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Title: Sugar Transport in Strawberry Fruit: Uptake Competition, and Developmental Changes in Invertase Levels

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Mechanisms by which plant organs accumulate photoassimilate are important in determining final sink size and may involve invertase activity. Because strawberry fruit are strong sinks for sugars, changes in invertase levels during development and mechanisms of sugar uptake within the fruit were examined.

Fruit from greenhouse-grown, day-neutral strawberries, (Fragaria X ananassa Duch. cv Brighton), were used for all studies. Fruit invertase levels were analyzed from 0 to 25 days post anthesis (DPA). Soluble and insoluble acid invertases were found, both with optimal activity at pH 4.6. Soluble invertase activity was highest at anthesis and decreased during development. Insoluble invertase activity was low at anthesis, but increased by 5 DPA where it remained for the following 20 days. Insoluble invertase was solubilized in 1 M NaCl and is presumably ionically bound to

the cell wall. At 15 DPA, histochemical staining showed invertase activity (mostly insoluble) throughout the tissue, but denser around vascular bundles; cortex tissue stained predominantly in the cell walls. Location of invertase near vascular bundles suggests a function in phloem unloading.

Sugar uptake kinetics of cortex protoplasts from 16-18 DPA fruit were similar to those found previously in tissue disks. Glucose uptake was higher than sucrose or fructose and exhibited simple saturation kinetics. Sucrose and fructose had similar uptake rates with biphasic curves; saturable kinetics was observed at low sugar levels (below 10 mM) and first-order (linear) kinetics at higher levels.

Apoplastic sucrose, glucose and fructose concentrations were estimated to be 80, 80, and 120 mM, respectively. Higher fructose levels may result from sucrose hydrolysis by cell wall invertase followed by more rapid uptake of glucose. The high apoplastic sugar concentrations indicate that the linear component of sucrose and fructose uptake is important in sugar uptake <u>in vivo</u>. No competition was seen among the three sugars, suggesting separate uptake pathways.

1'-Fluorosucrose, a sucrose analog, was not hydrolyzed by cell wall invertase and showed uptake kinetics similar to sucrose in cortex tissue. This, coupled with a lack of competition in sucrose uptake and demonstration of sucrose uptake by protoplasts, suggests that hydrolysis of sucrose by cell wall invertase is not a prerequisite for its uptake in strawberry fruit.

Sugar Transport in Strawberry Fruit: Uptake Competition and Developmental Changes in Invertase Levels

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CHAPTER I

Introduction

Strawberry production in Oregon is an important agricultural industry, accounting for several million dollars in revenue annually and employing thousands of full-time and seasonal workers. In 1987, farm receipts from strawberry production totaled approximately 31.5 million dollars; with processing, the value increased to about 54.8 million dollars (Crabtree et al., 1989). The majority of strawberries grown in Oregon are processed for eventual resale on retail or institutional markets; the fresh fruit market is relatively minor.

There is a continuing program to improve the yield and quality of strawberries. Yield has increased due to improved cultural practices, such as irrigation and fertilization scheduling and pest control, and through the introduction of new cultivars. There have been, however, reports of harvest yields below the potential yield because maximum fruit expansion does not occur (Abbott et al., 1970). This so-called "yield short-fall" represents

a possible increase in harvest yield if conditions causing it can be determined.

Strawberry fruit at different positions in the inflorescence have different growth rates (Forney and Breen, 1985b) and differ in their final size (Janick and Eggert, 1968; Moore et al., 1970). Fruit size declines with lower rankings.

Forney and Breen (1985b) demonstrated that the rate of sucrose uptake by cortex tissue of fruit was greatest in primary fruit and decreased as ranking of the fruit in the inflorescence declined. Similarly, rates of dry matter accumulation by fruit declined in parallel with decreasing inflorescence ranking (Forney and Breen, 1985b). While this suggests a limiting physiological difference between the different fruit positions, removal of flowers or young fruit at superior inflorescense rankings (e.g. primary) results in increased fruit size at inferior positions (Janick and Eggert, 1968). However, primary fruit will not attain a larger final size when secondary fruit are removed. This indicates that primary fruit size is probably limited genetically, whereas size of lower ranked fruit is limited by genetic and competitive factors. Possibly, lower ranked fruit are less efficient in competing for available photo-assimilates. Therefore, they fail to obtain sufficient sugars, or other nutrients, to allow them to attain their full potential size. The mechanism behind this competition is not known.

Photosynthesis is the ultimate source of energy and carbon skeletons needed for growth in the strawberry plant; therefore, the amount of photosynthesis and the partitioning of the photoassimilate are important components of yield. Improvements in yield for other crops have been due largely to changes in the increased partitioning of assimilate to the economically desirable sink organs. Increases in photosynthetic capability have not generally been noticed.

Sucrose is the primary sugar translocated in the strawberry (Forney and Breen, 1985a); therefore, knowledge of how sucrose is transported and stored by the fruit may give some insight into how fruit successfully compete with other tissues for available photoassimilate. This information may also be utilized in developing techniques to improve yield through either chemical treatments or genetic manipulations.

Total yield is not the only factor determining the value of a strawberry crop. Overall quality of the fruit is an important consideration for establishing a value of the harvest. Factors considered when assessing fruit quality include color, sweetness, flavor, texture, and firmness.

Sugar content is a determinant of fruit quality, a primary one for fruit grown for fresh market. Quality may be enhanced by increasing the amount of soluble sugars stored in the receptacle. Currently, the processes controlling import and storage of sugars by the strawberry fruit are poorly understood.

Invertase (β-fructofuranosidase, EC 3.2.1.26) catalyzes the hydrolysis of sucrose to form glucose and fructose. Work encompassing a variety of different plants and tissues has shown that invertase often is important in regulating movement, utilization, and storage of sucrose. Invertase activity changes with development in many organs. Changes in invertase activity have not been completely characterized for the developing strawberry fruit.

The objectives of this research were to refine characterization of invertase activity and its localization within developing strawberry fruit, and to examine the specificity of various sugar transport processes. Particular attention was given to processes related to transport of sucrose into fruit storage parenchyma. Because sucrose is the major sugar translocated in strawberries, the capacity of the fruit to unload sucrose from the phloem, and utilize it for energy and growth, is important in determining fruit size and harvest quality.

Phloem unloading may be thought of as those processes which, working together, stimulate the movement of sucrose out of the phloem and into the surrounding tissue. Phloem unloading may be either symplastic or apoplastic. In symplastic unloading, sugars are unloaded via plasmodesmatal connections between the vascular bundle and storage tissue, remaining in the living cells at all times. Apoplastic unloading involves movement of sugars from the phloem into the apoplastic spaces surrounding the cells where it is

taken up by storage tissue. The mechanism of phloem unloading in the strawberry fruit is unknown.

If unloading in strawberry fruit proceeds via an apoplastic pathway, as occurs in several storage organs, then the mechanism(s) for uptake of sucrose from the apoplast needs to be examined. The work of Forney and Breen (1986), using fruit cortex disks, has characterized the uptake kinetics of sucrose, glucose and fructose from the apoplast. Sucrose uptake occurs with approximately the same kinetics and rate as fructose, but glucose uptake is much more rapid. A cell wall invertase is known to be present in the strawberry fruit (Poovaiah and Veluthambi, 1985). Its function is unclear, but perhaps it aids in uptake of sucrose by cleaving it into glucose and fructose. Based upon the uptake kinetics observed by Forney and Breen (1986), this hydrolysis would allow for a more rapid accumulation of sucrose within the berry on a weight basis. This thesis attempts to examine the relationship between sucrose uptake and invertase activity.

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CHAPTER 2

REVIEW OF THE LITERATURE

Introduction

As plant breeders and physiologists attempt to improve crop varieties for higher yields, there is increased study of production, transport, metabolism, and storage of sugars within plants.

The introduction of new cultivars or varieties has dramatically increased the yield of several crops. Yields of soybean and wheat have increased by about 40% (Frey, 1981), and peanuts almost 100% (Duncan et al., 1978) in the last several decades. Advances in crop yields have resulted from improvements in management techniques and increases in the shoot harvest index (shoot HI) (Gifford et al., 1984). The shoot HI is the ratio of dry weight of the harvested crop to total above ground plant weight. If more sugars or other photoassimilates can be diverted to economically desirable plant structures, yields (improved HIs) may rise without an increase in photosynthesis. Carbon partitioning is driving the observed increases in HI.

Processes which control movement of photoassimilate throughout the plant are not well characterized. Currently, there is extensive research examining mechanisms which regulate movement of assimilate into sink tissues. Once

mechanism(s) have been identified and the limiting steps are known, this information can be used in developing new breeding or management strategies.

Sink Control of Assimilate Partitioning

The overall pattern of assimilate partitioning within a whole plant is determined by sink strengths of the various plant parts (Wyse, 1986); the source does not exert any control over the destination of assimilate (Gifford and Evans, 1981). Regulation of sink activity as a controlling point in assimilate partitioning has been reviewed recently by Wyse (1986).

The Münch hypothesis for explaining translocation of photoassimilate predicts that long distance transport is down a hydrostatic pressure gradient within sieve tubes (Giaquinta, 1983; Milburn, 1975). The gradient is produced by loading of sugars (often sucrose) into vascular strands in leaves and unloading of similar strands in sink regions. This process produces an osmotic potential gradient in the phloem which gives rise to the pressure gradient.

Phloem unloading may be via symplastic or apoplastic pathways. If the Münch hypothesis is correct, tissues with an apoplastic unloading pathway would have high levels of sucrose in the apoplast, unless sink cells possess efficient sugar uptake mechanisms. Without such mechanisms, the osmotic gradient between source and sink tissues would be reduced and unloading rates would decrease. A number of sink tissues have been shown to unload sucrose from the

phloem into the apoplast (Hawker and Hatch, 1965; Shannon and Dougherty, 1972; Thorne, 1979; Wyse; 1979; Jenner, 1986).

Regardless of the type of unloading pathway functioning in a given sink, the osmotic conditions of the receiving tissue will be altered as more sugars move from the phloem. This osmotic change may affect the metabolism of the tissue and its ability to accumulate sugars.

Turgor Sensitive Transport. As photoassimilate moves from the phloem into surrounding sink tissue, concentration gradients for sugars are established between the apoplast and symplast within the sink. These gradients may be produced by direct movement of sugars from the phloem to the apoplast or through leakage of sugars out of the cells. These gradients affect cell water potential (ψ) of the sink as determined by the turgor pressure (ψ_p) and the osmotic potential (ψ_s) of the cells (i.e. $\psi = \psi_p + \psi_s$). Recently, there have been a number of research groups investigating effects of changes in cell water potential on sugar transport in both source and sink tissues.

In legumes, it is possible to remove the embryo from the developing seed, keeping the seed coat intact, and forming an "empty ovule" which remains attached to the pod and continues to import photoassimilate (Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983). By filling the empty seed coat cup with solutions of various osmolarities, changes in translocate movement caused by

alterations in osmotic environment can be studied. Wolswinkel and Ammerlaan (1984) conducted pulse labelling experiments with pea (Pisum sativum L.) and found that solution osmolarity affected transport of sucrose and amino acids into the seed coat cup. Other reports show that the osmolarity of a solution bathing sink tissues affects sugar movement in broad bean (Vicia faba L.) seed coats (Wolswinkel and Ammerlaan, 1986) and stems (Aloni et al., 1986); sugar beet (Beta vulgaris L.) taproot (Wyse et al., 1986); castor bean (Ricinus communis L.) (Smith and Milburn, 1980) and Phaseolus coccinius L. (Daie and Wyse, 1985) leaves; and potato (Solanum tuberosum L.) storage tubers (Oparka and Wright, 1988a, 1988b).

To determine which of the components of cell water potential, turgor pressure or osmotic potential, affect sugar transport, tissues are incubated in solutions containing either permeating or non-permeating osmotica. Incubation in a non-permeating osmoticum, such as mannitol or polyethylene glycol, reduces the turgor pressure by causing water to move out of the cells (Daie and Wyse, 1985). Care must be taken in using mannitol because it is taken up by some tissues (Cram, 1984; Robinson and Loveys, 1986), especially during the isolation of protoplasts or in tissue where mannitol is translocated. To test for effects from reduced cell osmotic potential, a relatively permeable osmoticum such as ethylene glycol is used. After equilibration in such a solution, cell osmotic potential is

decreased, whereas turgor pressure is maintained at initial values (Greenway and Leahy, 1970; Daie and Wyse, 1985).

By comparing permeating vs. non-permeating osmotica, it appears that increases in sugar movement due to reduced cell water potential are caused by decreases in cell turgor pressure in source leaves (Smith and Milburn, 1980; Reinhold et al., 1984; Daie and Wyse, 1985) and sink organs (Wolswinkel, 1985; Aloni et al., 1986; Wolswinkel and Ammerlaan, 1986; Wyse et al., 1986; Oparka and Wright, 1988b). Turgor-sensitive sugar uptake has been shown to be a carrier-mediated, energy-requiring process (Wyse et al., 1986; Oparka and Wright, 1988b).

In potato storage tuber, the osmotic environment, specifically the turgor pressure, is important in regulating the synthesis of starch (Oparka and Wright, 1988a,b). In tuber disks, starch synthesis was optimal at a turgor of 80 kPa, whereas in intact tubers turgor was estimated to be approximately 320 kPa (Oparka and Wright, 1988b). Sucrose uptake and starch synthesis were found to increase as external mannitol concentrations were increased to approximately 250 mM. Above this level both were observed to decrease until plasmolysis occurred at approximately 400 mM mannitol. Here sucrose uptake increased (probably due to passive retention of sucrose in the increased apoplast resulting from plasmolysis), but starch conversion continued to decline (Oparka and Wright, 1988b). The authors were unable to demonstrate that ethylene glycol changed water

potential without altering turgor pressure in the potato tuber. Turgor was continually lost during 6 hr of incubation in ethylene glycol, indicating that an equilibrium had not been reached (Oparka and Wright, 1988b).

Turgor-sensitive transport is probably an important factor limiting the ability of a plant to transport material in the phloem and for sink tissue to unload the phloem. Patrick (1983) postulated that the osmotic environment of the apoplast may influence the rate of phloem unloading and thus determine the ability of various sinks to compete for assimilates. Many plants when subjected to drought stress undergo osmoregulation, a process whereby the plant accumulates solutes and maintains the positive turgor pressure essential for growth and other processes (Daie and Wyse, 1985). A decline in turgor pressure resulting from water stress potentially could have drastic effects on the ability of a plant to translocate sugars. Wyse et al. (1986) reported that osmotic concentrations in sugar beet taproot may vary between 200 mM in the seedling to 900 mM in the mature taproot. Because the sugar beet taproot exhibits increased sugar uptake at lower turgor pressures, the taproot must have some mechanism to regulate turgor in order to maintain sugar uptake (Wyse et al., 1986).

Daie and Wyse (1985) postulated that activation of the saturable transport system for sucrose in Phaseolus coccinius leaves is the site for turgor regulation of sucrose uptake. They found increases in the saturable

component of sucrose uptake curve at low turgors, but the linear non-saturable component appeared to be unaffected. The same results were found in sugar beet taproot (Wyse et al., 1986). The authors also found that the ability of taproot tissue to acidify an external bathing solution was dependent upon cell turgor pressure, and theorized that a plasmalemma-bound $^{+}$H-ATPase is turgor sensitive and may regulate activity of the sucrose carrier in the cell membrane. Similar results were seen in Senecio mikaniodes (Reinhold et al., 1984). Both proposals are similar in that the site of turgor detection is associated with the cell membrane (Daie and Wyse, 1985).

Sugar Uptake in Plant Tissue

Processes by which plant cells take up sugars from the apoplast have received increasing attention and our knowledge in the area has advanced greatly over the past decade. There have been several reviews of this field in recent years (Baker, 1978; Komor, 1982; Reinhold and Kaplan, 1984). Other reviews (Humphreys, 1980; Giaquinta, 1983a; Delrot, 1987) have dealt specifically with phloem loading, which, if apoplastic, is a special case of sugar uptake in plants. Studies of sugar uptake by plant cells have generally emphasized kinetics of sucrose uptake because sucrose is the most commonly translocated sugar.

Protoplast Studies.

The use of plant cell protoplasts to study sugar transport has grown rapidly with the development of better

isolation techniques. While protoplasts are in many ways an artificial experimental system (Galun, 1981), they do allow for analysis of sugar uptake in the absence of cell walls and associated enzymes. By comparing sugar uptake of tissue disks with that of protoplasts, it is possible to determine if the presence of a cell wall influences the uptake kinetics of various sugars from the apoplast.

This technique has been especially useful for investigating the hydrolysis and uptake of extracellular sucrose. The presence of a cell wall-bound invertase has been demonstrated in several systems (Hatch and Glasziou, 1963; Shannon and Dougherty, 1972; Vattuone et al., 1981; Maclachlan and Singh, 1983; Giaquinta et al., 1983; Poovaiah and Veluthambi, 1985; Schaffer, et al., 1987). Giaquinta et al. (1983) demonstrated uptake of apoplastic [^{14}C]-sucrose in sections of corn root tissue; but found that protoplasts obtained from the same tissue had a much lower capacity for sucrose uptake. They concluded that in corn root tissue the breakdown of extracellular sucrose by cell wall-bound invertase is necessary for efficient "sucrose uptake". Apparent sucrose transport is actually uptake of glucose and fructose resulting from sucrose hydrolysis. This type of system has been well documented in sugar cane parenchyma storage cells (Glasziou, 1960). Here the hydrolysis of sucrose by an extracellular invertase appears to be a prerequisite for its uptake by parenchyma cells. A similar requirement for sucrose cleavage before uptake has been

observed in elongating apical pea stem tissue (Maclachlan and Singh, 1983). The authors found that sucrose was taken up by apical pea stem protoplasts much slower than either glucose or fructose, and that the [^{14}C]-label from [^{14}C-glc]-sucrose was taken up much more rapidly than from [^{14}C-fru]-sucrose. This suggests that sucrose is hydrolyzed and the glucose and fructose moieties taken up by their separate carrier systems. In addition to pea stem and young corn roots a requirement for sucrose hydrolysis before uptake has been observed in cell suspensions of sugar cane stem (Komor, et al., 1981), and pea leaf mesophyll (Guy et al., 1981).

Uptake of sucrose without hydrolysis has been seen in protoplasts isolated from soybean cotyledons (Lin et al., 1984; Schmitt et al., 1984) and beetroot (Getz et al., 1987). True sucrose uptake has also been seen in tissue from tomato fruit (Damon et al., 1988).

In examining sucrose uptake curves in both protoplast and tissue disk systems, researchers have used a two-component model to describe the uptake kinetics in soybean cotyledons (Lichtner and Spanswick, 1981), strawberry fruit (Forney and Breen, 1986), and red beet root protoplasts (Getz et al., 1987). The two components are the saturable and the linear nonsaturable.

Lin et al. (1984a) have shown that sucrose uptake by protoplasts from developing soybean cotyledons exhibits biphasic kinetics. There is a saturable component specific for sucrose, and a linear uptake component which dominates

at higher substrate levels. After examining the effects of pH, metabolic inhibitors, and temperature on sucrose uptake into cotyledons protoplasts, Schmitt et al. (1984) suggested that sucrose uptake has three distinct components: (a) saturable- and (b) nonsaturable-sulfhydryl reagent-sensitive, and (c) diffusive. At low concentrations (below 12 mM) the saturable component was dominant. Uptake at higher sucrose concentrations was dominated by the linear component.

Invertase Activity.

Robinson and Brown (1952) published one of the first studies dealing with acid invertase in plants. They reported acid invertase activity in Vicia faba roots and found the greatest activity in a zone 4-9 mm behind the growing root tip. Weatherley (1953) demonstrated invertase activity in leaf disks of Atropa belladonna placed in a 10% sucrose solution under acidic conditions. When slices of sugar beet root (Bacon 1961), red beet (Bacon et al., 1965), Jerusalem artichoke (Edelman and Hall, 1964), carrot root, and potato tuber (Vaughan and MacDonald, 1967) are washed and aerated for a few hours or days, an increase in acid invertase activity is observed. This increased activity results from de novo enzyme synthesis (Vaughan and MacDonald, 1967; Wray and Brice, 1973). As tissues mature their ability to synthesize new invertases decreases (Engel and Kholodova, 1969).

Invertase has been isolated from many plant tissues, especially those showing active growth and development (Ap Rees, 1974). In fully differentiated tissue, invertase levels are known to be influenced by wounding (Matsushita and Uritani, 1974), light (Howard and Witham, 1983; Krishnan et al., 1985; Zouaghi, 1976; Zouaghi et al., 1979; Zouaghi and Rollin, 1976) and hormones (Howard and Witham, 1983; Kaufman et al., 1968, 1973; Poovaiah and Veluthambi, 1985).

Invertase occurs in at least two forms within many plant tissues, a soluble form found in the cytoplasm or vacuole (or both), and an insoluble form associated with the cell wall (cell wall-bound invertase). Cell wall-bound invertase activity seen in some tissues is likely an artifact resulting from binding of soluble invertase to cell walls when cells are ruptured during extraction (Hawker, 1969b). In other tissues, however, bound and soluble forms of invertase have different properties (i.e. pH optima, susceptibility to inhibitors, molecular weight,) (Krishnan et al., 1985; Poovaiah and Veluthambi, 1985; Sacher, 1966; Hawker and Hatch, 1965).

Wheat coleoptiles (Krishnan et al., 1985) and strawberry fruit (Poovaiah and Veluthambi, 1985) possess two acid invertases: one soluble, the other insoluble. Other tissues, such as sugar cane (Hatch et al., 1963; Sampietro et al., 1980), carrot (Ricardo and Ap Rees, 1970), and onion (Lercari, 1982) possess both a neutral intracellular soluble invertase and an acid extracellular invertase.

Soluble Invertase Activity. Soluble neutral invertases are thought to occur within the cytoplasm, while soluble acid invertases are assumed to be localized within the vacuole. The physiological significance (if any) in a plant possessing two acid invertases, rather than an acid and a neutral invertase, is unknown.

Sugar beet root also contain a soluble acid invertase, which decreases in activity from initially high to almost undetectable levels as the tissue matures (Giaquinta, 1979). The author observed that sucrose storage increased as soluble invertase levels decreased, and postulated that soluble invertase present early in development prevented accumulation of sucrose in storage root tissue. The soluble invertase level in a sink may be important in determining when, and to what concentration, sucrose accumulates.

Morris and Arthur (1984b) have reviewed the findings that, in a variety of plant tissues, high levels of acid invertase activity accompanied by low sucrose concentrations. The authors infer that acid invertase acts as a regulating factor on tissue growth in these plants. Sucrose accumulates in apple fruit (Golden Delicious) only after acid invertase decreased to nearly undetectable levels (Berüter, 1985). Yelle et al. (1988) found that in fruit of the tomato species Lycopersicon chmielewskii, low invertase levels were associated with high levels of sucrose. Conversely, fruit of L. esculentum had higher levels of the hexoses, glucose and fructose, lower levels of sucrose

(Yelle et al., 1988), and higher levels of invertase than fruit of L. chmielewskii.

Soluble invertase activity seen during early fruit development may also be important in establishing the fruit as an effective competitive sink for photoassimilate. Eschrich and Eschrich (1987) found that in the C_4-plant Gomphrena globosa, conditions which prevented the activation of soluble invertase in sink leaves also prevented the unloading of sucrose in that leaf. In tomato fruit, the hydrolysis of sucrose by invertase was important to the maintenance of high carbon import rates (Walker and Thornley, 1977). Walker and Ho (1977) and Walker et al. (1978) provided evidence of this association. Invertase activity is thought to control sucrose import in the grape berry (Hawker, 1969a), and alkaline invertase controls storage and utilization of sucrose in the mature tissue of sugar cane stalk (Glasziou and Gayler, 1972b). Soluble acid invertase activity was correlated with expansion rates of developing bean (Phaseolus vulgaris L.) leaves, suggesting that it somehow regulates the import of materials necessary for leaf development (Morris and Arthur, 1984a).

As indicated above, Zamski and Wyse (1984) and Wyse et al. (1986) suggest that cell turgor regulates sink activity. A relatively high cell turgor inhibits the saturable component of sucrose uptake in sugar beet taproot tissue (Wyse et al., 1986) and in wheat endosperm (Rijven and Gifford, 1983). Because sucrose would produce lower cell

turgor than hexoses at equivalent soluble carbohydrate levels (e.g., g CHO/unit tissue wt.) (Yelle et al., 1988), one could predict that intracellular invertase activity would have a negative effect on the sink activity, unless the resulting hexoses are rapidly metabolized and/or incorporated into products with low osmotic activity.

Cell Wall Invertase Activity. If pollination is prevented in strawberry, salt-extracted invertase activity remains low in the receptacle (fruit), which fails to expand (Poovaiah and Veluthambi, 1985). This suggests that the salt-extracted (i.e., cell wall-bound) invertase plays a role in the growth of receptacle tissue.

In several plant tissues, hydrolysis of apoplastic sucrose by cell wall-bound invertase appears to regulate the tissue's ability to metabolize assimilate by controlling the levels of hexoses available for uptake (Giaquinta et al., 1983; Russell and Morris, 1982; Shannon, 1968; Hawker and Hatch, 1965). If extracellular invertase has such a regulatory role, then factors affecting its activity would be important in determining growth rates. Levels of cell wall-bound invertase activity may be regulated by one or more of the following: (1) synthesis of invertase inhibitors, (2) changes in intercellular pH, or (3) synthesis and turnover rates.

Red beet, sugar beet and sweet potato roots all synthesize invertase inhibitors, which control the levels of invertase activity (Pressey, 1968). In carnations, an

invertase inhibitor is produced which controls sucrose transportation from petals to other flower parts (Halaba and Rudnicki, 1985). Suppression of invertase activity near vascular bundles by a proteinaceous inhibitor is necessary for transport of sucrose out of sugar beet leaves (Dubinina et al., 1984). If invertase activity remains high, transport is inhibited and sucrose concentrations in the phloem are low.

Most cell wall-bound invertases show optimum activity at acidic pH's. The activity of cell wall-bound invertase may be regulated by changes in the apoplastic pH (Eschrich, 1980; Ugalde et al., 1988b). Ugalde et al. (1988a) found that peach mesocarp tissue had high levels of extracellular invertase, as measured in vitro, throughout development and postulated that it did not play a regulatory role in utilization of sucrose. However, pH of the apoplast was observed to change from neutral to acidic and was correlated with an increase in fruit growth rate (Ugalde et al., 1988b). The authors postulate that the change in extracellular pH controls the activity of extracellular invertase, in vivo, which in turn regulates the mesocarp growth.

Little is known about control of invertase synthesis or its turnover rate within plant tissue. Changes in levels of invertase have been seen in many tissues but the mechanisms regulating the enzyme levels are unclear. Auxin is known to stimulate the synthesis of cell wall-bound invertase in

strawberry fruit (Poovaiah and Veluthambi, 1985), but nothing is known regarding the turnover of invertase.

Invertase Localization

Although plant tissues contain several types of invertases, their distribution and location within tissues has not been extensively studied. Improved techniques for histochemical localization of plant enzymes are becoming available (Gahan, 1984; Spruce et al., 1987). Cytological localization of invertase may give a clue to its physiological function within tissues.

Krishnan et al. (1985) used a histochemical technique developed for animal tissue (Lojda et al., 1979) to examine the localization of invertase in wheat coleoptiles. They found invertase in both cell wall and cytoplasmic locations with higher activity associated with the vascular strand than with other regions. They postulate that invertase may be functioning in some aspect of phloem unloading and utilization of sucrose at the vascular strand.

Invertase activity in developing maize kernels is associated with the cell wall and localized in the basal endosperm and pedicel tissues (Doehlert and Felker, 1987). In tissues which exhibit apoplastic phloem unloading, cell wall invertase may function to maintain a gradient of sucrose between the vascular strand and surrounding tissue. The gradient would be maintained by hydrolysis of sucrose to glucose and fructose and uptake of the hexoses via separate

transport systems. Low apoplastic sucrose levels would allow continued unloading of sucrose from the phloem.

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Chapter 3

CHANGES IN SOLUBLE AND INSOLUBLE INVERTASE ACTIVITY DURING STRAWBERRY FRUIT DEVELOPMENT

Abstract

Fruit of 'Brighton' strawberry (Fragaria X ananassa Duch.) were analyzed for invertase (β-fructofuranosidase, EC 3.2.1.26) activity from 0 to 25 days post anthesis. Fruit contained a soluble and an insoluble invertase, both with optimum activity at pH 4.6. Insoluble invertase was solubilized by 1M NaCl for 24 hr and presumably was attached to cell walls by ionic bonds. Soluble invertase activity (per unit fresh weight) was highest at anthesis and decreased as development progressed. Insoluble invertase activity was very low at anthesis, but increased by 5 days post anthesis to a level that was maintained throughout the remainder of fruit development. The location of invertase in immature fruit was examined using tissue slices and a coupled glucose oxidation and nitro blue tetrazolium assay. At 15 days post anthesis, when the cell wall-bound invertase was predominant ($\approx$ 80% of total activity), invertase activity was detected throughout the tissue, with highest intensity observed around vascular bundles. This suggests that, in intact fruit, cell wall invertase may play a role in the uptake of sucrose supplied or released to the apoplast.

Introduction

The strawberry fruit accumulates sugars during its development. Greater than 95% of the photosynthate translocated in a strawberry plant is in the form of sucrose (Forney and Breen, 1985); therefore, if the fruit is to compete effectively for photosynthate, it must possess effective mechanisms for unloading sucrose from vascular tissue and metabolizing it. Forney and Breen (1986) demonstrated that sucrose and fructose are taken up by strawberry fruit disks with similar biphasic kinetics, but glucose uptake is much faster and shows only a saturable component.

While there has been increased interest in processes by which sink tissues stimulate sugar transport, the mechanism(s) are still poorly understood. However, enzymes involved in metabolism or storage of sucrose within sink tissue appear to play an important role in determining a sink's ability to accumulate photosynthate. Invertase (β-fructofuranosidase, EC 3.2.1.26) activity has been postulated to be a factor controlling phloem unloading of sucrose (Eschrich, 1980; Bowen and Hunter, 1972; Sacher et al., 1963).

Invertase hydrolyzes sucrose to the hexose sugars glucose and fructose. Invertase has been isolated from a number of flowers and fruit (Hawker et al., 1976; Manning and Maw, 1969; Russell and Morris, 1982; Walker and Hawker,

1976), suggesting that sucrose hydrolysis may provide the hexoses necessary for growth in these tissues.

Giaquinta et al. (1983) found that whereas intact corn root segments readily accumulate sucrose, protoplasts from roots take up sucrose poorly. They hypothesize that sucrose hydrolysis by a cell wall invertase is necessary for sucrose uptake by corn root cells. The apparent sucrose uptake is actually the uptake of glucose and fructose produced by sucrose hydrolysis. A similar uptake system is seen in immature sugar cane stalk (Glasziou and Gayler, 1972). Invertase activity appears to be necessary for the transport of sucrose in some but not all tissues (Daie, 1985).

Strawberry fruit have two acid invertases: one soluble, the other insoluble (Poovaiah and Veluthambi, 1985). The soluble form decreases in activity during fruit development, whereas activity of insoluble invertase increases rapidly after fertilization. This increase is thought to be regulated in the receptacle by auxin produced by fertilized achenes. Changes in invertase activity in sugar cane are associated with fluctuations in auxin and levels of certain carbohydrates (Sacher and Glasziou, 1962; Sacher et al., 1963).

Invertases may be present in several different organs or tissues within a plant (Ap Rees, 1974). Few studies have attempted to characterize the tissue distribution of observed invertase activity. Information on the location of invertase within sink tissues could aid in deciding whether

invertase is directly involved in the process of phloem unloading. Krishnan et al. (1985) observed, in wheat coleoptiles, higher levels of invertase activity around vascular bundles relative to the bulk of parenchyma cells. There is no information available on the localization of invertase within the strawberry fruit.

The objectives of this study were to determine the types and levels of invertases present in the strawberry fruit throughout its development, and to determine, histochemically, the location of invertase.

Materials and Methods

Plant material. Plants of the day-neutral strawberry 'Brighton' (Fragaria X ananassa Duch.) were grown in a greenhouse under a 16 hr photoperiod. Supplemental lighting was supplied by high pressure sodium lamps (165 ± 20 μmol·m^{-2}·s^{-1}, 400-700nm). Fruit and runners were removed during the first month to allow plant establishment. Fully opened flowers were hand pollinated from anthesis to petal fall (about 4 days post anthesis).

Invertase determination. Invertase activity was assayed in primary and secondary fruit from 0 to 25 days after anthesis. Achenes were removed from harvested fruit by gently scraping with a spatula. The entire receptacle was used for analysis on days 0 and 5; for all succeeding times only cortex tissue was used.

Invertase extraction was performed using the methods of Poovaiah and Veluthambi (1985). Tissue was homogenized at 4°C using a mortar and pestle in a solution containing 50 mM 2-[N-morpholino]ethanesulfonic acid (MES)-NaOH, pH 7.0, 0.1 mM $MgCl_2$, 1 mM EDTA, and 0.5% 2-mercaptoethanol (v/v). For each gram of tissue, 6 ml of homogenizing buffer, 1 g sand, and 0.4 g of prewetted polyvinylpolypyrrolidone (PVPP) were used. The homogenate was centrifuged at 8000 x g for 10 min and the resulting supernatant was recentrifuged at 60,000 x g for 10 min. The final supernatant was called the soluble protein fraction. The pellet from the initial 8000 x g

centrifugation was resuspended and washed twice in homogenizing buffer and centrifuged at 8000 x g. The resulting pellet was suspended in homogenizing buffer containing 1 M NaCl and stirred on a rotary shaker at 4°C for 24 hr. The suspension was recentrifuged at 8000 x g and the supernatant designated the salt-extracted (i.e., insoluble) protein fraction. Both protein fractions were dialyzed against four changes of a 10mM acetate buffer, pH 4.6 for a total of 24 hr.

Invertase activity was assayed for 30 min at 30°C in 2 ml of a 10 mM acetate (pH 4.6) and 40 mM sucrose solution (assay buffer). Glucose produced by the hydrolysis of sucrose was assayed using a coupled glucose oxidase system (Sigma Technical Bulletin No. 510). Heat-killed extract samples were used as controls. Protein levels were determined by the Lowry method as modified by Peterson (1983), using bovine serum albumin as the protein standard.

The pH optimum for each of the isolated invertases was determined by incubating aliquots of the soluble and insoluble extracts in a solution of 40mM sucrose at various pH's for 30 min. Invertase activity was determined as described above.

The linearity of the reaction was tested for both the soluble and insoluble invertase. Soluble and insoluble forms were obtained from fruit 5 and 15 days post anthesis, respectively. Aliquots of protein fractions were incubated in assay buffer at 30°C. Samples were removed at intervals

for glucose assay.

Histochemical Localization of Invertase. Fully opened flowers were pollinated as described above and covered with aluminum foil to prevent exposure to light. This was an attempt to prevent the light-activated synthesis of phenols within the fruit. Studies were done using fruit at 5 and 15 days post anthesis.

Achenes were removed from the fruit and freehand sections made using a razor blade. Sections were washed in solution of 4% formalin, 3% polyvinylpyrrolidone (PVP) solution pH 6.0 for 30 min, then rinsed in 3% PVP for 4 hr with 10 changes of solution to remove any endogenous sugars. In addition to PVP, nitrogen was bubbled through the rinse solution in an attempt to minimize possible interference oxidation of endogenous phenolic compounds. Preliminary experiments showed that browning of fruit tissue could mask color changes resulting from invertase activity. Also, phenolic compounds are known to effectively inhibit enzymatic activity by covalently binding to enzymes (Anderson and Rowan, 1967; Mason and Wasserman, 1987).

Invertase activity was localized in tissue sections by a modified version of a coupled glucose oxidation with nitro blue tetrazolium assay described by Doehlert and Felker (1987). Sections were incubated for 40 min at room temperature in a reaction mixture containing 0.38M sodium phosphate (pH 5.5), 0.24 $mg \cdot ml^{-1}$ nitro blue tetrazolium, 0.14 $mg \cdot ml^{-1}$ phenazine methosulfate, 25 $units \cdot ml^{-1}$ glucose

oxidase and 5 $mg \cdot ml^{-1}$ sucrose. Deposition of an insoluble blue formazan product was assumed to occur in areas possessing invertase activity. The presence of any nonspecific accumulation of reaction product was monitored by incubating disks in the above reaction mixture minus sucrose. After incubation, sections were rinsed in water and post-fixed in 4% formalin (pH 7.0). The fixed and stained tissues were photographed with a Leitz photomicroscope using Kodak Ektachrome ASA 400 slide film.

Results and Discussion

Both soluble and insoluble (salt-extracted) invertases had pH optima of 4.6 (Figure 3-1) and therefore would be classified as acid invertases. This is in agreement with the results of Poovaiah and Veluthambi (1985) obtained using the cultivar 'Ozark Beauty'. Krishnan et al. (1985) reported that wheat coleoptiles also possess a soluble and insoluble invertase. While the strawberry fruit is similar to wheat coleoptiles in this respect, it differs from sugar cane (Hatch et al., 1963; Sampietro et al., 1980) carrot (Ricardo and Ap Rees, 1970) and onion (Lercari, 1982). These plants possess both a neutral intracellular invertase and an acid extracellular invertase. Presumably, soluble acid invertase is localized in the vacuole, while soluble neutral invertase is in the cytoplasm. The physiological significance of plants possessing two acid invertases rather than an acid and a neutral invertase is unknown.

Under the assay conditions used, both the soluble and insoluble invertases show linear activity for up to 2 hr in a 40 mM sucrose solution (Figure 3-2). The reaction was reproducible enough to allow invertase activity to be routinely analyzed using a 30 min incubation period.

To determine whether the invertases isolated were actually distinct, or just the same protein occurring in two locations due to the isolation procedure, the extracts were incubated in the presence of 2 μM Hg^{++} or 100 μM I_2.

Soluble invertase activity decreased by 74% and insoluble activity by 3% in the presence of Hg^{++}. Incubation in I_2 gave 57% inhibition of soluble activity and no decrease in insoluble activity. The differential inhibition of the two invertases suggests that they are two distinct forms of the enzyme.

Soluble Invertase. The soluble invertase showed high activity at anthesis followed by a rapid decline as receptacle development proceeded (Figure 3-3). Sucrose is not detected in 'Brighton' fruit until about 10 days post anthesis (Forney and Breen, 1986), at which time the soluble invertase activity is relatively low. Giaquinta (1979) found that the sugar beet root also contained a soluble acid invertase which decreased in activity from initially high to almost undetectable levels as the tissue matured. Sucrose storage occurred only after the soluble invertase activity was very low. High levels of soluble acid invertase activity are often accompanied by low sucrose concentrations in a variety of plant tissues, and may act as a regulating factor of tissue growth in these plants (Morris and Arthur, 1984b). Sucrose levels in cells are probably dependent upon levels of soluble acid invertase because they are both localized in the vacuole (Schaffer et al., 1989). Such a dependency could be a characteristic of all organs in which sucrose is stored in the vacuole (Schaffer et al., 1989).

Red beet, sugar beet and sweet potato all synthesize invertase inhibitors which control invertase activity within

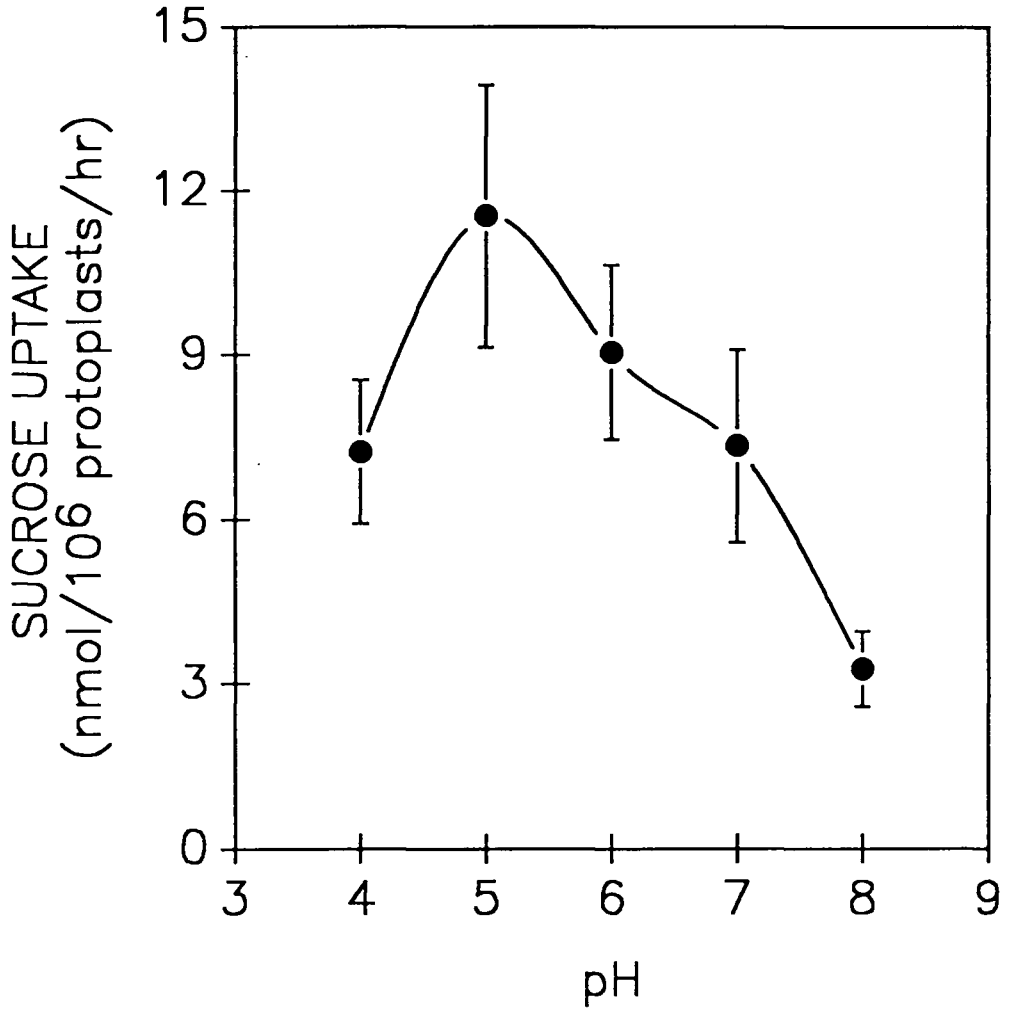

Figure 3-1. Effect of pH on invertase activity in the soluble and insoluble fractions of strawberry fruit. Buffers used were 20 mM citrate-phosphate (pH 3.5-5.0), 2(N-morpholino) ethanesulfonic acid (MES, pH 5.5-6.), 3(N-morpholino) propanesulfonic acid (MOPS, pH 7) and N-2-hydroxyethylpiperazine propane sulfonic acid (EPPS, pH 8).

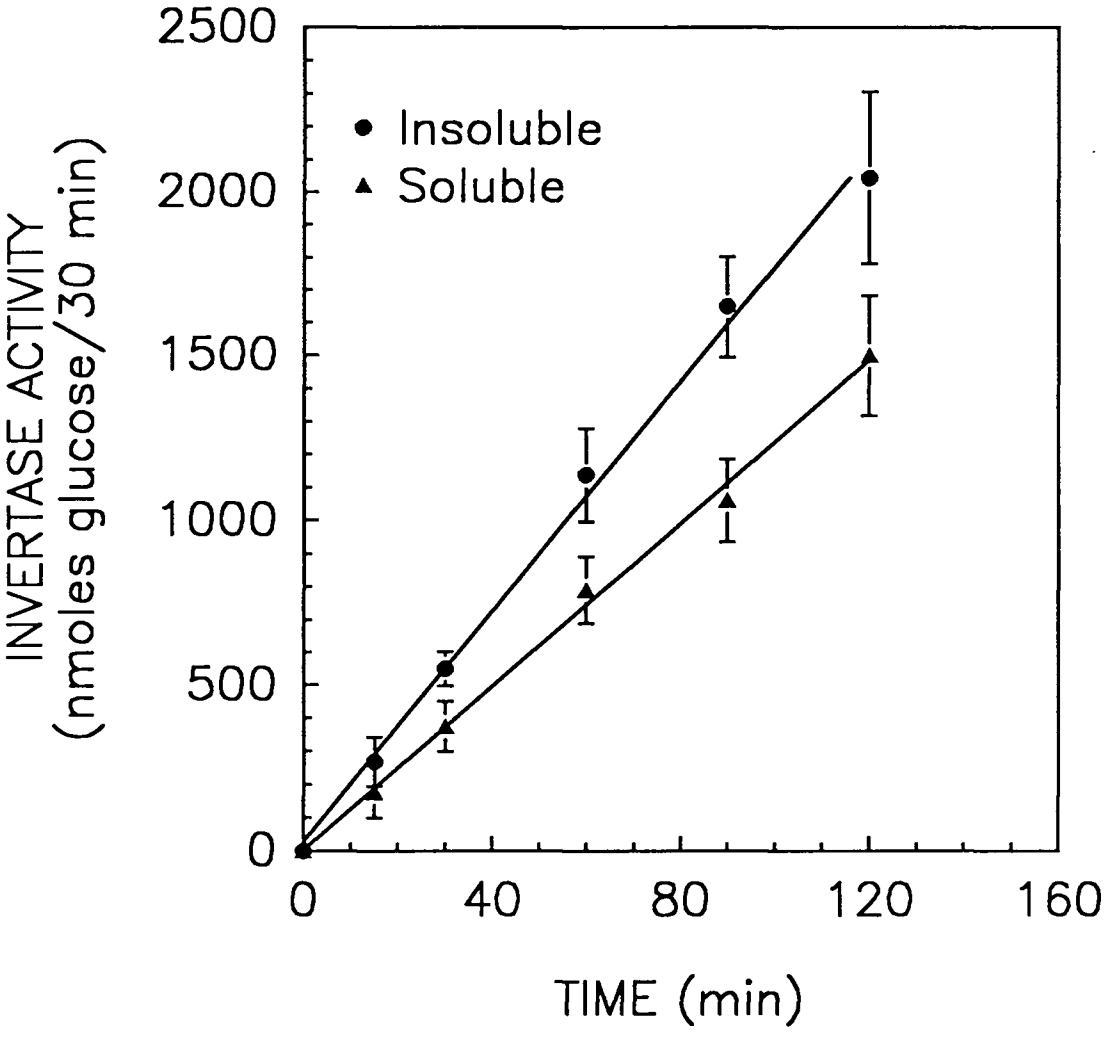

Figure 3-2. Invertase activity with time as measured by the production of glucose in soluble and insoluble strawberry fruit extracts. Points represent the mean ± S.E. of three replications.

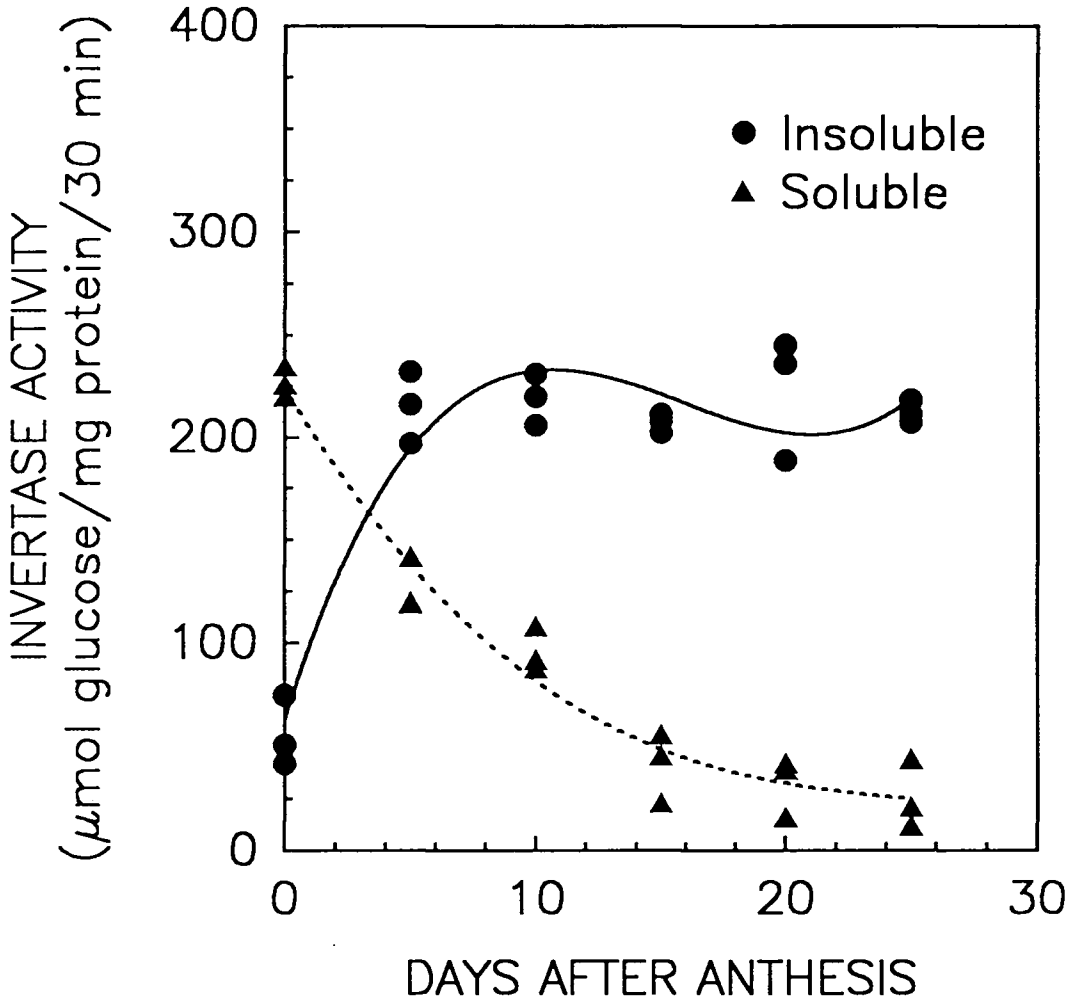

Figure 3-3. Activity of soluble and insoluble invertase in the developing strawberry fruit.

the roots (Pressey, 1968). In carnation flowers, an invertase inhibitor is produced which controls sucrose translocation from petals to other flower parts (Halaba and Rudnicki, 1985). It is not known if invertase inhibitors were responsible for the decline in soluble invertase activity observed in developing strawberry fruit (Figure 3-3). Addition of aliquots of the soluble fraction from 15 day old fruit did not inhibit soluble invertase activity in 5 day old fruit.

Zamski and Wyse (1984) and Wyse et al. (1986) suggest that cell turgor may regulate sink activity. In sugar beet taproot tissue, a relatively high cell turgor inhibits the saturable component of sucrose uptake (Wyse et al., 1986). This result has also been reported in wheat endosperm (Rijven and Gifford, 1983). Because sucrose would give a lower cell turgor than hexoses for equivalent soluble carbohydrate levels (Yelle et al. 1988), intracellular invertase activity may have a negative effect on sink activity unless the hexoses produced are rapidly consumed or metabolized into products of low osmotic activity. Assuming sugar accumulation is turgor-sensitive in strawberry fruit, the decline in soluble invertase activity may enhance sugar transport by lowering cell turgor pressure.

While a decline in soluble invertase activity may be necessary for sucrose to accumulate in strawberry fruit, high activity early in fruit development (Figure 3-3) may assist is establishing the fruit as an effective sink for

photoassimilate. Eschrich and Eschrich (1987) found that conditions which prevented the activation of soluble invertase in sink leaves of Gomphrena globosa also inhibited unloading of sucrose. Similarly, soluble acid invertase activity was correlated with the rate of expansion of developing leaves in beans (Morris and Arthur, 1984a). In tomato fruit, the metabolism of sucrose by invertase was important to the maintenance of high carbon import rates (Walker and Thornley, 1977). Initial high soluble invertase activity may be necessary to maintain a gradient of sucrose between the phloem and storage parenchyma, and therefore sustain high phloem unloading rates.

Cell Wall-Bound Invertase. Salt-extracted invertase, presumably originally cell wall-bound, was low in activity at anthesis when compared with the soluble invertase (Figure 3-3). After pollination the activity increased rapidly to a nearly constant level.

Fertilization may be required for the development of insoluble invertase activity since Poovaiah and Veluthambi (1985) found that if pollination was prevented the activity of invertase remained low and the receptacle did not expand. The maintenance of high insoluble invertase activity through the period of rapid fruit growth (Forney and Breen, 1986) and accumulation of sucrose (Forney and Breen, 1986) suggests that extracellular invertase plays a role in sugar import.

The level of cell wall-bound invertase may be low at anthesis because the receptacle is not actively growing at this time and level of soluble invertase is sufficient to meet its needs for glucose and fructose. As the receptacle expands and soluble invertase activity decreases, cell wall-bound activity may increase to satisfy the growing demand for hexoses for metabolism and to sustain phloem unloading.

Hydrolysis of apoplastic sucrose by extracellular invertase, as observed in several plant tissues, appears to regulate metabolism of translocated assimilate by controlling the levels of hexoses available for uptake (Giaquinta et al., 1983; Russell and Morris, 1982; Shannon, 1968; Hawker and Hatch, 1965). If extracellular invertase has such a regulatory role, then factors affecting its activity would be important in determining growth rates. In intact fruit, the activity of cell wall-bound invertase may be regulated by one or more of the following: (1) synthesis of invertase inhibitors, (2) changes in intercellular pH, or (3) synthesis and turnover rates of invertase. As previously noted, the role of inhibitors in controlling invertase activity in strawberry fruit is unknown.

Cell wall-bound invertase of strawberry fruit shows optimum activity at a acidic pH. Eschrich (1980) and Ugalde et al. (1988b) put forth the idea that the activity of existing cell wall-bound invertase may be regulated by changes in apoplastic pH. Ugalde et al. (1988a) found that peach and apricot mesocarp tissue had high levels of

extracellular invertase throughout development and postulated that it did not play a regulatory role in utilization of sucrose. The pH of the apoplast was observed to change from neutral to acidic (Ugalde et al. 1988b) and was correlated with an increase in the growth rate. They postulate that the change in extracellular pH is controlling the activity of the extracellular invertase which in turn regulates the mesocarp's growth rate. In 'Brighton' fruit the cell wall invertase activity (e.g., insoluble) is stable after day 5 suggesting that regulation of its activity, rather than its synthesis or degradation may be important in vivo. Changes in fruit cell wall pH may regulate cell wall invertase activity.

Changes in the levels of invertase have been seen in many tissues but the mechanism regulating the levels of the enzyme are unclear. In strawberry fruit, auxin is known to stimulate the synthesis of cell wall-bound invertase (Poovaiah and Veluthambi, 1985), but nothing is known regarding the turnover of invertase within the fruit.

Histochemical Localization of Invertase. In strawberry fruit at 15 days after anthesis, invertase activity appeared throughout the tissue but was highest near the vascular bundles (Figure 3-4). Tissue incubated in the absence of sucrose showed no formation of a blue formazan product (Figure 3-5). Since soluble invertase levels at this stage in development were relatively low, (≈ 20% of the total activity), the activity visualized in the tissue sections

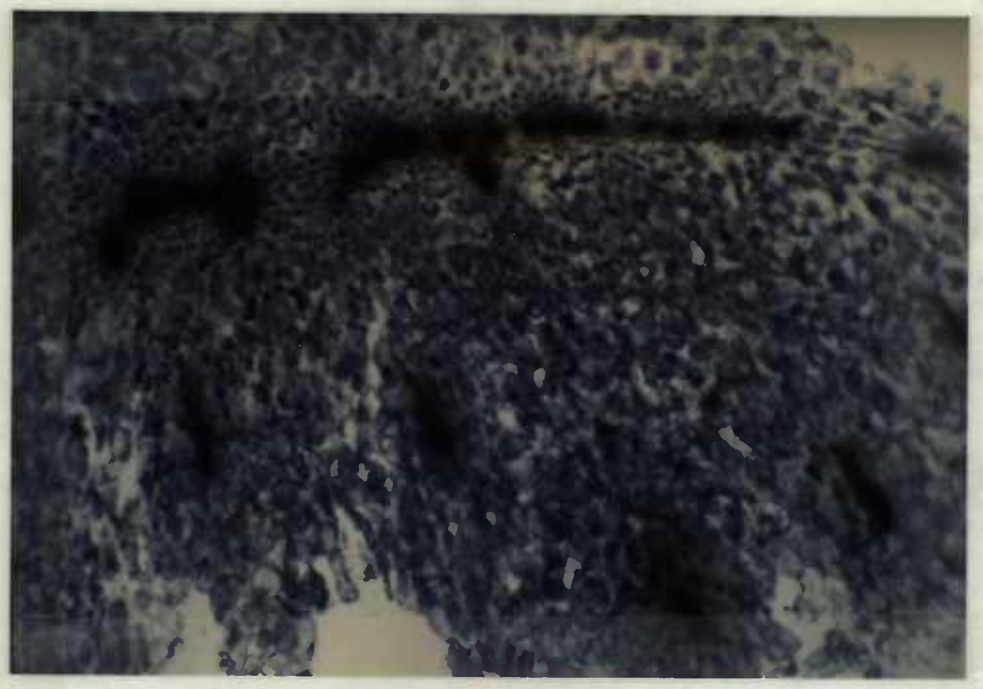

Figure 3-4. Histochemical localization of invertase in strawberry fruit tissue 18 days after pollination. Deposition of the blue formazan product represent areas of invertase activity.

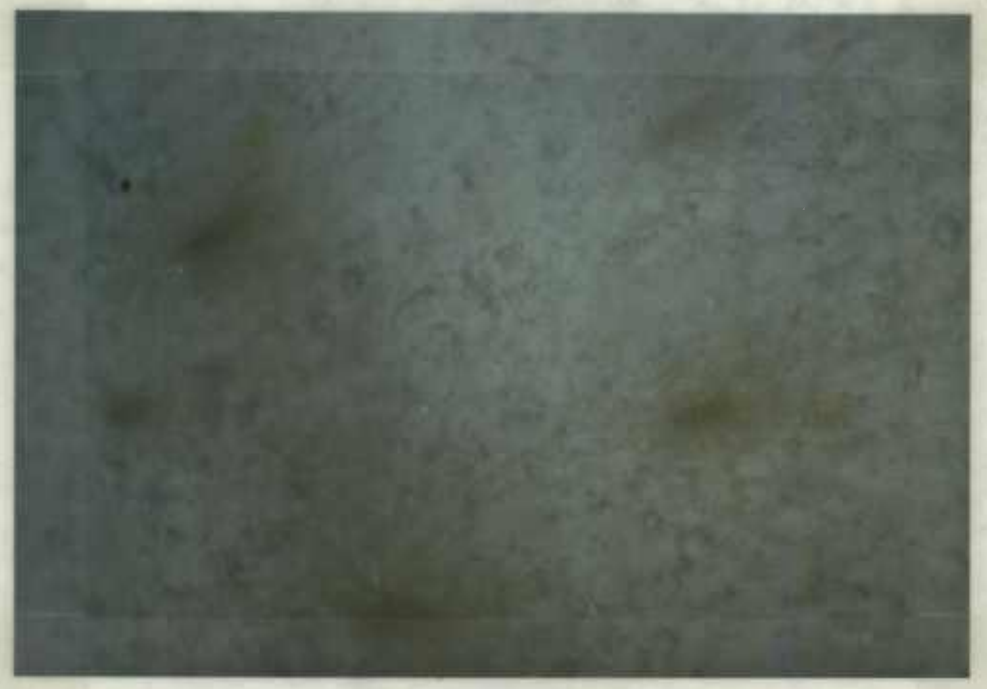

Figure 3-5. Control incubation. Tissue incubated in localization mixture minus sucrose.

most likely represented cell wall-bound invertase (Figure 3-5). If precautions were not taken to minimize tissue browning, the blue formazan product was partially masked by large amounts of oxidized phenolics (data not shown). Covering the fruit during development minimized the difficulty. Attempts to locate invertase activity in 5 day old fruit were unsuccessful due to extensive browning despite the above precautions.

Invertase activity is also higher near vascular bundles than in the bulk parenchyma of wheat coleoptiles (Krishnan et al., 1985). Doehlert and Felker (1987) stained developing maize coleoptiles for invertase and found the majority of activity to be in the basal endosperm and pedicel tissue, and predominantly in the cell wall. Invertase activity is higher in both coleoptile tissues where phloem unloading is occurring, suggesting that invertase is associated with this process. Results with strawberry fruit are consistent with this idea (Figure 3-4).

In summary, soluble invertase levels declined as strawberry fruit development progressed and corresponded with the onset of sucrose accumulation reported by Forney and Breen (1986). Soluble invertase may regulate the storage of sucrose in fruit cells. Insoluble invertase activity increased after pollination and may play a role in sustaining phloem unloading in the fruit. High invertase activity observed around the vascular bundles supports the idea of its association with phloem unloading.

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CHAPTER 4

UPTAKE OF SUCROSE, GLUCOSE AND FRUCTOSE BY STRAWBERRY FRUIT PROTOPLASTS

Abstract

Protoplasts were isolated from cortical tissue of 16-18 day old strawberry fruit (Fragaria X ananassa Duch. cv Brighton) and uptake of exogenous [^{14}C]-labeled-sucrose, glucose and fructose determined. Sucrose uptake was optimal at pH 5.0. Uptake rates for each of the sugars were linear for at least 1 hr at a concentration of 10 mM. Sucrose and fructose uptake rates were similar, but lower than that of glucose. Glucose uptake exhibited simple saturable kinetics throughout the concentration range examined (0-50 mM). Sucrose and fructose showed biphasic uptake curves with saturable kinetics at low sugars levels (below 10 mM) and first-order kinetics at higher concentrations. These results are in agreement with previous kinetic studies utilizing cortical tissue disks in the strawberry fruit; however, K_M values were lower for all three sugars in the protoplast studies. Sucrose accumulation by strawberry fruit does not appear to require prior sucrose hydrolysis by cell wall invertase as evidenced by its uptake by protoplasts lacking cell walls.

Introduction

The ability of a sink tissue to attract assimilates is an important component in determining the yield of a crop (Gifford and Evans, 1981; Gifford et al., 1984). To a large extent improvement in yield seen with newer cultivars has been due to increases in the shoot harvest index (Gifford et al., 1984), that is, the ratio of harvested dry weight to total above ground dry weight. Although differences in net photosynthesis between cultivars have been documented (Ozbun, 1978), often there is no correlation found between net photosynthesis (per unit leaf area) and yield (Wittwer, 1980). Currently there is much interest in understanding the mechanisms by which plants allocate available assimilates. Such knowledge may lead to ways to efficiently increase allocation of assimilates to economically desirable sink tissues through improved cultural, genetic, or chemical practices (Schmitt et al., 1984; Daie, 1985).

There is considerable evidence that, in plants which translocate sucrose, phloem loading in source leaves occurs from the apoplast utilizing a proton-sucrose co-transport system (Giaquinta, 1980). However, there is also some evidence for symplastic loading (Madore and Lucas, 1986; and references therein). Mechanisms by which plants unload the phloem in sink regions are not as well characterized. Transport of sugars in sugar cane stem tissue is probably the best understood system. It has been reviewed elsewhere

(Glasziou and Gaylor, 1972), but briefly, in the immature stem tissue sucrose is unloaded from the phloem into the apoplast where cleavage by a cell wall invertase is required for uptake by the surrounding parenchyma storage cells. Activity of this invertase appears to control the availability of sucrose for storage.

Studies on various plants and tissues have shown that different unloading pathways exist which are tissue-dependent (Geiger and Fondy, 1980; Giaquinta et al., 1983). Unloading may occur into the apoplast with subsequent uptake by storage cells or it may be via the symplast where material moves from the phloem into surrounding tissue through plasmodesmatal connections.

Strawberry fruit are a strong sink for translocated assimilates (Forney and Breen, 1985b). The fruiting trusses of 'Brighton' plants comprise 40% of the total plant dry weight by 80 days after planting (Forney and Breen, 1985b). During the peak of fruiting, strawberry fruit accumulate more dry matter than the plant assimilates (Forney and Breen, 1985b), indicating that fruit have such a high sink activity that reserve carbohydrates are mobilized to meet their growth demands. Strawberry fruit cortex tissue takes up sucrose and fructose at much slower rates than glucose (Forney and Breen, 1986), even though sucrose is the primary sugar translocated by the strawberry (Forney and Breen, 1985a). The strawberry fruit possesses a cell wall acid invertase (Poovaiah and Veluthambi, 1985) whose activity

increases after pollination (Chapter 3). If pollination is inhibited the cell wall invertase activity remains low and the fruit does not expand (Poovaiah and Veluthambi, 1985). It is not known whether sucrose must be hydrolyzed by cell wall invertase before it can be taken up by cortical cells of the receptacle. Asymmetrically-labeled sucrose fed to strawberry leaves showed partial randomization of the label when fruit contents were analyzed (Forney and Breen, 1986). Similar randomization was seen in fruit disk experiments (Forney and Breen, 1986), suggesting that invertase hydrolysis of sucrose occurs somewhere in the translocation pathway. In sugar cane, inhibition of invertase by antibodies prevents the uptake of extracellular sucrose (Bowen and Hunter, 1972).

Protoplasts have recently been used to examine sugar uptake in a variety of plants and tissues (Giaquinta et al., 1983; Lin et al., 1984; Schmitt et al., 1984; Lin, 1985a,b; Singh and Maclachlan, 1986; Getz et al., 1987a,b; Martinoia et al., 1987; Stanzel et al., 1988a,b). They are especially useful in studying sucrose uptake because, lacking cell walls and associated invertase, it is possible to determine if sucrose is transported into cells without extracellular hydrolysis (Lin et al., 1984).

This study was undertaken to examine the uptake kinetics of sugars into strawberry protoplasts in the absence of cell wall enzymes. We especially wished to

determine if sucrose uptake occurred in the absence of cell wall-bound invertase.

Materials and Methods

Plant Material. Strawberry plants (Fragaria X ananassa Duch. cv Brighton) were grown in the greenhouse under a 16 hr photoperiod. Supplemental lighting was provided using high pressure sodium lamps (165 ± 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 400-700nm). Fully opened flowers were hand-pollinated daily until petal fall (about 4 days post anthesis). Berries 16-18 days post anthesis were used for all studies.

Protoplast isolation. Cortex tissue was obtained by cutting plugs of receptacle tissue using a 7 mm cork borer. The plug was cut into disks approximately 3 mm thick and rinsed in a solution of 25 mM K-acetate and 25 mM $CaCl_2$ (pH 5.8).

Protoplasts were obtained by a modified version of the method reported by Yamaki (1984). About 10 g of fruit disks were shaken (30 cycles per min) for 30 min at 30°C in 40 ml of a solution containing 0.5% Macerase, 0.3% Pectinase, 0.1% bovine serum albumin (BSA), 2 mM dithiothreitol (DTT), 25mM $CaCl_2$, 0.6 M sorbitol, and 50 mM K-acetate (pH 5.8). The tissue was then placed in 50 ml of new medium and incubated as above for 2 hr with solution replacement every 40 min.

The mixture was centrifuged at 100xg for 2 min and the pellet resuspended in a wash solution composed of 0.6 M sorbitol and 25 mM K-acetate (pH 6.0). Intact cells were collected in the upper layer of a mixture made up of two volumes of the suspension and one volume of Ficoll pack

containing 0.6 M sorbitol (pH 6.0) (Yamaki, 1985). The intact cells were washed twice with the wash solution and then incubated in a digestion mixture consisting of 2.5% cellulase, 25 mM $CaCl_2$, 0.6 M sorbitol, 0.1% BSA and 25 mM acetate (pH 5.6) at 30°C for 1 hr. The digestion mixture was layered onto wash solution containing 5% Ficoll and centrifuged at 100xg for 2 min. Protoplasts were found at the interface between the two mixtures. They were removed with a Pasteur pipette and washed twice with wash solution and finally suspended in the wash solution.

The density of protoplasts was determined using a Neubauer hemacytometer. The presence of cell wall remnants was tested for using calcofluor white ST (Nagata and Takebe, 1970). Viability was assessed using the exclusion of Evan's blue stain (Gaff and Okong'O-ogala, 1971) and respiration as criteria.

Respiration of the protoplasts was monitored using a infrared gas analyzer and the technique described by Clegg et al. (1978). Approximately 10^5 protoplasts in 0.5 ml of wash solution containing 25 mM $CaCl_2$, 20 mM sucrose, and 25 mM acetate (pH 5.0) were placed in 10 ml Erlenmeyer flasks. The flasks were sealed with a serum stopper and incubated at 25°C. Periodically, 2 ml air samples were removed with a syringe. The flasks were flushed with air and resealed. Samples were injected into the IRGA for analysis of CO_2 content.

Sugar Uptake. Sugar uptake was measured by adding approximately 5 x 10^5 protoplasts (about 50 μl) to 500 μl of a solution containing 25 mM citrate, 0.6 M sorbitol, 25 mM $CaCl_2$, and ^{14}C-labeled sugar (1480 Bq/mmol) at pH 5.0. The mixture was incubated for 60 min with constant shaking (30 cycles/min) in a 25^oC water bath. After 60 min the reaction mixture was diluted with 30 ml of buffer containing unlabeled sugar, centrifuged at 100xg for 5 min and the supernatant discarded. The pellet was rinsed twice more and the final protoplast pellet lysed with 1.0 ml of hot distilled water (Giaquinta et al., 1983). Aliquots of the resulting solutions were added to 15 ml of scintillation cocktail (Beckman Ready Solv MP) and the radioactivity determined using a Beckman LS 7000 scintillation counter. Lineweaver-Burk plots were analyzed to determine the kinetics of the uptake curves. If two components were observed, the first order rate constant for the linear component was calculated as the slope of the regression line for points on the total uptake curve at or above 10 mM sugar concentration (Forney and Breen, 1986).

Results and Discussion

During the preparation of protoplasts, it was observed that the vascular strands within the cortex tissue were relatively resistant to digestion. Therefore, isolated protoplasts most probably represent storage parenchyma cells of the cortex. The protoplasts obtained appeared to be free of cellulose contamination (less than 5% showed cell wall remnants) when stained with calcofluor white ST. Protoplasts excluded Evan's blue indicating that the plasma membrane had maintained its integrity. With the incubation conditions used, respiration rates were relatively constant for 60 min but then slowly decreased (Figure 4-1). Uptake rates for sucrose, glucose and fructose were linear for at least 60 min (Figure 4-2). Thereafter, protoplast preparations appeared to deteriorate as uptake rates decreased corresponding to the decrease in respiration. A higher percentage of the protoplasts were also observed to take up Evan's blue dye after 1 hr indicating a progressive loss of membrane stability. It is not known why the protoplasts were stable for such a relatively short period of time. Their comparatively large size ($\approx$ 100 μm in diameter) may make them very fragile and more susceptible to rupture. Cortex tissue disks are stable up to 6 hr (Forney and Breen, 1986).

Sucrose uptake was optimal at approximately pH 5.0 (Figure 4-3); the stability of the protoplast decreased

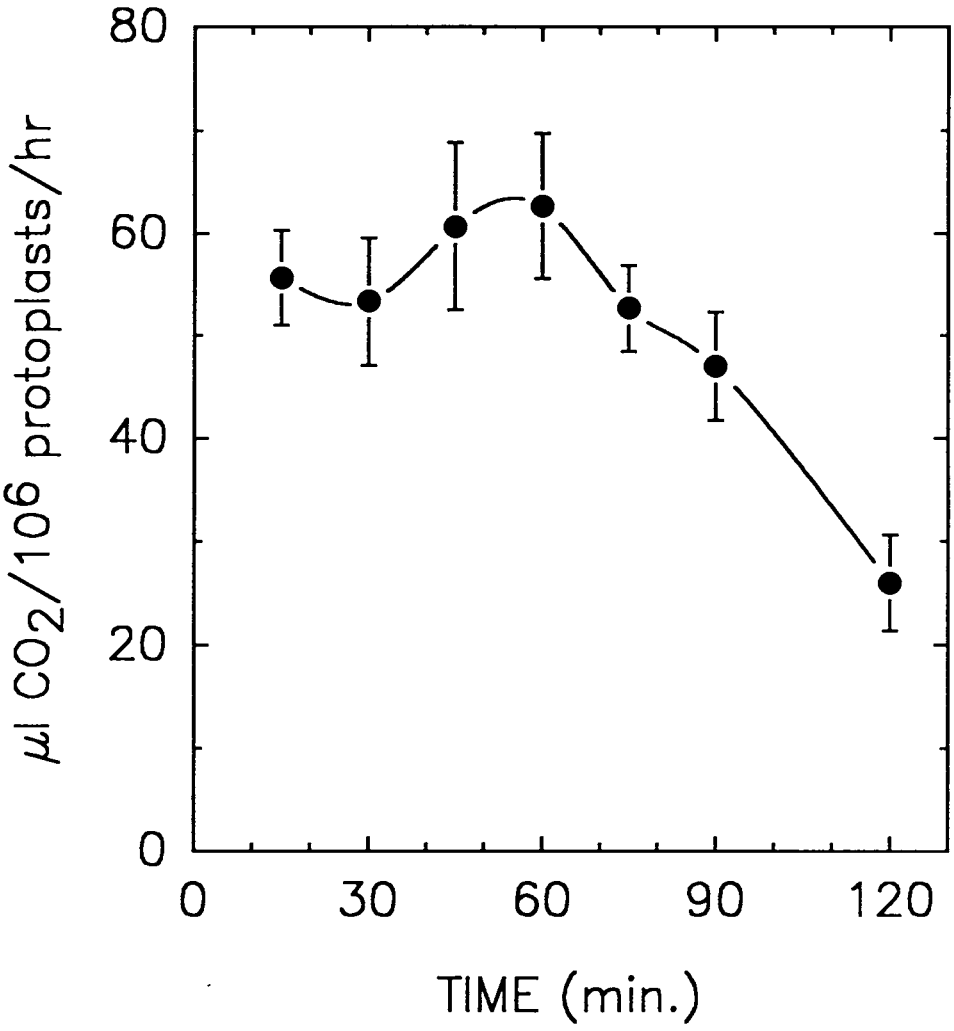

Figure 4-1. Respiration rate for 'Brighton' fruit cortex protoplasts at various times after isolation. Each point represents the mean ± S.E. of two replicates.

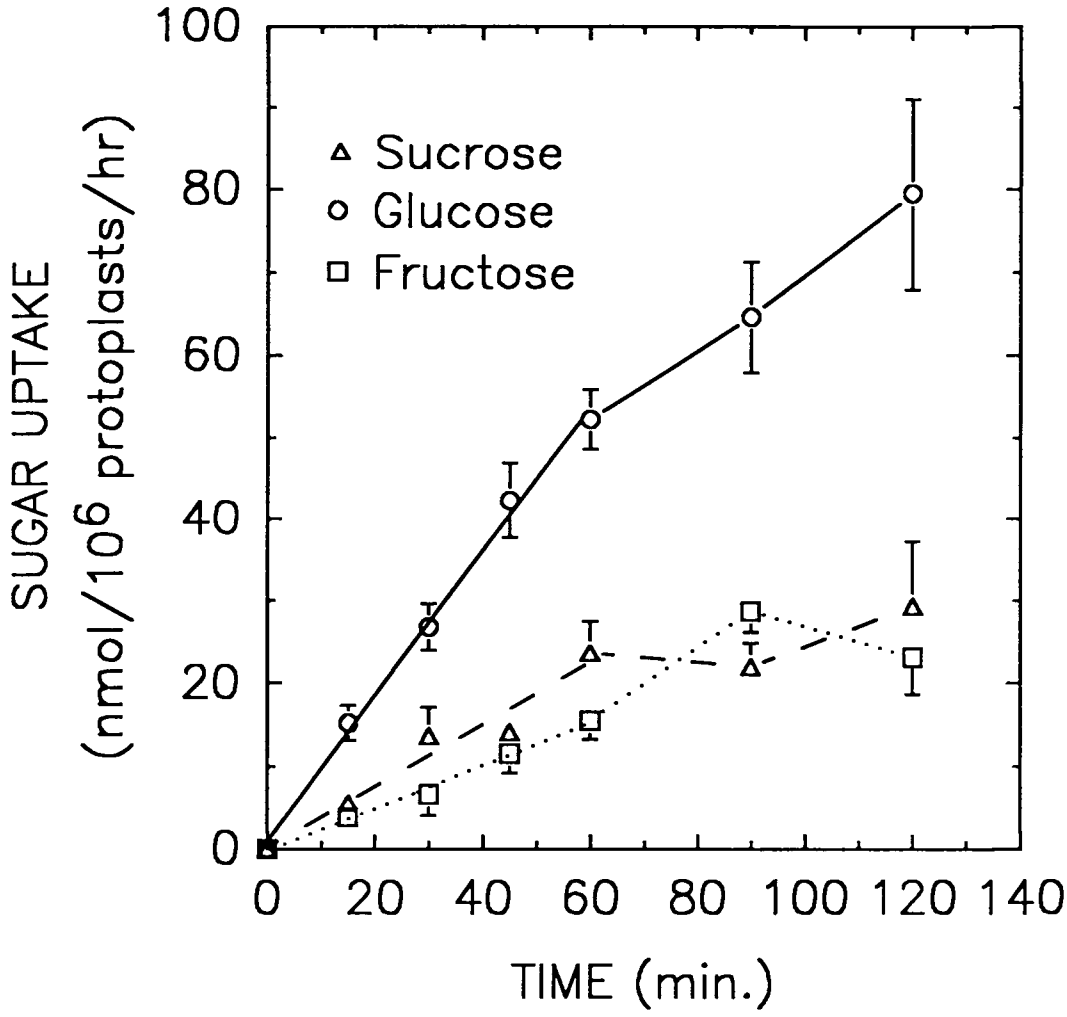

Figure 4-2. Uptake of sucrose, glucose or fructose by 'Brighton' fruit cortex protoplasts. Incubation conditions were 5 x 10^5 protoplasts in a solution containing 25 mM citrate, 0.6 M sorbitol, 25 mM $CaCl_2$, and 25 mM ^{14}C-labeled sugar (40 nCi/mmol) at pH 5.0. Each point represents the mean ± S.E. of three replicates.

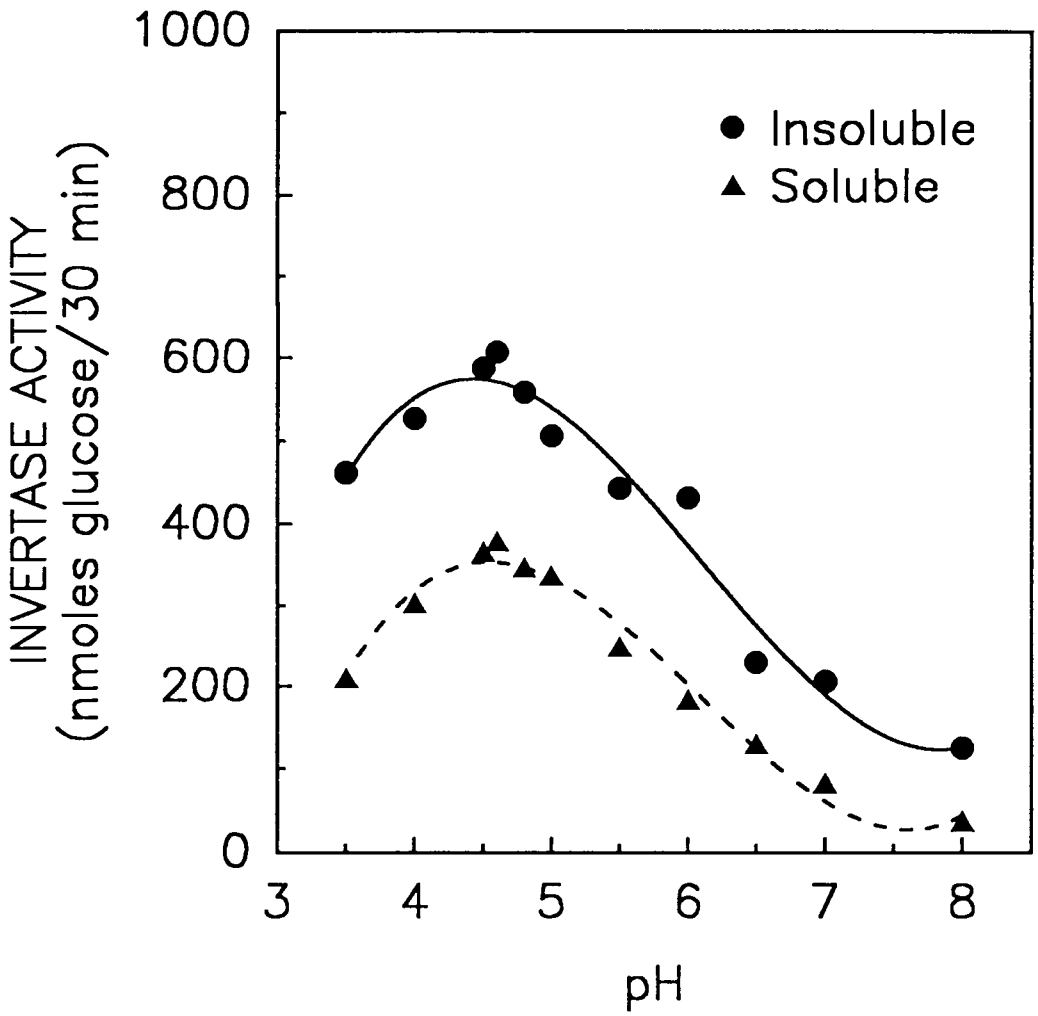

Figure 4-3. The pH dependence of sucrose uptake by strawberry fruit cortex protoplasts. The buffers used consisted of 0.6 M sorbitol, 25 mM $CaCl_2$, and 25 mM of citrate-phosphate (pH 4 and 5), 2(N-morpholino) ethanesulfonic acid (MES, pH 6), 3(N-morpholino) propanesulfonic acid (MOPS, pH 7) and N-2-hydroxy-ethylpiperazine propane sulfonic acid (EPPS, pH 8). Each point represents the mean ± S.E. of three replicates.

below pH 4.0 and above approximately pH 8.0. This is in agreement with the work of Forney and Breen (1986) who found that cortex tissue exhibited the greatest uptake of exogenous sucrose when in an incubation solution of pH 5.0. Protoplasts therefore appear to be a valid system for examining apoplastic sugar uptake in the strawberry.

The uptake kinetics for glucose, sucrose and fructose into protoplasts are shown in Figure 4-4. Glucose uptake was highest over the range studied with sucrose and fructose exhibiting much lower rates. Glucose uptake only showed saturable kinetics indicating the presence of a carrier-mediated uptake pathway. The V_{max} and K_M for glucose uptake calculated from the Lineweaver-Burk plot were 72 nmol$\cdot$(10^6 protoplasts)$^{-1}\cdot hr^{-1}$, and 27 mM, respectively.

Kinetic analysis for sucrose and fructose demonstrated two components (Figures 4-5 and 4-6, respectively). At low sucrose or fructose concentrations a saturable component, which appeared to obey Michaelis-Menton kinetics, was prevalent. Sucrose and fructose uptake kinetics can be modeled using the equation $v=[V_{max}S/(S+K_M)]+kS$ (Maynard and Lucas, 1982) where v is the rate of sugar uptake, V_{max} and K_M are the Michaelis-Menton constants, and k is the first-order rate coefficient. For sucrose uptake the values calculated for V_{max}, K_M, and k were 5.9 nmol$\cdot$(10^6 protoplasts)$^{-1}\cdot hr^{-1}$, 2.2 mM, and 0.508 nmol$\cdot$(10^6 protoplasts)$^{-1}\cdot hr^{-1}\cdot mmole^{-1}$, respectively. For fructose V_{max} was 6.4 nmol$\cdot$(10^6 protoplasts)$^{-1}\cdot hr^{-1}$, K_M was 2.9 mM,

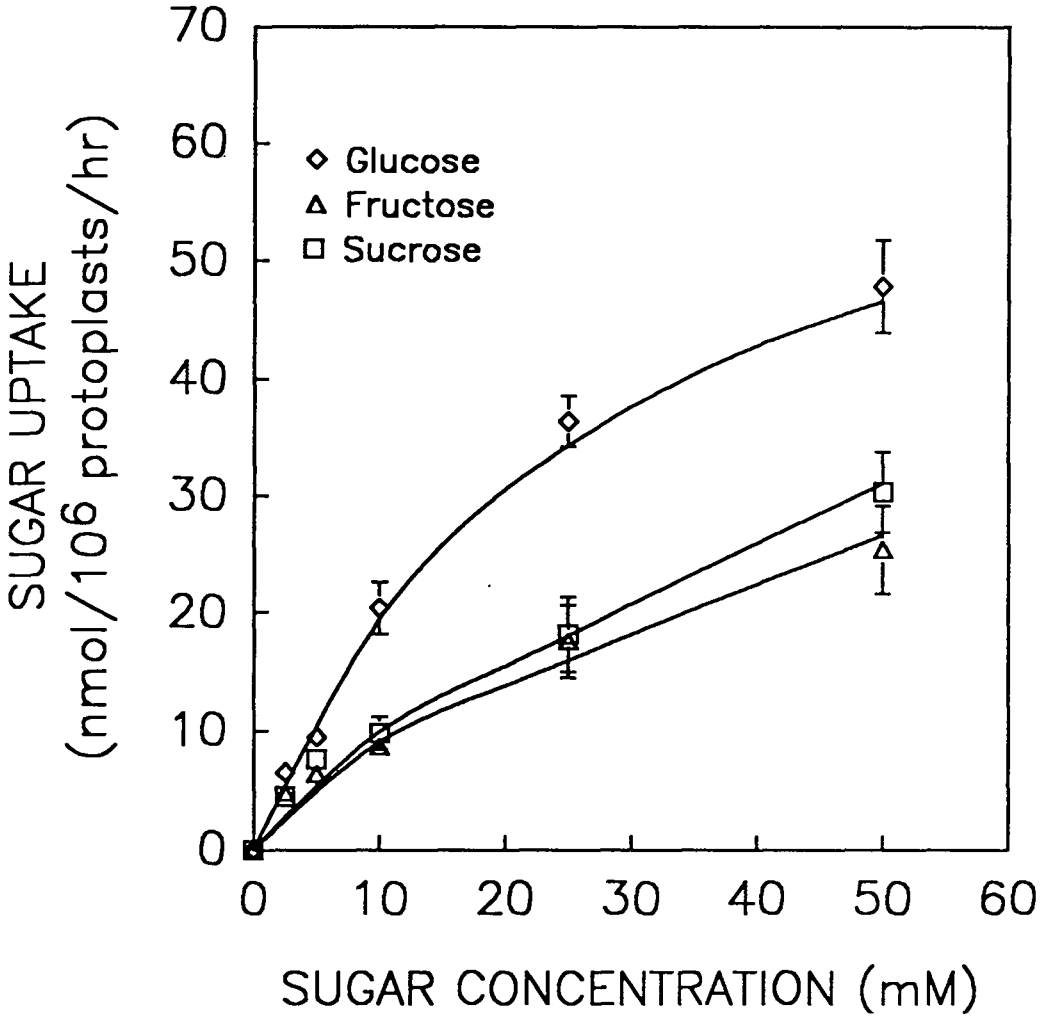

Figure 4-4. Kinetics of sucrose, glucose and fructose uptake by strawberry fruit cortex protoplasts. Each point represents the means ± S.E. of three replicates.

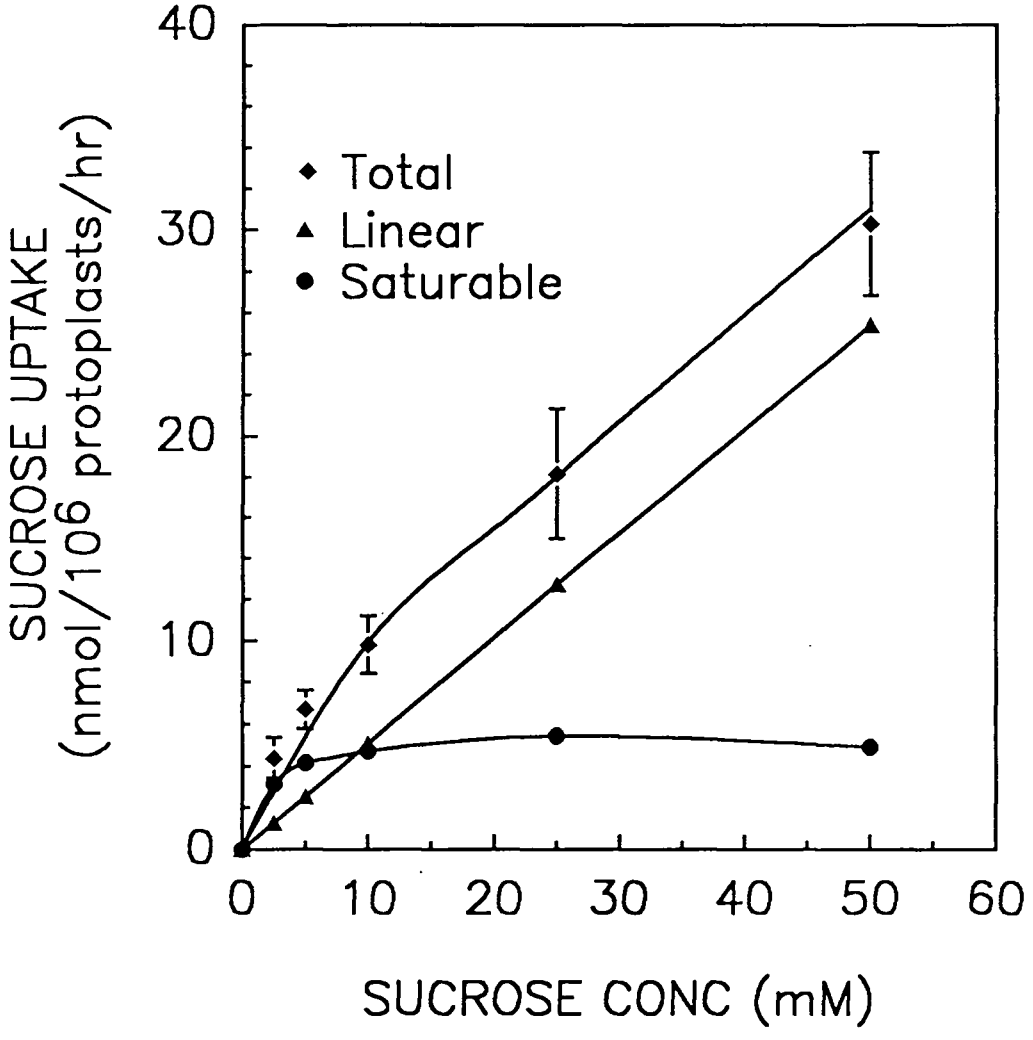

Figure 4-5. Concentration dependence of the total, linear and saturable components of sucrose uptake.

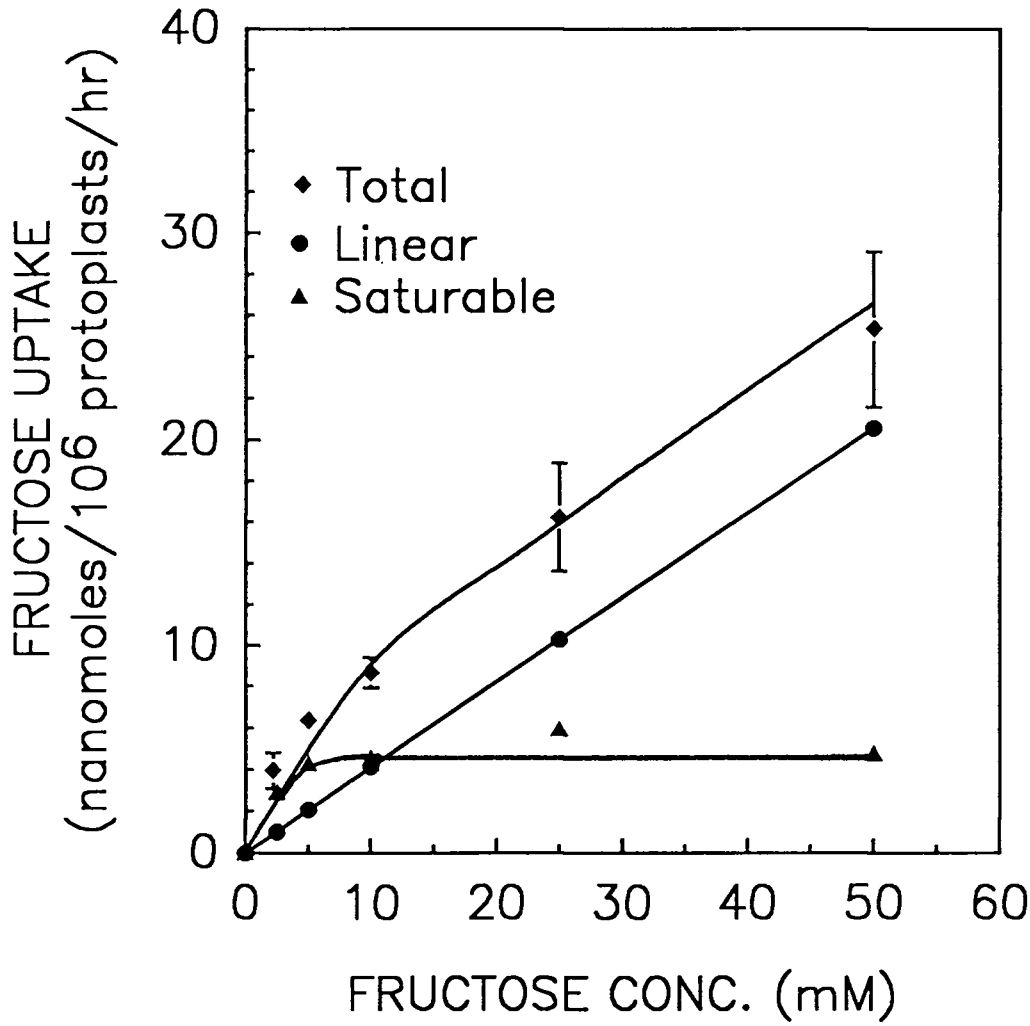

Figure 4-6. Concentration dependence of the total, linear and saturable components of fructose uptake.

and k was equal to 0.411 nmol·(10^6 protoplasts)$^{-1}$·hr^{-1} ·mmole^{-1}.

The shapes of sugar uptake curves for protoplasts were similar to those seen using strawberry fruit cortex tissue disks (Forney and Breen, 1986). However, the Michaelis-Menton constants (K_M) calculated for the uptake curves are not equal between the two systems (see Table 4-1). The protoplast system had lower K_Ms for all three sugars. There may be several reasons for this variation.

As noted earlier, the protoplast isolation procedure appeared to select for a given population of cells, e.g. parenchyma cells. Vascular tissue tended to not form protoplasts. Therefore, the protoplasts obtained may be thought of as coming from "vascular poor" tissue. The uptake kinetics of protoplasts and tissue disks may differ because each contains a different population of cell types. Differences in sugar uptake kinetics for "vascular rich" vs "vascular poor" cells derived from the same organ have been observed in red beet taproot (Getz et al., 1987a) and onion leaves (Wilson et al., 1985).

The incubation conditions were quite different between protoplasts and tissue disks. The protoplast solution by necessity contained a high concentration of sorbitol to prevent protoplasts from rupturing. Also, protoplasts were isolated in a solution of high osmolarity, conditions which allow uptake of the osmoticum into protoplasts (Robinson and Loveys, 1986). The protoplast isolation procedure may have

Table 4-1. A comparison of the Michaelis-Menton constant (K_M) calculated for glucose, sucrose or fructose uptake into protoplasts and tissue disks derived from the cortex of 'Brighton' strawberry fruit.

Sugar	Protoplasts K_M (mM)	Tissue Disks K_M[a] (mM)
Glucose	27	88
Sucrose	2.2	12.5
Fructose	2.9	13.2

[a]From Forney and Breen, 1986

altered characteristics of the plasma membrane, rendering it more permeable to the sugars tested, thus lowering the observed K_M values.

The tissue disk system contains the cell wall and intercellular spaces through which the incubation solution must diffuse. If the diffusion period is significant in relation to the incubation time, then the tissue may appear to have a lower than actual K_M. Also, in tissue disk systems it is possible to have retention of the sugars in the apoplastic spaces even after period of 'washout', leading to over estimations of cellular uptake by the tissue.

The K_M for glucose was several fold larger than that of either sucrose or fructose in both tissue disk and protoplast studies. Over the range of sugar concentrations studied, glucose uptake does not show any type of linear component. At physiological levels of glucose present in the fruit, diffusive or carrier mediated nonsaturable uptake of glucose is a probably negligible path for glucose uptake. (Chapter 5).

Both sucrose and fructose had uptake curves which were dominated by diffusive or carrier mediated nonsaturable components at concentrations above about 10 mM (Figures 4-5 and 4-6, respectively). It is probable that in intact fruit passive uptake is a major pathway for accumulation of these sugars from the apoplast.

Uptake curves similar to those observed for strawberry fruit tissue have been observed elsewhere. Glucose uptake into disks of sugar beet taproot tissue exhibits simple saturation kinetics whereas sucrose uptake is characterized by biphasic kinetics with a linear component dominant at higher substrate levels (Wyse et al., 1986). However, uptake of both glucose and sucrose show biphasic kinetics in protoplast from red beet taproot (Getz et al., 1987a) and developing soybean cotyledons (Lin et al., 1984). Thus it appears that sucrose uptake kinetics, in tissues that can take up sucrose, are generally biphasic in nature, while glucose uptake may show either simple saturation or biphasic kinetics. Fructose uptake has not been studied in many tissues but appears to exhibit biphasic uptake kinetics (Getz et al., 1987a).

The uptake curve for sucrose by protoplasts was similar in shape to that reported in tissue disks (Forney and Breen, 1986). This indicates that sucrose uptake by strawberry fruit cells at about 16 days post anthesis is not dependent upon hydrolysis of the sucrose by a cell wall invertase. This is in contrast to growing corn roots (Giaquinta et al., 1983), sugar cane stem (Komor et al., 1981) and growing pea stem (Singh and Maclachlan, 1986). In these tissues, uptake of supplied sucrose by cells requires that it first be hydrolyzed to glucose and fructose.

Examples of tissues which do not require hydrolysis of apoplastic sucrose for its uptake include sugar beet taproot

(Wyse, et al., 1986), developing soybean cotyledons (Lin et al., 1984) and tomato fruit (Damon et al., 1988).

Singh and Maclachlan (1986) postulate that growing tissues can't take up apoplastic sucrose directly, but depend upon hydrolysis of sucrose by cell wall invertase. These tissues may regulate sucrose uptake and their own energy metabolism by controlling invertase activity.

Tissues in which sucrose-specific uptake systems have been seen are all sucrose storage organs (Singh and Maclachlan, 1986). Here it is desirable to take up sucrose directly and therefore bypass the necessity of resynthesizing it for storage, and to also have a mechanism for retrieving sucrose which may leak from the tissue. In storage organs such as the sugar beet, which store large amounts of sucrose, leakage from the cell may be a problem. Sugar cane, however, does not appear to take up sucrose directly but does store large amounts of sucrose (Glasziou and Gaylor, 1972). Plants probably possess a variety of mechanisms for coping with the same process.

The strawberry fruit, at the stage studied here, is accumulating sucrose for storage (Forney and Breen, 1986). Therefore, a mechanism to take up sucrose directly from the apoplast would be advantageous if phloem unloading is apoplastic, or as a retrieval system if it is symplastic. At early stages in development, before sucrose accumulates, the system would aid in transporting more sugar into the fruit for use in metabolism.

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CHAPTER 5

APOPLASTIC SUGAR CONCENTRATIONS, AND UPTAKE COMPETITION IN STRAWBERRY FRUIT TISSUE

Abstract

Apoplastic concentrations of sucrose, glucose and fructose were estimated in cortex tissue of 16-18 days old strawberry fruit (Fragaria X ananassa Duch. cv Brighton). Sucrose and glucose were present in approximately equal levels (≈ 80 mM) whereas the concentration of fructose was estimated at 120 mM. Higher apoplastic fructose levels could result from hydrolysis of sucrose by an extracellular invertase followed by more rapid uptake of glucose. The apoplastic concentrations of sucrose and fructose were at levels where linear nonsaturable uptake is dominant in tissue disks, and possibly reflecting the importance of this component in vivo. Competition between the three sugars for uptake into cortex tissue disks was also examined. No competition was observed between any of the sugars tested indicating the presence of separate uptake systems for each. A sucrose analog, 1'-deoxy-1'-fluorosucrose, was not hydrolyzed by the fruit's cell wall invertase, and exhibited uptake kinetics similar to those of sucrose in cortex tissue disks. The results suggest that invertase hydrolysis of sucrose is not required for its uptake by the strawberry fruit.

Introduction

During the strawberry fruiting season, developing fruit are probably the strongest sink for photoassimilates. In established plants, over 40% of plant dry weight is recovered in the fruit at harvest (Olsen et al., 1985). How the fruit is able to attract photoassimilates so effectively is unknown. The in vitro kinetics of sucrose, glucose and fructose uptake by the strawberry fruit has been characterized (Forney and Breen, 1986). Sucrose and fructose have similar biphasic uptake kinetics. A saturable component is important at lower sugar levels (below 25 mM), with linear nonsaturable uptake dominating at higher concentrations. Glucose has much higher uptake rates than either sucrose or fructose and exhibits only saturable kinetics up to 200 mM concentration. It is not known, however, if sugars compete for uptake by a common carrier system or possess separate uptake pathways. This information is important if we are to understand the role apoplastic sugars play in sugar movement within fruit.

Sugar analogs are useful tools in examining the uptake kinetics and specificity of sugar transport systems. Uptake of sucrose across a cell membrane may either be directly via a sucrose-specific carrier (Delrot, 1981) or by extracellular hydrolysis by a cell wall invertase with uptake of the resulting hexoses (Giaquinta et al., 1983). The sucrose analog, 1'-fluorosucrose, is a useful compound

for studying sucrose uptake because it is recognized and transported by sucrose carrier proteins but poorly hydrolyzed by invertases (Hitz et al., 1985). Use of 1'-fluorosucrose makes it possible to determine whether hydrolysis of apoplastic sucrose by cell wall invertase is a prerequisite for its uptake by plant tissue.

The apoplastic sugar levels within the strawberry fruit have not been characterized, and so it is difficult to evaluate where in vivo conditions are described by uptake curves derived for the strawberry fruit. The objectives of this study were to characterize any competition between sucrose, glucose and fructose for uptake into strawberry fruit cells; to examine the role of cell wall invertase in the uptake of apoplastic sucrose; and to estimate the in vivo apoplastic concentrations of the three sugars.

Materials and Methods

Plant Material. Plants of the day-neutral strawberry 'Brighton' (Fragaria X ananassa Duch.) were grown in a greenhouse under a 16 hr photoperiod. Supplemental lighting was provided using high pressure sodium lamps (165 ± 20 $\mu mol \cdot m^{-2}s^{-1}$, 400-700 nm). Flowers and runners were removed for the first month to allow the plants to become well established. Thereafter, plants were allowed to flower but runners were still removed. All fully opened flowers were hand pollinated at anthesis and each succeeding day until petal fall (about 4 days post anthesis). Primary or secondary fruit, 16-18 days post anthesis (DPA), were used for all studies.

Sugar Competition Studies. Achenes were removed from harvested fruit by gentle scraping with a spatula. A 7 mm diameter plug of cortex tissue was removed from the fruit with a cork borer and sliced into 1.1 mm thick disks using a sharp razor blade and a plexiglass jig (Forney and Breen, 1986). The disks were equilibrated in a buffer solution consisting of 50 mM $CaCl_2$ and 20 mM Na-citrate (pH 5.0) for 30 min at 25°C.

^{14}C-sugar uptake measurements were performed using the method of Forney and Breen (1986). Ten tissue disks (approximately 0.52 g) were placed in a 50 ml Erlenmeyer flask containing 5 ml of equilibration buffer. To examine competition for uptake between sugars, ^{14}C-labeled sugars

(1480 Bq/mmol) and unlabeled competitor sugars were added to the solution. The disks were incubated for 2 hr at 25°C in the presence of ^{14}C-sugars and then rinsed for a total of 30 min in ice-cold buffer containing the same concentrations of unlabeled sugars. Flasks were shaken (60 revolutions per minute) throughout the incubation and rinse process. Disks were extracted overnight in 80% ethanol at 80°. The ethanol fraction was dried at 50° under vacuum and redissolved in 1 ml of distilled H_2O. The water was added to 15 ml of scintillation fluor (Beckman Ready Solv MP) and counted by liquid scintillation spectrometry. Counts obtained were corrected for background and internal quenching.

Preparation of 1'-Deoxy-1'-Fluorosucrose. Synthesis of 1'-deoxy-1'-fluorosucrose (1'-fluorosucrose, FS) was accomplished using a technique described by Hitz (Hitz et al., 1985; personal communication, 1988). The 1-deoxy-1-fluorofructose (1-fluorofructose) used was a generous gift from W.D. Hitz and E.I. du Pont de Nemours and Co.

A crude preparation of sucrose synthase (UDP-glucose:D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13) was used to synthesize [^{14}C]-glucosyl-FS from [^{14}C]-UDP-glucose and 1-deoxy-1-fluorofructose (Card and Hitz, 1984) (Figure 5-1). Wheat germ was used as a source of sucrose synthase.

Five grams of wheat germ were defatted by extracting three times with hexane, dried and ground thoroughly in 7 ml of ice-cold buffer (100 mM Hepes-KOH pH 7.0, 5mM EDTA, 5 mM DTT) in a prechilled mortar and pestle. The mixture was

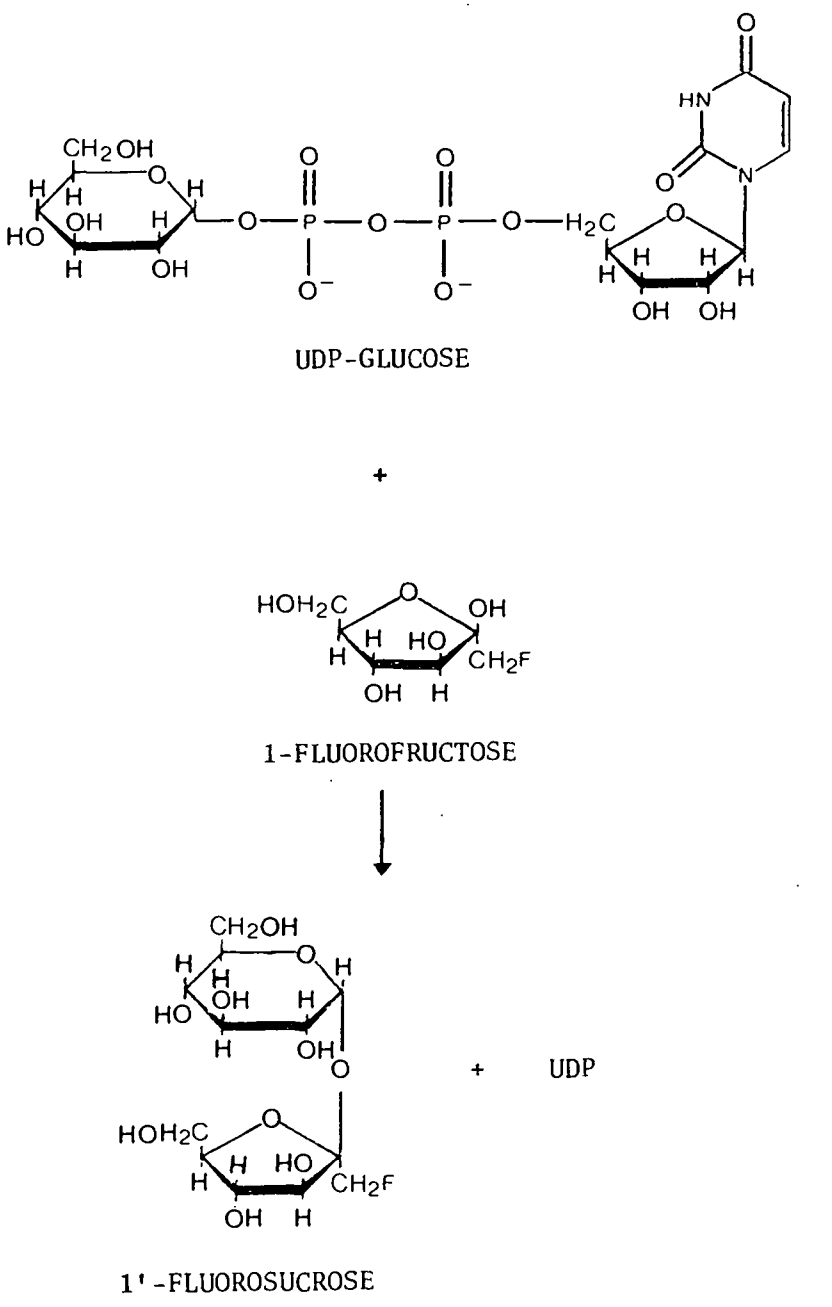

Figure 5-1. Synthesis of 1'-deoxy-1'-fluorosucrose.

squeezed through Miracloth and the supernatant collected and centrifuged at 10,000 x g for 10 min (Schmalstig and Hitz, 1987). The supernatant was recentrifuged for 30 min at 40,000 x g. The crude extract was precipitated with 70% saturation ammonium sulfate and centrifuged at 40,000 x g for 15 min. The pellet was washed with the same saturation, recentrifuged, redissolved in buffer, and de-salted by passage through a Sephadex G-25 column.

The coupling reaction was run at pH 7.5 in 50 mM Tris containing 5mM $CaCl_2$. A 3 molar excess of 1-fluorofructose was dissolved in 50 μl of reaction buffer containing 2.5 μCi (9.25 kBq, 0.011 μmoles) of [^{14}C]-UDP-glucose and added mixed with 50 μl of enzyme preparation in the same buffer. After 5 hr at 25^{o}C the reaction mixture was dried under vacuum and extracted twice with 95% ethanol to remove proteins. The extract was de-ionized by passage through 0.5 ml each of Dowex-50 (H^+) and Dowex-1 (OH^-). Extract (1 μl) was separated by thin layer chromatography along with unlabeled UDP-glucose on cellulose in 5:2 ethanol:(1 M ammonium acetate, pH 3.2) to make sure no radioactivity remained as UDP-glucose (Hitz, personal communication 1988). The UDP-glucose spot was visualized under UV, scraped and counted by liquid scintillation spectrometry (Beckman LS 7000). The recovered radioactivity was found to be all in 1'-fluorosucrose.

Invertase Cleavage of 1'-Fluorosucrose. Strawberry cell wall invertase was isolated from 16-18 DPA fruit using

the method of Poovaiah and Veluthambi (1985) (Chapter 3). Invertase activity was assayed at 30°C in a buffer containing 10 mM acetate (pH 4.6) and 10 mM sucrose with either [^{14}C]-sucrose or [^{14}C]-glucosyl-FS (40 nCi/mmol) added. Controls containing heat denatured invertase were also assayed. Aliquots were removed at given times and the reaction terminated by incubation in a boiling water bath for 5 min. The aliquots were cooled, evaporated to dryness under vacuum, and redissolved in 0.5 ml of 0.38M sodium phosphate buffer (pH 6.5). Samples were then added to 2 ml of a solution containing 25 units·ml^{-1} glucose oxidase in 0.38M sodium phosphate buffer and incubated at 25°C for 60 min. This incubation converted any glucose produced through invertase cleavage of sucrose or 1'-fluorosucrose to gluconic acid. The reaction was terminated by incubation in boiling water for 5 min, cooled, and passed through a Dowex 1-8X anion exchange column. The column was eluted with 2 N HCl and the eluent dried under vacuum. Samples were redissolved in distilled water, added to 15 ml of scintillation fluor and their radioactivity measured by liquid scintillation spectrometry.

Tissue Uptake of Sucrose and 1'-Fluorosucrose. Strawberry cortex tissue disks from fruit were obtained as described by Forney and Breen (1986). Sucrose uptake studies also followed the methods developed by Forney and Breen (1986) except radioactivity was increased to 40 nCi/mmol (1480 Bq/mmol). Sucrose was used as the unlabeled

sugar in uptake studies with [^{14}C]-glucosyl-FS or [^{14}C]-sucrose. Calculations for absolute rates of uptake were corrected for differences in the specific activity of the [^{14}C]-sugars used.

Apoplastic Sugar Determination. The concentrations of sucrose, glucose and fructose in the apoplast of the strawberry fruit was estimated using the null-balance technique of Gifford and Thorne (1985). Cortex tissue disks were obtained as described above. Disk thickness was increased to 3 mm. Ten disks were quickly rinsed in distilled water (for 5 sec), dab-dried for 10 sec between paper towels (Gifford and Thorne, 1985), and placed in 10 ml of 80% ethanol. These were later analyzed for initial sugar levels. The remaining disks were also rinsed quickly in distilled water and placed, in groups of ten, into bathing solutions containing increasing amounts of either sucrose, glucose or fructose. The samples were incubated at 25^{o}C and 60 revolutions per minute for 2 hr. Disks were then removed from the solutions and rinsed and dab-dried as described above. The rinsed and initial disks were extracted in three 10 ml changes of 80% ethanol at 80^{o} for 24 hr. Ethanol extracts from each sample were pooled, evaporated at 50^{o} under vacuum, and redissolved in distilled water.

The water solution was assayed for glucose levels using the glucose oxidase, peroxidase and o-dianisidine system from Sigma (Sigma Technical Bulletin No. 510). Sucrose levels were determined by first analyzing for glucose

concentration. Invertase (Sigma), 15 units per ml, was added and the solution heated for 2 hr at 50°C to hydrolyze any sucrose to glucose and fructose. The glucose level was then reanalyzed and the concentration of sucrose present was calculated from the amount of glucose produced after addition of invertase. Fructose was analyzed using a colorimetric method described by Ting (1956).

Initial sugar levels in disks were subtracted from the levels present after incubation and the change expressed as a relative proportion to the initial value.

Results and Discussion

The uptake rates of exogenously applied sucrose, glucose or fructose were not affected by the presence of the other two sugars (Tables 5-1, 5-2, and 5-3). This would indicate that the three sugars have separate mechanisms for uptake in strawberry fruit. The uptake kinetics of these sugars has been characterized by Forney and Breen (1986). Glucose uptake exhibits simple saturation kinetics up to 200 mM; however, sucrose and fructose uptake shows biphasic kinetics. A saturable component is dominant below approximately 20 mM with a linear nonsaturable component becoming predominant as exogenous sucrose and fructose concentrations increase (Forney and Breen, 1986). Our results indicated that neither the saturable nor linear components were competitively inhibited by the presence of glucose, fructose or sucrose.

Similar studies conducted with other plants have given a variety of results. Separate sucrose and hexose carriers were reported in Vicia faba leaf tissue (Delrot, 1981) and cotton hypocotyls (Hampson et al., 1978) while competition between sucrose and hexoses has been seen in sugar beet taproot (Wyse, 1979) and lily pollen (Deshusses et al., 1981).

Glucose and fructose have been observed to possess separate pathways for uptake in pea stem (Singh and Maclachlan, 1986), and Streptanthus tortuosus suspension

Table 5-1. Uptake of ^{14}C-sucrose into strawberry fruit disks in the presence of increasing levels of either glucose or sucrose. The data represents the mean ± S.E. of four replications.

^{14}C-Labeled Sucrose (mM)	Unlabeled Sugar (mM)	^{14}C-Uptake (μmol/gFW/hr)
	Glucose	
10	0	0.47 ± 0.08
10	10	0.41 ± 0.05
10	100	0.42 ± 0.10
100	0	2.25 ± 0.13
100	10	2.19 ± 0.11
100	100	2.14 ± 0.15
	Fructose	
10	0	0.47 ± 0.08
10	10	0.48 ± 0.04
10	100	0.44 ± 0.06
100	0	2.25 ± 0.13
100	10	2.21 + 0.10
100	100	2.18 ± 0.17

Table 5-2. Uptake of ^{14}C-glucose into strawberry fruit in the presence of increasing levels of either sucrose or fructose. The data represents the mean ± S.E. of four replications.

^{14}C-Labeled Glucose (mM)	Unlabeled Sugar (mM)	^{14}C-Uptake (μmol/gFW/hr)
	Sucrose	
10	0	0.97 ± 0.04
10	10	0.95 ± 0.06
10	100	0.92 ± 0.06
100	0	4.53 ± 0.27
100	10	4.51 ± 0.19
100	100	4.46 ± 0.23
	Fructose	
10	0	0.97 ± 0.04
10	10	1.00 ± 0.08
10	100	0.96 ± 0.09
100	0	4.53 ± 0.27
100	10	4.42 ± 0.18
100	100	4.47 ± 0.17

Table 5-3. Uptake of ^{14}C-fructose into strawberry fruit in the presence of increasing levels of either glucose or sucrose. The data represents the mean ± S.E. of four replications.

^{14}C-Labeled Fructose (mM)	Unlabeled Sugar (mM)	^{14}C-Uptake (μmol/gFW/hr)
	Glucose	
10	0	0.39 ± 0.05
10	10	0.36 ± 0.06
10	100	0.34 ± 0.09
100	0	1.91 ± 0.09
100	10	1.87 ± 0.11
100	100	1.88 ± 0.06
	Sucrose	
10	0	0.39 ± 0.05
10	10	0.37 ± 0.07
10	100	0.33 ± 0.09
100	0	1.91 ± 0.09
100	10	1.92 ± 0.06
100	100	1.85 ± 0.08

cells (Stanzel et al., 1988). Wyse (1979) postulated the presence of separate carrier systems for glucose and fructose in sugarbeet taproot but showed competition between the two sugars for uptake in sugar beet suspension cells (from hypocotyl tissue) (Zamski and Wyse, 1985). This variation in mechanisms makes it difficult to generalize regarding sugar uptake in plants. The types of transport systems functioning are probably tissue specific and may change during plant growth. Singh and Maclachlan (1986) postulate that specific sucrose uptake system are only found in those sinks which store sucrose. The strawberry fruit is such a sink (Selvaraj et al., 1976; Forney and Breen, 1985.).

1'-Fluorosucrose Uptake and Cleavage. Uptake of [^{14}C]-1'-fluorosucrose also supported the idea of the existence of a separate sucrose uptake system in strawberry fruit cortex. 1'-Fluorosucrose was not hydrolyzed by the fruit's cell wall invertase to an appreciable extent (Figure 5-2), however; uptake rates of tracer levels of [^{14}C]-1'-fluorosucrose by cortex tissue disks, in the presence of unlabeled sucrose, were only slightly lower than those of [^{14}C]-sucrose under similar conditions (Figure 5-3). The lower uptake of 1'-fluorosucrose may be due to a slightly lower affinity for the sucrose carrier which predominates at low sucrose concentrations.

The ability of tissue disks to accumulate 1'-fluorosucrose without hydrolysis to hexoses is evidence for

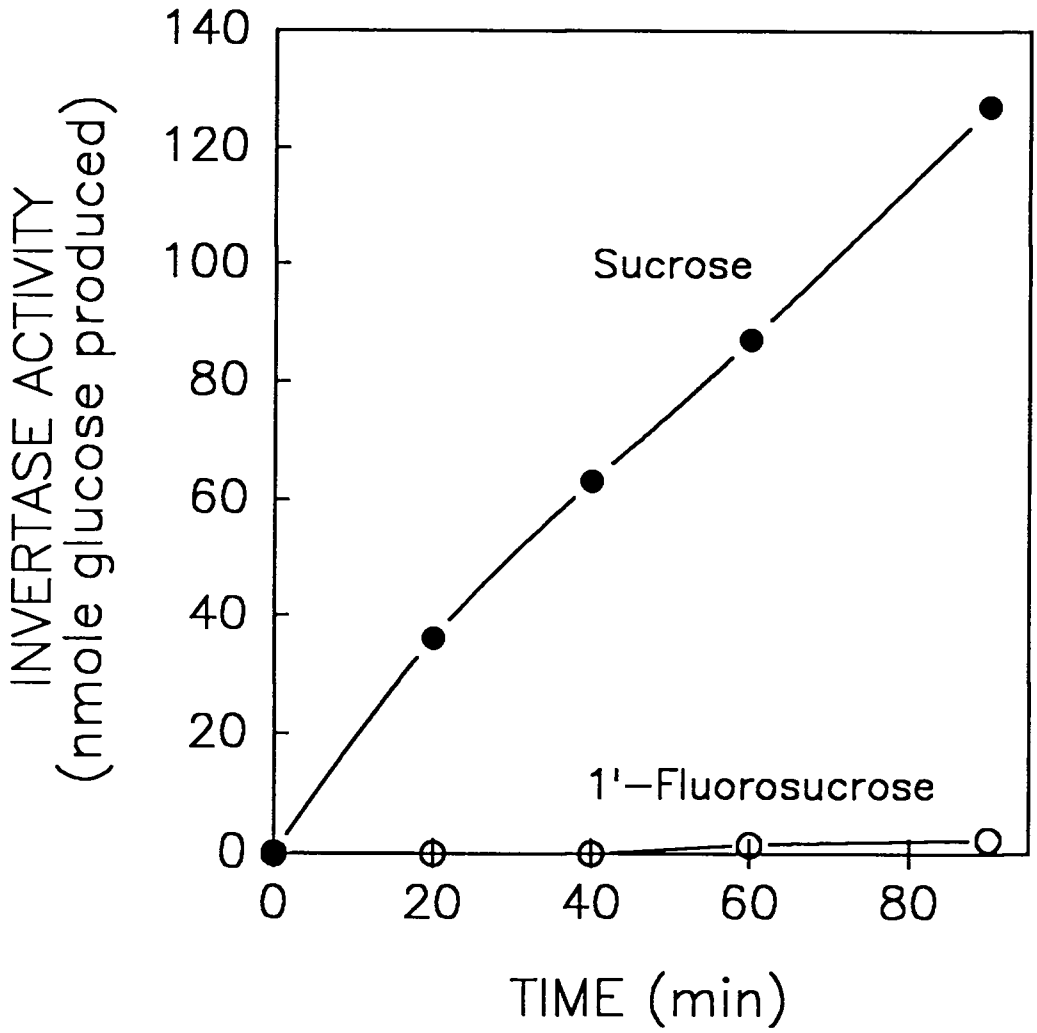

Figure 5-2. Cleavage of sucrose and 1'-fluorosucrose by strawberry fruit cell wall invertase.

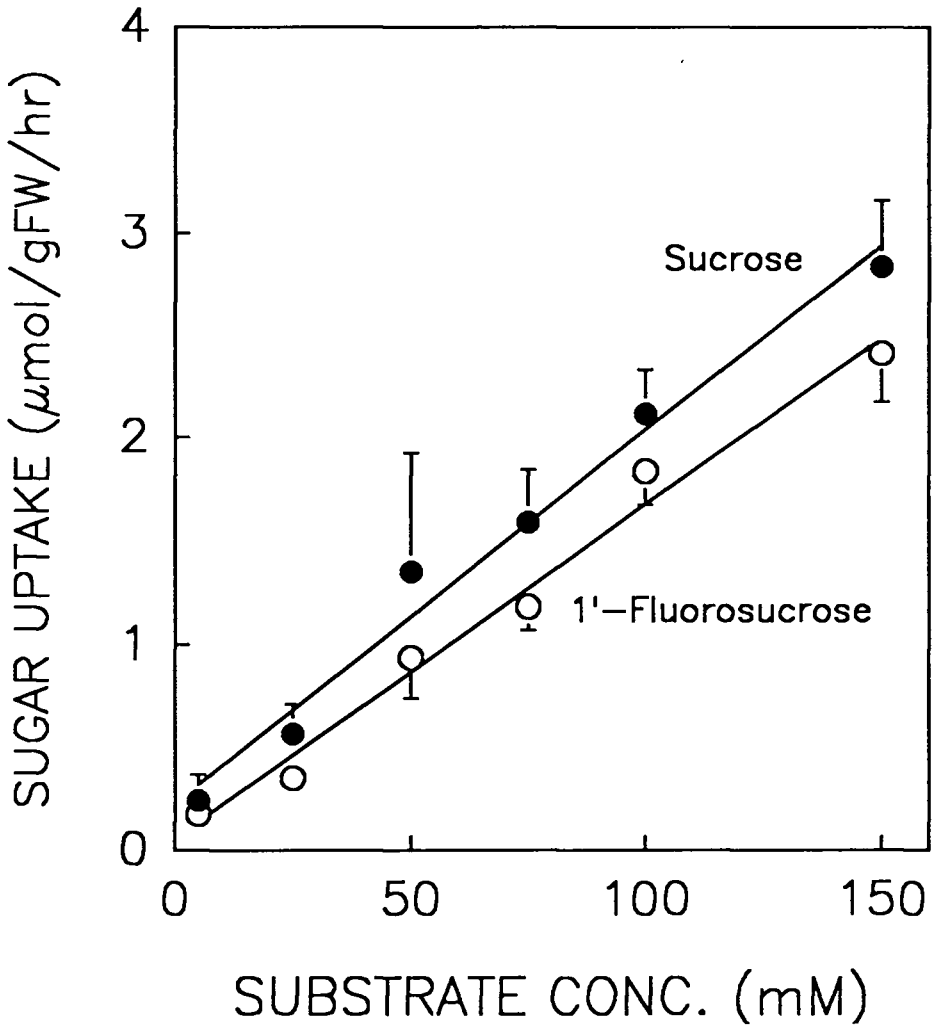

Figure 5-3. Uptake of sucrose and 1'-fluorosucrose by 'Brighton' fruit cortex disks. Points represent the mean ± SE of three replications.

a separate sucrose carrier system operating in the strawberry cortex. Similar results are seen in soybean cotyledon protoplasts (Hitz et al., 1985) and tomato fruit (Damon, et al., 1988) where 1'-fluorosucrose uptake into the tissues does not require its hydrolysis. In soybean cotyledon protoplasts, the rate of 1'-fluorosucrose uptake was higher than that of sucrose (Hitz et al., 1985).

Apoplastic Sugar Levels. The work of Forney and Breen (1986) has characterized the uptake of sucrose, glucose and fructose over a range of 0-200 mM. However, the in vivo apoplastic concentrations of the three sugars in the strawberry fruit are not known; thus, it is difficult to decide how important the different uptake components are to the developing plant. We have attempted to estimate the apoplastic sugar concentrations using the null-balance method described by Gifford and Thorne (1985). The developing strawberry fruit has a certain pool-size for each of the three sugars. This pool-size should be relatively constant over short periods of time (a few hours) in vivo. If the tissue is placed in a bathing solution having a sucrose concentration equal to the in vivo apoplastic concentration, then the tissue should remain in equilibrium and the concentration of sucrose within the tissue should remain relatively constant. The same rationale should apply for glucose and fructose. If the sugar concentration of a bathing solution is lower than the apoplastic concentration, a net diffusion of sugar out of the tissue should result.

If the bathing medium has a higher sugar concentration than that *in vivo*, then a net movement of sugar into the tissue would be expected. By comparing tissue sugar levels pre- and post-incubation and graphing the proportional change in levels, a line fitted through the data should indicate a sugar concentration where no net change in tissue sugar levels would occur. This point is interpreted to equal the apoplastic sugar concentration present in the tissue.

The null-balance method gave an estimate of about 80 mM for the apoplastic concentrations of both glucose and sucrose (Figures 5-4 and 5-5, respectively). The fructose level was estimated to be about 120 mM (Figure 5-6). In developing soybean cotyledons, the same technique predicts an apoplastic sucrose concentration of about 150 mM (Gifford and Thorne, 1985).

If these concentrations accurately described apoplastic sugar levels of intact fruit, then using the uptake curves found by Forney and Breen (1986), both sucrose and fructose uptake are dominated by a linear nonsaturable component. Glucose uptake does not show a linear component presumably because the glucose carrier is not saturated at levels below 200 mM (Forney and Breen, 1986). The observed apoplastic glucose concentration of about 80 mM was close to calculated K_Ms of 88mM using tissue disks (Forney and Breen, 1986) and 77mM for cortex protoplasts (Chapter 4).

One question raised by these results is why is the apoplastic concentration of fructose higher than those of

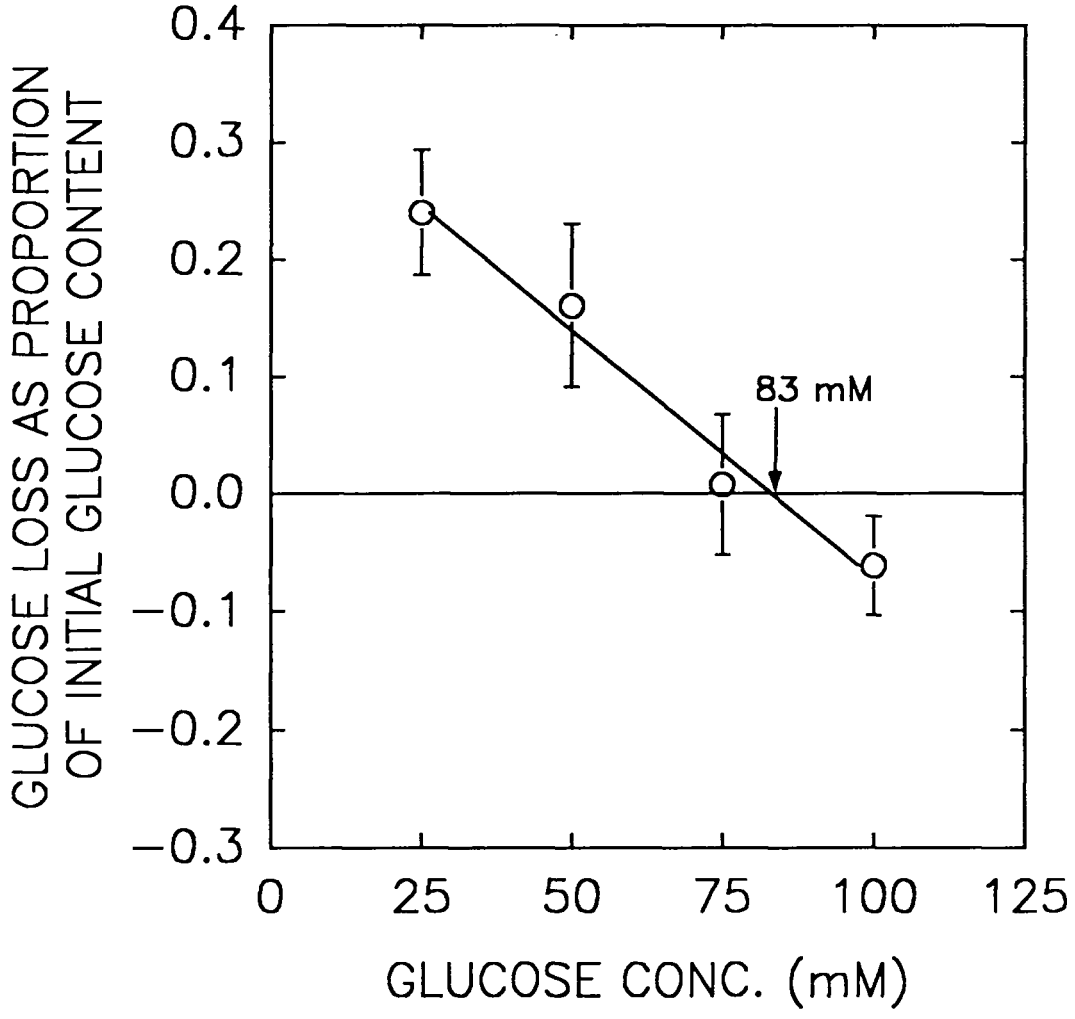

Figure 5-4. The apoplastic glucose concentration in 'Brighton' strawberry fruit as determined by the null-balance method. Points represent the mean ± SE of three replicates.

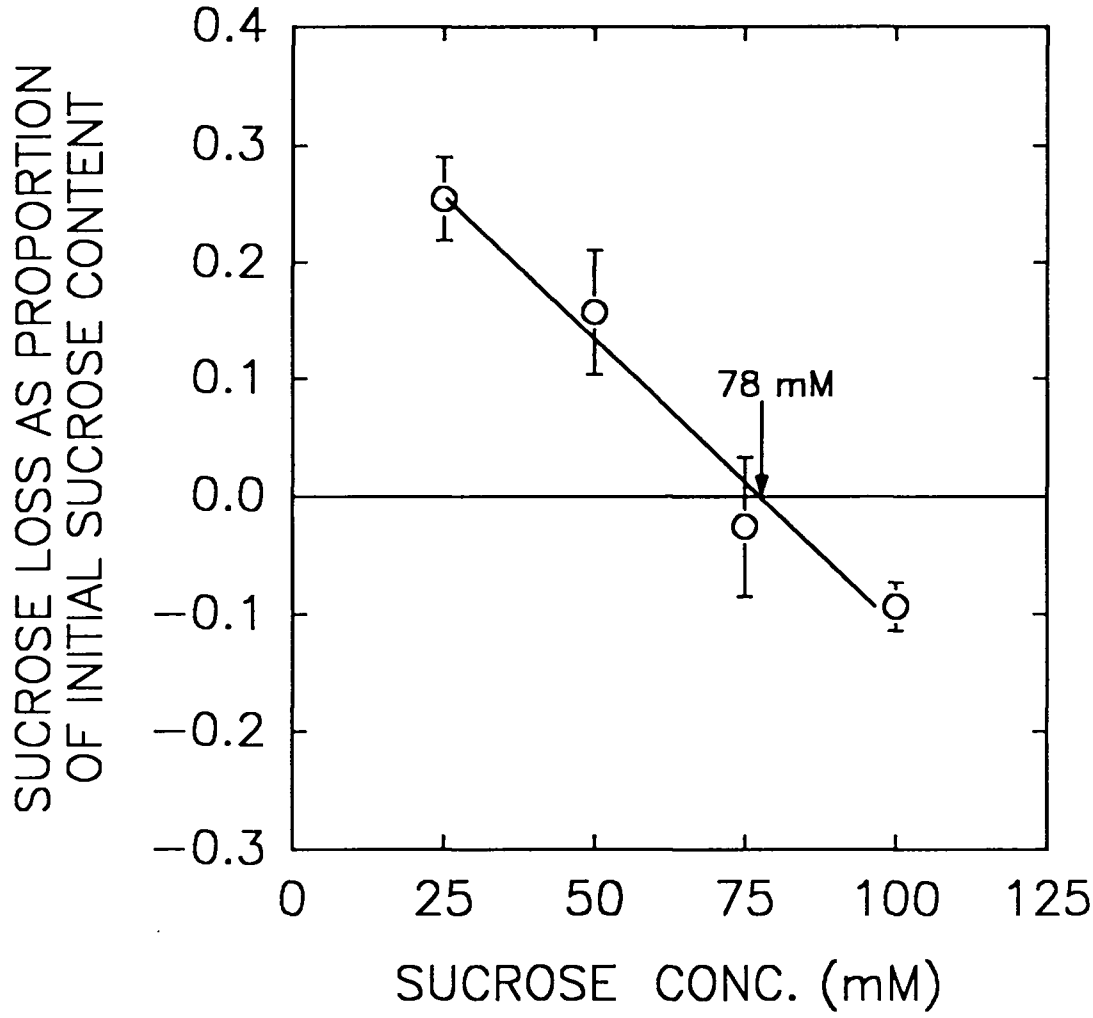

Figure 5-5. The apoplastic sucrose concentration in 'Brighton' strawberry fruit as determined by the null-balance method. Points represent the mean ± SE of three replicates.

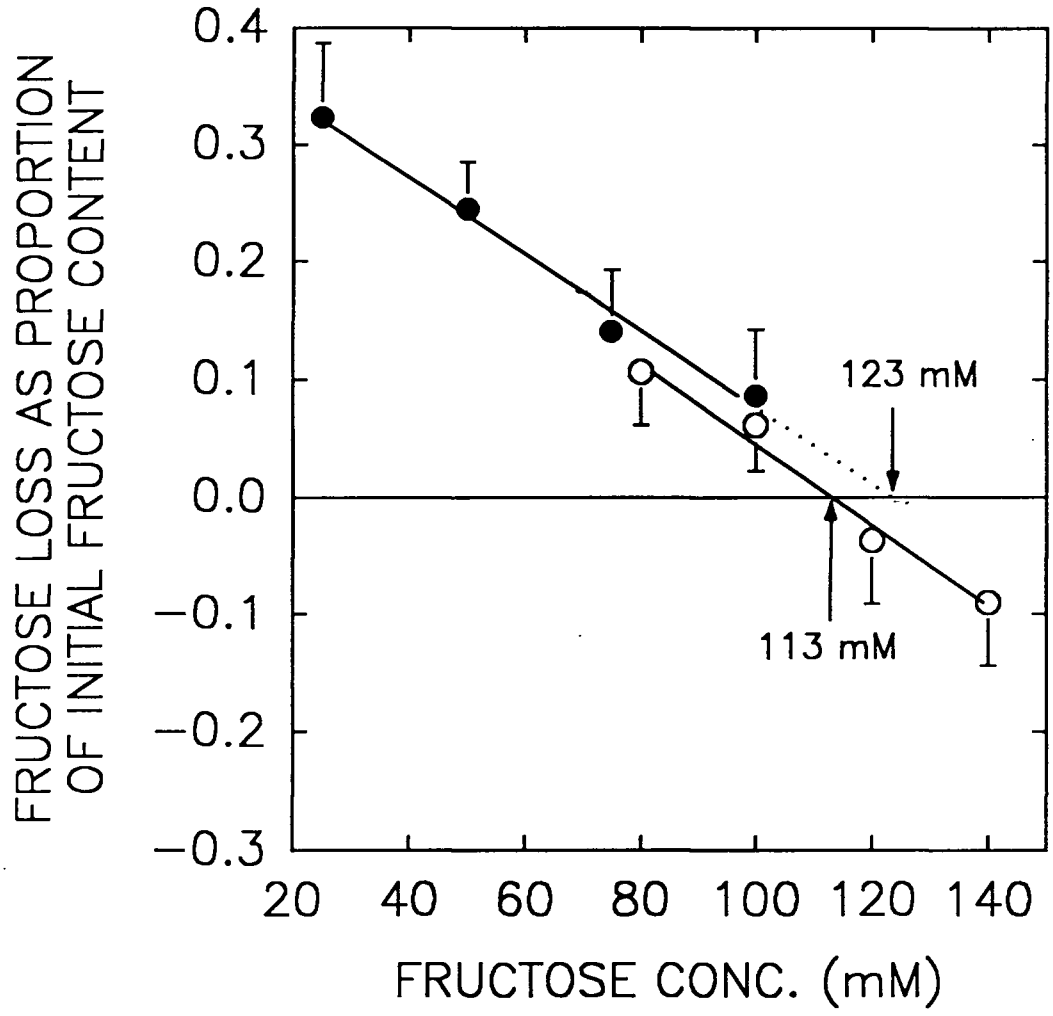

Figure 5-6. The apoplastic fructose concentration in 'Brighton' strawberry fruit as determined by the null-balance method. Points represent the mean ± SE of three replicates.

glucose or sucrose? The strawberry fruit possesses a cell wall-bound invertase (β-fructofuranosidase, EC 3.2.1.26) (Poovaiah and Veluthambi, 1985; Chapter 3), and presumably, a portion of the sucrose present in the apoplast is hydrolyzed to glucose and fructose. Since glucose uptake by cortex tissue is more rapid than fructose (Forney and Breen, 1986; Chapter 4), it could be expected to accumulate in the apoplast to a greater extent than glucose. The total fructose present in 'Brighton' fruit at 16-18 days post anthesis is slightly higher than glucose or sucrose levels (Forney and Breen, 1986), which may also partially explain the measured differences in apoplastic levels.

Sugar uptake systems in strawberry fruit almost certainly function to retrieve sugars which may "leak" from the cells, and as such are important in maintaining the internal sugar supply. Whether these systems also play a direct role in phloem unloading in the strawberry is unknown. It can be argued that any system which influences the movement of sugars within the fruit is, either directly or indirectly, involved in phloem unloading. Sugar uptake systems likely exert some control over the apoplastic concentrations of osmotically active sugars. The osmotic environment of sink tissues is known to influence the rate of sugar movement within a variety of sinks (Wyse et al., 1986; Oparka and Wright, 1988a,b). Sugar uptake, in strawberries, may also effect turgor and therefore exert control over sugar transport within the fruit.

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CHAPTER 6

EPILOGUE

The quality and yield of the strawberry is determined by many factors acting in concert. Environment, cultural practices, and genetic characteristics all interact to affect the plant's ability to produce a "good" strawberry. While the environment is difficult to control in a commercial operation, improved growing techniques and new cultivars offer opportunities for enhancing the crop.

Little is known about the processes which, in total, make the strawberry fruit a competitively successful sink organ. Since sugars, particularly sucrose, are the primary organic material translocated by the plant, an understanding of the fruit's mechanisms for assimilating sugars may give fresh ideas for approaches to improving production.

In these studies, invertase levels were followed during development of the fruit. Soluble invertase activity decreased as development progressed; this decrease may be important in determining when, and to what extent, sucrose accumulates within storage cells. Manipulations of soluble invertase activity could be one method of increasing the final sucrose content within fruit.

Unfortunately, the mechanisms regulating soluble invertase activity in strawberry fruit, as in other plants, are unknown. Proteins which inhibit invertase activity are

present in some plant parts and have dramatic effects in terms of sucrose transport and accumulation. These inhibitory proteins have not been investigated in strawberries. Experiments to determine if soluble invertase activity is controlled by its rate of synthesis, destruction, or by inhibitor proteins (or a combination of the above) would be valuable.

Cell wall invertase is found to increase rapidly after pollination, but then stabilizes at days 5 through 25 (near ripeness). It has been postulated that extracellular invertase is important in promoting the fruit's expansion. Auxin, which promotes receptacle expansion early in development, also stimulates the synthesis of extracellular invertase (Poovaiah and Veluthambi, 1985). Previous work has demonstrated uptake of sugars from the apoplast in strawberry fruit tissue and suggests that apoplastic phloem unloading may occur (Forney and Breen, 1986). In this case, cell wall invertase could be important in maintaining a sucrose gradient between the phloem and surrounding tissue by hydrolyzing sucrose in the apoplast.

Cell wall invertase activity was found to be markedly affected by pH with an optimum at 4.6. Because its activity <u>in vitro</u> is stable from day 5 through 25 it would be interesting to see if the apoplastic pH changes over this time period. Changes in apoplastic pH could regulate the enzyme's activity <u>in vivo</u>.

Studies in which apoplastic unloading has been observed have generally shown poor sucrose uptake by cells in the absence of cell wall invertase. Strawberry fruit protoplasts were found to take up sucrose and had uptake curves similar in shape to those of tissue disks. Uptake of the sucrose analog, 1'-fluorofructose, into tissue disks also had kinetics very similar to sucrose, and no competition for uptake was observed between sucrose, glucose or fructose. These results would indicate that sucrose uptake occurs by a pathway separate from that of hexose uptake. This does not rule out a role for cell wall invertase in phloem unloading in the fruit; it just can not be confirmed by these studies.

Histochemical staining shows an apparent increased concentration of invertase near the vascular bundles compared to the parenchyma tissue as a whole. This result again suggests some role in phloem unloading for cell wall invertase. If problems with excessive tissue oxidation in young fruit can be overcome, staining of younger fruit would be valuable to determine if invertase distribution is constant or changes with development. By using controlled enzymatic digestion of strawberry fruit, it may be possible to isolate vascular strands from the rest of the fruit tissue. It would be interesting to compare the uptake kinetics for sucrose, glucose and fructose between "vascular rich" and "vascular poor" tissue. Differences in uptake kinetics could indicate specialized sugar transport system

for phloem unloading in tissue near the vascular bundle. Using such a system would allow invertase levels surrounding the vascular strand to be more accurately determined and could confirm a localization of invertase near the vascular system.

Sugar concentrations in the fruit apoplast were determined to be approximately 80 mM for both sucrose and glucose, and 120 mM for fructose. Higher fructose levels could be the result of extracellular hydrolysis of sucrose by cell wall invertase and a more rapid uptake of the glucose produced. The apoplastic solution appears to have a relatively high osmolarity, which may lower the cell turgor in the fruit. Decreased cell turgor has been seen to enhance sugar transport and uptake in other plants. If phloem unloading is apoplastic in the strawberry fruit, cell wall invertase could have a dual purpose: 1) to stimulate passive sucrose unloading by maintaining lower apoplastic sucrose levels and 2) to promote sugar uptake into storage cells, producing hexoses which would lower cell turgor pressure more effectively than a equivalent weight of sucrose. Research geared towards examining cell turgor effects on sugar uptake and metabolism would be valuable.

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APPENDIX

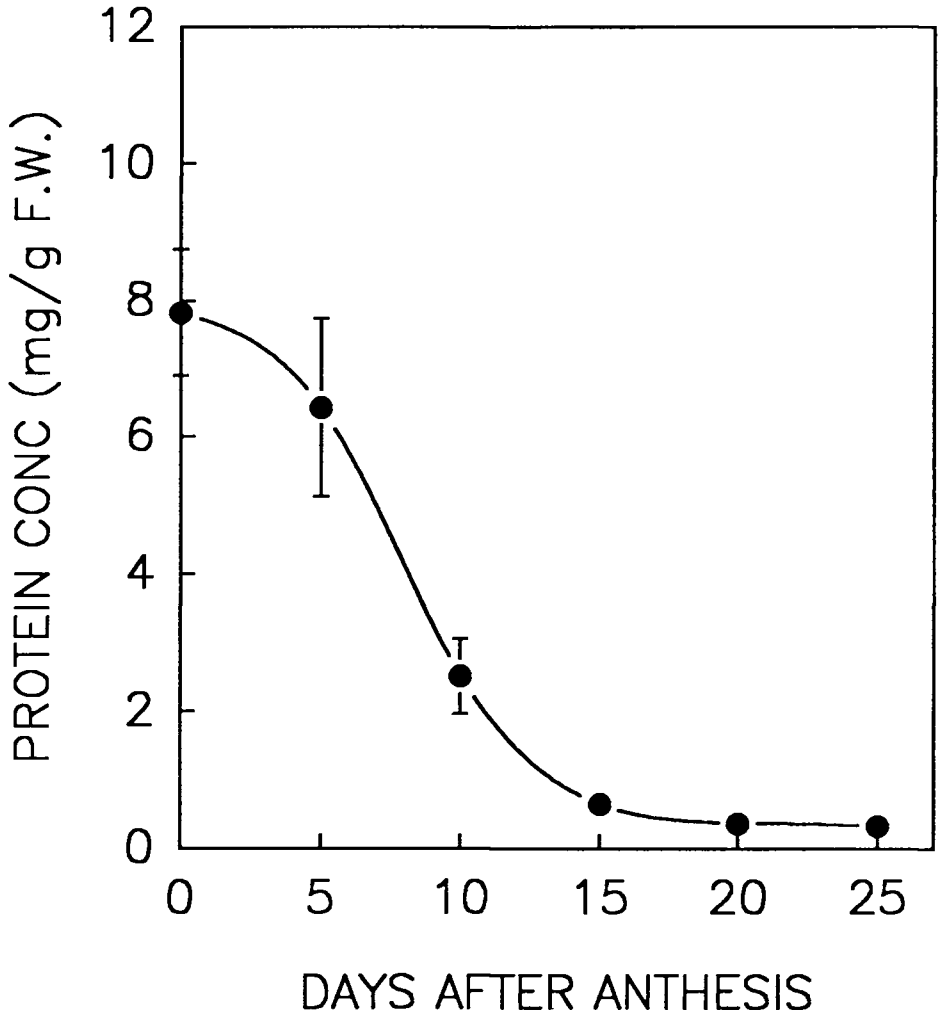

Figure A-1. Changes in protein levels in 'Brighton' strawberry fruit during development. Each point represents the mean ± SE of four replicates.