrus at low but nonfreezing temperatures. A comparison between the changes in chilling susceptibility of Fortune mandarin fruits and its flavedo starch content during the season indicates that starch appears not to play a physiological role in defending the fruits of this cultivar against chilling when they are stored at low temperatures. Light-induced increases in the starch of tomato seedlings were, however, concomitant with its beneficial effect in reducing CI (King et al., 1987).

Enzymes of Sugar Metabolism. The activity of acid invertase increased concomitantly with reducing sugars levels in Fortune mandarins flavedo tissue throughout the fall and winter of the 1996/1997 season (Figures 5A and 3). This result agrees with that in the flavedo of grapefruit reported by Purvis and Rice (1983). In the present work, we have further shown that other sucrolytic enzymes rose in the flavedo of the sink fruit

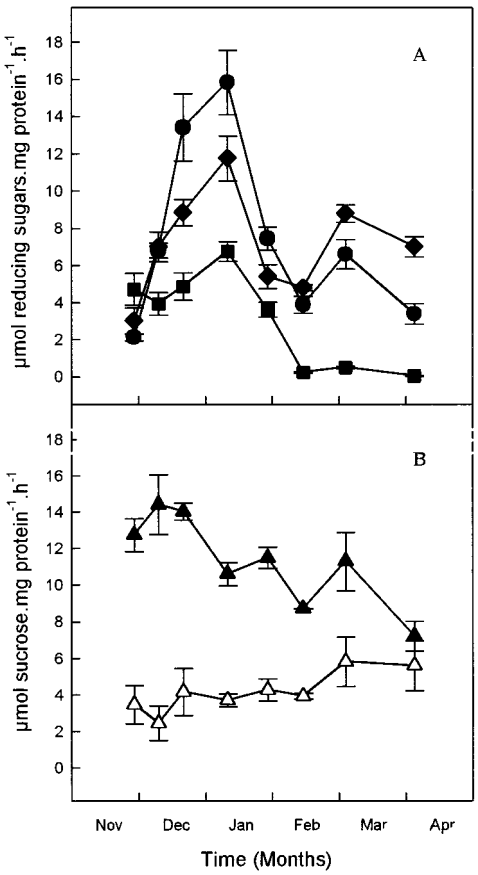

Figure 5. Extractable activities of acid (■) and alkaline (◆) invertase and sucrose synthase (SS) (●), all as production of reducing sugars, and of sucrose phosphate synthase (SPS), as production of sucrose, all on a fresh mass basis, in the flavedo of Fortune fruit during the growing period. SPS enzyme activity was assayed with V_{max} (▲) and limiting-substrate conditions (△) as described under Materials and Methods. The data are from fruit harvested during the season 1996/1997. Values are the mean of three replicate samples containing 10 fruits ± SE.

following a similar pattern of changes to that of acid invertase (Figure 5A). In addition, the data presented here demonstrate that the SS and alkaline invertase activities were greater than the acid invertase activity in the flavedo extract and may, therefore, play a more important role than acid invertase in sucrose cleavage contributing to the accumulation of glucose and fructose. Acid invertase was, however, the primary enzyme responsible for sucrose catabolism in the developing *Citrus* sink leaf (Schaffer et al., 1987). A close association has been found between this enzyme and the rate of cell division and expansion in other plant tissues, including the albedo of grapefruit (Lowell et al., 1989). In this tissue, acid invertase activity was considerably higher than SS or alkaline invertase activity in stage I of fruit development when cell division and expansion were maximal. Reducing sugars showed little change in the flavedo of Fortune mandarins after mid-January 1997 (Figure 3), when sucrose-degrading enzymes sharply declined (Figure 5A). It is important to point out that other factors such as reduction in the utilization of hexoses at low temperature and nonenzymic cleavage of sucrose at low pH (Echevarria and Burns, 1989) may affect hexose accumulation.

In Fortune mandarin sucrose was not inversely related to reducing sugars as occurred in grapefruit (Purvis and Rice, 1983). Sucrose is the main form of translocated sugar in most plants. Purvis and Yelenosky (1983) showed in grapefruit that sucrose does not appear to interchange between different tissues of the fruit at low temperature but instead is translocated into the fruit from other parts of the plant and that defoliation of branches inhibited the accumulation of sucrose in the flavedo tissue during hardening but had little effect on the level of reducing sugars. Koch (1984) demostrated that peel (albedo plus flavedo) accumulated the greatest portion of [^{14}C]photosynthates (~35%). The fact that sucrose levels did not decrease in the flavedo of Fortune mandarins despite the increase in sucrolytic enzymes paralleling the increase in reducing sugars could be due to the unloading of the sucrose by the phloem. The lack of correlation between both events could be also related to the differential cellular compartmentation of those enzymes and sucrose. Furthermore, other factors or enzymes of the carbohydrate metabolism may contribute to the changes in sugars occurring in the flavedo during the development and ripening of the fruit.

Besides the sucrolytic enzymes, we have examined seasonal changes in SPS in the flavedo of the chilling sensitive Fortune mandarins. We have found that, in general, SPS (V_{max}) activity decreased as the fruit ripened, whereas SPS (limiting assay) was much lower and showed little change (Figure 5B). The most important decrease in the activity of SPS occurred from December to February, during the coolest months in the season in the citrus area of Valencia. This pattern of changes agrees with previous results showing that SPS activity may be reduced in plants under cool conditions (Rufty et al., 1985; Khayat and Zieslin, 1987). It is also important to point out that SPS is more active in chlorophyllous tissues (Lingle and Dunlop, 1987) and that chlorophyll content in the flavedo of citrus decreases as fruit ripens. In this case, the photosynthate formation should be lower and probably sucrose is translocated from the leaves of the tree. Our results may well indicate that the maintenance of sucrose levels in the flavedo of Fortune mandarin is related to sucrose translocation rather than to its synthesis by SPS. In tomato fruit, the sucrose concentration and SPS remained relatively constant throughout fruit development (Islam et al., 1996), whereas SPS increased concomitantly to sugar accumulation during banana ripening (Cordenunsi and Lajolo, 1995).

Many carbohydrate sinks degrade translocated sugars and accumulate hexoses, whereas in others the sugars are degraded and converted into starch. Starch increased during fruit ripening in Fortune mandarins (Figure 4) despite sucrose showing little change. This increase also paralleled the changes occurring in the sucrose-degrading enzymes from the end of November until mid-January. Therefore, those enzymes may contribute to starch accumulation in the flavedo of the Fortune mandarins during fruit ripening. The data of this work suggest that if sucrolytic enzymes contribute to starch accumulation, SS and alkaline invertase, rather than acid invertase, are involved in the accumulation process in the more mature Fortune mandarins. It has been suggested that the enzyme SS is responsible for providing the carbon for starch biosynthesis in other organs accumulating starch such as bananas (Cordenunsi and Lajolo, 1995) and potatoes (Ross et al., 1994). Our results also indicate that the accumulation of starch was concomitant with the decline in the activity of the enzyme SPS (Figure 5). Irving et

al. (1997) showed that the maturation phase of *Curcubita maxima* D. Delica was characterized by the cessation of starch accumulation and the beginning of sucrose accumulation, which appear to be mainly related to the increase in SPS. During banana ripening, SPS increased concomitantly to starch degradation (Cordenunsi and Lajolo, 1995).

In conclusion, the time course changes in the activities of the enzymes of the carbohydrate metabolism studied in the flavedo during Fortune fruit ripening, together with the fact that sucrose did not change and reducing sugars increased until January, indicate that sucrose accumulation did not occur at the expense of the hexose pool; rather, sucrose is translocated into the fruit during ripening, and, in turn, broken down to hexoses by SS or acid and alkaline invertases. On the other hand, the accumulation of starch could be related to the decline in SPS activity and to the increase in the sucrolytic activities during fruit ripening.

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