Starch Degradation in Buttercup Squash (*Cucurbita maxima*)

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ABSTRACT. Extractable activities of α -amylase, β -amylase, and starch phosphorylase were investigated in order to understand the mechanism of starch degradation in buttercup squash (*Cucurbita maxima* Duchesne ex Lam. 'Delica') with the ultimate goal of improving the conversion of starch into sweet sugars. During rapid starch synthesis (0 to 30 days after flowering), extractable activities of α -amylase and β -amylase were low, but those of starch phosphorylase increased. After harvest, during ripening at 12 °C, or in fruit left in the field, activities of α -amylase and β -amylase increased. Starch contained 20% to 25% amylose soon after starch synthesis was initiated and until 49 days after harvest irrespective of whether the crop remained in the field or in storage at 12 °C. Maltose concentrations were low prior to harvest, but levels increased during fruit ripening. Data suggest starch breakdown is hydrolytic in buttercup squash, with α -amylase being the primary enzyme responsible for initiating starch breakdown.

Buttercup squash (*Cucurbita maxima*) is a significant export crop of New Zealand. New Zealand-grown squash competes with Mexican-grown squash in the Japanese market, and Mexican squash seems to be sweeter than the New Zealand product and commands a higher price. We chose to conduct research on buttercup squash believing that the market position of New Zealand squash can be improved with a better understanding of starch breakdown.

Buttercup squash is a starchy-fruit vegetable, and most of the starch accumulates during the 'maturation' phase of fruit growth (Irving et al., 1997). The major sugar influencing flavor is sucrose, but glucose and fructose also make a contribution (V.K. Corrigan et al., unpublished). These sugars accumulate during postharvest storage (the ripening phase, Irving et al., 1997), and it is during this time, when the starch is degrading, that the true flavor and sweetness of cooked squash comes to the fore (V.K. Corrigan et al., unpublished).

The breakdown of starch in plant storage organs is thought to involve, at least initially, α -amylase, because this is the only enzyme known to attack raw starch granules (Beck and Ziegler, 1989; Sivak and Preiss, 1998). The cooperative action of a range of other degrading enzymes, including β -amylase, debranching enzyme, starch phosphorylase, and α -glucosidase, result in the dissolution of the stored starch through the production of a number of oligosaccharides, leading finally to maltose, glucose or glucose-1-phosphate. Sugar synthetic enzymes, such as sucrose phosphate synthase (Cordenunsi and Lajolo, 1995; Hill et al., 1996; Irving et al., 1997; MacCrae et al., 1992), would continue the passage of carbon from starch into sucrose, thus giving rise to the major flavor component in banana (*Musa × paradisiaca* L.), kiwifruit (*Actinidia deliciosa* C. F. Liang and A. R. Ferguson), Irish potato (*Solanum tuberosum* L.), and buttercup squash.

Starch phosphorylase catalyzes a reversible reaction, and can

degrade glucose polymers to glucose-1-phosphate or synthesize starch polymers, depending on the levels of substrates and products (Stitt and Steup, 1985). Some researchers have provided evidence that starch phosphorylase has a major role in starch breakdown in banana (Hill and ap Rees, 1994), even though amylases have been detected (Garcia and Lajolo, 1988). MacCrae et al. (1992) provided data to support phosphorolytic starch breakdown in kiwifruit, but subsequent studies (Wegryzen and MacCrae, 1995) revealed an active α -amylase. Morrell and ap Rees (1986) concluded potato starch was degraded by phosphorolytic attack because they could measure no amylase, but since then, other researchers have detected amylolytic activity (Nielsen et al., 1997). Phillips (1946) found no evidence for maltose in samples taken between harvest and 6 months storage, in 'Blue Hubbard', a cultivar of *C. maxima*, taking this as evidence that starch breakdown was catalyzed by phosphorylase rather than amylase. Nevertheless, Nagao and Indou (1991) detected α -amylase activity during early fruit development, which increased along with higher concentrations of sugars, some 40 to 45 d after flowering when starch content began to decline. Their data suggest amylolytic enzymes may catalyze starch degradation in *C. maxima*. In this communication, we report on changes in extractable activities of some starch degrading enzymes, both to increase our knowledge of starch degradation in buttercup squash, and to test the hypothesis of Phillips (1946), that starch breakdown is phosphorolytic.

Materials and Methods

PLANT MATERIAL. Seeds of 'Delica' squash were purchased from Yates Seeds (Auckland, New Zealand) and sown 19 Nov. 1997, 0.5 m apart within rows with 3 m between rows, as described previously (Irving et al., 1997). The rows were 30 m long. During the season, the crop was irrigated twice, and weeds were controlled by hand tillage. At flowering, the expanded ovaries of at least 200 flowers were tagged and six fruit were harvested at frequent intervals, and samples of mesocarp removed for maltose and starch extraction and analysis, or enzyme extraction and assay. Samples of the edible mesocarp were taken

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from the fruit equator, on the side that was exposed to the light during crop growth. At 50 d after flowering, the fruit were mature, and half of those that remained in the field were harvested, washed, dried, and stored at 12 °C for up to 49 d. The other half of the crop remained intact in the field. The average daily temperatures in the field ranged from 15 to 10.7 °C from harvest until the end of storage. At regular intervals after harvest, fruit were removed from storage and the field for biochemical analyses.

SUGAR EXTRACTION AND ASSAY. Freeze-dried squash samples (10 mg, dry weight) were ground and extracted in 1 mL of distilled water for 40 min at 60 °C. Maltose was quantified using a maltose kit (Boehringer Mannheim, Germany, catalogue no. 1 113 950), and results were converted into a fresh weight basis from measurement of the dry matter content. Maltose was also identified in methanol extracts (62.5% v/v) of squash mesocarp using paper chromatography (Harborne, 1984).

ENZYME EXTRACTION AND ASSAYS. Frozen squash samples (2 g, fresh weight) were ground, in a mortar on ice, in 10 ml extraction buffer composed of 50 mM Hepes-KOH (pH 7.5), 10 mM MgCl₂, 10% (v/v) glycerol, 2 mM Na₂EDTA, 25 mM dithiothreitol, 1 g·L⁻¹ bovine serum albumen, 3 mM CaCl₂, and 1.0 M NaCl. The supernatant was desalted through Sephadex G-25 mini-columns, frozen in liquid nitrogen, and stored at -80 °C. Activities in all assays were proportional to the volumes of extract added, and were linear for the duration of the assays.

Assays were carried out using the Megazyme (Sydney, Australia) Ceralpha kit (catalogue no. K-CERA) for α -amylase, and the Betamyl kit (catalogue no. K-BETA) for β -amylase. These kits are designed for measurement of α -amylase and β -amylase in cereal flours, and consist of a blocked p-nitrophenyl maltoheptaoside (BPNPG7), glucoamylase and α -glucosidase (K-CERA), and a nonblocked p-nitrophenyl maltopentaoside (PNPG5) and α -glucosidase (K-BETA). In preparation for assay, the pH of the extracts was adjusted from 7.5 to 6.1 with 1.0 M Mes pH 5.8. Then 0.1 mL (or 0.05 mL as required) of pH-adjusted extract was added to 0.1 mL α - or β - substrates and the solutions incubated at 40 °C for 10 min. Trizma base (1% w/v) was then added to stop the reaction, the absorbance read at 410 nm, and the enzyme activity calculated as directed by the manufacturer's instructions (using an extinction coefficient for p-nitrophenol (PNP) in Trizma base of 17.8 L·mmol⁻¹·cm⁻¹). For the reaction blanks, Trizma base was added prior to the squash extract.

The assay for starch phosphorylase activity consisted of 100 mM Hepes-KOH (pH 7.2), 1.2 mM NAD, 20 mM potassium phosphate, 0.04 mM glucose 1,6 biphosphate, 5 mM NaMoO₄, 2.1 U glucose-6-phosphate dehydrogenase, 1.2 U phosphoglucosmutase, and 0.05 mL extract. The assay was started with 0.1% (w/v) soluble starch, and the absorbance at 340 nm was recorded continuously at 30 °C for 15 min. Reaction blanks did not contain potassium phosphate.

AMYLOSE/AMYOPECTIN RATIOS. The method used was based on Magel (1991), and relied upon extraction of starch with HCl, staining with KI/I₂, and measuring the absorbance at 530 and 625 nm (rather than 605 nm). Ground freeze-dried squash samples were first decolorised in several washes of 12 methanol : 5 chloroform : 3 water (by volume) followed by 10 min centrifugations at 3000 g_n. After each centrifugation, the pigmented supernatant was removed. Finally, the samples were rinsed with 5 mL methanol, centrifuged for 10 min at 3000 g_n, the supernatant removed, and the residue dried under vacuum. The dry colorless samples were then dissolved in 18% (w/v) HCl, left for 10 min at

20 °C, centrifuged at 3000 g_n, and then diluted with 18% (w/v) HCl. A 0.06 mL sub-sample was taken and diluted to 1.8% HCl (w/v) with distilled water. Following treatment of the diluted solution with the KI/I₂ solution, absorbance was read against a blank at 530 and 625 nm, and the ratio of the absorbances (A_{625}/A_{530}) calculated. The ratios were also measured for a standard curve (50 mg·L⁻¹ starch), which was a mixture of amylose and amylopectin in varying proportions from 100% amylose to 100% amylopectin in 1.8% (w/v) HCl. The sample results were plotted on the standard curve and the percentage amylose was determined by regression. All results presented are means of six samples $\pm$ SE.

Results

Extractable activities of α -amylase (Fig. 1A) declined from flowering until about 21 d later. Thereafter, α -amylase activities increased steadily during fruit development and maturation, and especially so during storage at 12 °C. Extractable activities of β -amylase (Fig. 1B) changed very little throughout fruit development and maturation, and increased dramatically when fruit were harvested and stored but not when fruit remained in the field. There was a gradual increase in extractable activities of starch phosphorylase (Fig. 1C) during early growth and maturation and activities were higher after harvest and storage, although differences between attached and stored fruit were not large. Percentage amylose in starch (Fig. 1D) increased rapidly after flowering to near 20%, and the composition ranged between 20% to 25% during fruit maturation, ripening, and storage. Some maltose was detected using paper chromatography at 18 and 76 d (days chosen at random, data not presented), and this maltose was quantified using the enzymic method (Fig. 1E). Maltose concentrations were generally low, and increased mainly after harvest to maximal concentrations at 90 d. By 50 d after harvest (99 d after flowering), levels of maltose had declined again to very low levels.

Discussion

Only total extractable activities of several enzymes were identified. However, we confirmed the presence of α -amylase in buttercup squash (Nagao and Indou, 1991), and reported active β -amylase and starch phosphorylase in enzyme extracts.

Starch synthesis in buttercup squash first takes place from flowering until just after harvest (Irving et al., 1997), which is $\approx$ 50 d after flowering in our environment. Extractable activities of α -amylase increased dramatically around harvest, and to a lesser extent when fruit remained in the field. The large increase may be related to the act of harvest in that carbohydrates are no longer being translocated from leaves into the fruit, and cessation of carbohydrate translocation into the fruit may trigger the onset of starch degradation. The lesser increase, when fruit remained in the field, may be due to continued translocation of carbohydrates, although by harvest time, leaves are usually beginning to senesce from powdery mildew (*Sphaerotheca fuliginea* Schl.) and the cooler night temperatures.

If starch loss, as hexose equivalents, is about 13.3 μ mol·d⁻¹·g⁻¹, fresh weight (Irving et al., 1997), rates of α -amylase activity measured in vitro, as PNP released, ($\approx$ 0.4 μ mol·min⁻¹·g⁻¹, fresh weight or 576 μ mol·d⁻¹·g⁻¹, fresh weight), are far in excess of the measured rates of starch breakdown. These figures also suggest a large measure of in vivo control over α -amylase activity. The pattern of increase in α -amylase activity is consistent with that

Fig. 1. Extractable activities of (A) α -amylase, (B) β -amylase, and (C) starch phosphorylase; (D) percentage amylose in starch; and (E) maltose concentrations in buttercup squash fruit during growth in the field. Attached fruit (solid symbols) were compared with fruit harvested 50 d after flowering and stored at 12 °C (open symbols). Measurements (means of six samples $\pm$ SE) are on a fresh weight basis, except for D.

found by Nagao and Indou (1991). Since α -amylase is the only enzyme known to attack raw starch granules (Beck and Ziegler, 1989), these data provide evidence that α -amylase is the primary enzyme of starch degradation in buttercup squash.

In Irish potato, β -amylase is induced upon storage at 3 °C (Nielsen et al., 1997). In buttercup squash, activity of β -amylase increased dramatically during storage at 12 °C, but levels of maltose, the major end product of β -amylase activity, were not greatly altered by harvest (Fig. 1E). There is some discussion in the literature about the role of β -amylase in starch degradation, particularly in relation to its cellular location. The enzyme is reported to be extrachloroplastic (Beck and Ziegler, 1989), and in the wood of 'Robusta' poplar (*Populus $\times$ canadensis* Moench), the enzyme is soluble, rather than being starch-bound as is the case for α -amylase (Witt et al., 1995). As such, β -amylase may not be attached to starch granules and may not have a primary role in starch degradation, but could instead, produce maltose and glucose from maltooligosaccharides. In sweetpotato (*Ipomoea batatas* L.), β -amylase is present in excess (Hagenimana et al., 1994), but variations in activity are not synchronized with rates of starch degradation, again suggesting an indirect influence on starch breakdown. Nevertheless, the presence of maltose is evidence for a role of β -amylase in degradation of malto-oligosaccharides in buttercup squash, but as yet we cannot explain why β -amylase activity is high in stored squash, but not in squash which remained in the field. We speculate that synthesis of β -amylase is induced by the overall warmer temperatures in storage, but there is no effect on starch breakdown because β -amylase is limited by the amount of maltooligosaccharides, which in turn, is limited by α -amylase activity. Low levels of free maltose might be explained by the presence of active α -glucosidase (maltase). The contribution that maltose makes to the flavor of cooked buttercup squash will need to be determined in sensory experiments. Its presence could explain the more 'rounded' flavor of high-starch cultivars of squash (V.K. Corrigan et al., unpublished), and it might be a factor in differentiation of Mexican- and New Zealand-grown squash.

Percentage amylose in the starch did not change appreciably after harvest and during storage (Fig. 1D). This indicates that the starch degrading enzymes are capable of hydrolyzing both α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) glycoside bonds in a coordinated manner.

We assessed activity of the amylases with synthetic substrates (BPNPG7 for α -amylase and PNPG5 for β -amylase) rather than with authentic raw starch (Hallett et al., 1995). Although these substrates are widely used, α -amylase from Irish potato tubers was found to react with both PNPG5 and BPNPG7 (Nielsen et al., 1997), and at about the same rate. If there was such a cross reaction in the present study, the rate of α -amylase activity was only $\approx$ 10% that of β -amylase in buttercup squash (Figs. 1A and B), i.e., low enough to satisfy us that most of the activity with PNPG5 is in fact, β -amylase activity.

The presence of maltose and significant α - and β -amylase activity, strongly suggest starch degradation is hydrolytic in buttercup squash, and not phosphorolytic as Phillips (1946) suggested. Nevertheless, it is possible that cultivar differences exist in modes of starch breakdown, so the final judgement will need to await further studies on a range of genotypes.

The presence of starch degrading enzymes during early buttercup squash development, when starch synthesis is high, poses the question of what role they may be performing. Wegrzyn and MacCrae (1995) also found α -amylase activity throughout kiwi-fruit development. They speculated that concerted action of a

range of enzymes could contribute to the overall balance between starch synthesis and degradation, with the balance in favor of synthesis until ripening is initiated. Witt et al. (1995) found α -amylase in poplar wood needed to bind to raw starch before degradation started, and that binding was reversible and dependent upon the maltose concentration. Thus, maltose concentration or other inhibitors or effectors could be the means of mediating the synthesis/degradation balance during fruit development, and allowing the granule-to-granule variation in rates of starch breakdown during ripening reported by Hallett et al. (1995). A role in balancing starch synthesis/degradation may also explain why starch phosphorylase activity increased so much during the first 40 d after flowering (Fig. 1C), when starch synthesis was so active (Irving et al., 1997).

Literature Cited

- Beck, E. and P. Ziegler. 1989. Biosynthesis and degradation of starch in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40:95–117.
- Cordenunsi, B.R. and F.M. Lajolo. 1995. Starch breakdown during banana ripening: Sucrose synthase and sucrose phosphate synthase. *J. Agr. Food Chem.* 43:347–351.
- Garcia, E. and F.M. Lajolo. 1988. Starch transformation during banana ripening: The amylase and glucosidase behaviour. *J. Food Sci.* 53:1181–1186.
- Hagenimana, V., R.E. Simard, and L-P. Vézina. 1994. Amylolytic activity in germinating sweetpotato (*Ipomoea batatas* L.) roots. *J. Amer. Soc. Hort. Sci.* 119:313–320.
- Hallett, I.C., T.F. Wegrzyn, and E.A. MacCrae. 1995. Starch degradation in kiwifruit: In vivo and in vitro ultrastructural studies. *Intl. J. Plant Sci.* 156:471–480.
- Harborne, J.B. 1984. *Phytochemical methods: A guide to modern techniques of plant analysis*. 2nd ed. Chapman and Hall, London.
- Hill, S.A. and T. ap Rees. 1994. Fluxes of carbohydrate metabolism in ripening bananas. *Planta* 192:52–60.
- Hill, L.M., R. Reimholz, R. Schröder, T.H. Nielsen, and M. Stitt. 1996. The onset of sucrose accumulation in cold-stored potato tubers is caused by an increased rate of sucrose synthesis and coincides with low levels of hexose-phosphates, and activation of sucrose phosphate synthase and the appearance of a new form of amylase. *Plant Cell Environ.* 19:1223–1237.
- Irving, D.E., P.L. Hurst, and J.S. Ragg. 1997. Changes in carbohydrates and carbohydrate metabolizing enzymes during the development, maturation, and ripening of buttercup squash (*Cucurbita maxima* D. 'Delica'). *J. Amer. Soc. Hort. Sci.* 122:310–314.
- MacCrae, E.A., W.P. Quick, C. Benker, and M. Stitt. 1992. Carbohydrate metabolism during postharvest ripening in kiwifruit. *Planta* 188:314–323.
- Magel, E. 1991. Qualitative and quantitative determination of starch by a colorimetric method. *Starch* 43:384–387.
- Morrell, S. and T. ap Rees. 1986. Control of the hexose content of potato tubers. *Phytochemistry* 25:1073–1086.
- Nagao, A. and T. Indou. 1991. Studies on changes in components of squash fruit during maturation to determine harvest time. *Hokkaido Prefecture Agr. Expt. Sta. Bul.* 62:63–68.
- Nielsen, T.H., U. Deiting, and M. Stitt. 1997. A β -amylase in potato tubers is induced by storage at low temperature. *Plant Physiol.* 113:503–510.
- Phillips, T.G. 1946. Changes in the composition of squash during storage. *Plant Physiol.* 21:533–541.
- Sivak, M.N. and J. Preiss. 1998. Starch degradation, p. 153–161. In: M.N. Sivak and J. Preiss (eds.). *Starch, basic science and biotechnology*. Academic Press, San Diego.
- Stitt, M. and M. Steup. 1985. Starch and sucrose degradation, p. 347–390. In: R. Douce and D.A. Day (eds.). *Encyclopedia of plant physiology* (new series). Vol. 18. Higher plant cell respiration. Springer-Verlag, Berlin.
- Wegrzyn, T.F. and E.A. MacCrae. 1995. Alpha-amylase and starch degradation in kiwifruit. *J. Plant Physiol.* 147:19–28.
- Witt, W., A. Buchholz, and J.J. Sauter. 1995. Binding of endoamylase to native starch grains from poplar wood. *J. Expt. Bot.* 46:1761–1769.