

Starch Content and Amylase Activity in Avocado Fruit Pulp

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ABSTRACT. Amylase activity in detached avocado fruit (*Persea americana* Mill. cv. Fuerte) was directly correlated with ripening processes such as the climacteric rise in respiration, ethylene evolution, and softening. The term amylase designates the total amylolytic activity of avocado fruit but its exact nature was not studied. Amylase activity was higher in young than in mature fruits. After harvest, amylase activity started to rise with the onset of the respiratory climacteric. Parallel to the increase in amylase, a decrease in the starch content of the fruit pulp was observed. The disappearance of starch during softening was also demonstrated by electron microscopy. The possible role of starch as substrate and that of amylolytic activity as energy supplier, for metabolic processes in the fruit, is discussed.

The metabolic activity of avocado during ripening is one of the highest known in fruits. Biale and Young concluded (3) that, despite the high fat content of the mature avocado, available evidence does not support the idea that lipids are utilized as respiratory substrates during the course of the climacteric. Sugars disappear with ripening (1, 5) and so do insoluble pectins (7), but whether they account for all the substrates required in respiration remains to be determined (3).

Hydrolysis of starch during ripening is a common feature in fruits such as banana (11), mango (2) and pear (14). Starch was reported to be present in avocado pericarp plastids (4, 16). Studies of starch-hydrolyzing enzymes have been described in some fruits such as pear (14), mango (12) and tomato (6). The main amylolytic enzymes in plants are α and β amylases and phosphorylase. The reaction products of α -amylase are known to be dextrans, oligosaccharides, maltose and glucose; that of β -amylase is maltose, and the product of phosphorylase activity is glucose-1-phosphate (9). In order to test the hypothesis that amylase activity might contribute to the pool of substrates for respiration, changes in amylolytic activity during development and ripening of avocado

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pericarp were studied, and some characterization of this activity has been attempted. The possible role of starch as a substrate and that of amylolytic activity as an energy supplier for metabolic processes in the fruit is discussed.

Materials and Method

PLANT MATERIAL. Fruits of the 'Fuerte' cultivar were harvested periodically, starting in June with very young fruit (62 g and 1.6% oil content) and ending in Dec. with completely mature fruit (313 g and 14.9% oil content). Fruits were stored at 20°C. Ethylene (8 µl/liter) was applied for 48 hr at 20°, starting on the day of harvest. Ethylene-free air was applied to the controls.

ENZYME EXTRACTION. Acetone powders were prepared from fruit pulp, as described previously (15). The powder (400 mg) was extracted with 10 ml of 0.02 M phosphate buffer at pH 6.6 for 1 hr at 1°C. The slurry was centrifuged for 20 min at 30,000 x *g* at 1°C. The supernatant was decanted and then filtered through a Millipore filter (1.2 µm). One ml of the filtrate was applied on a 1.7 x 18.0 cm G-25 Sephadex column and eluted with 0.02 M phosphate buffer at pH 6.6. Fractions of 3 ml were collected and assayed for amylase activity, and protein was determined by measuring optical density at 280 nm; the bovine serum albumin calibration curve was used. The fractions containing the main amylase activity were combined to give the enzyme solution.

AMYLASE ACTIVITY ASSAY. Dinitrosalicylic acid reagent was used to detect the reducing groups liberated by the amylase activity (17). The reaction mixture contained 0.5 ml of enzyme solution and 1 ml of 1% soluble starch (Fisher) in 0.1 M sodium phosphate at pH 6.9, containing 0.002 M CaCl₂. For determination of the optimal pH for the reaction, 3 different buffers (0.1 M) were used: citrate, phosphate and borate, containing 0.002 M CaCl₂ each. The reaction mixture was incubated at 37°C for 5 min. A reaction mixture to which the enzyme solution was added at the end of the incubation time, just prior to the addition of the dinitrosalicylic acid reagent, served as a blank. Amylase activity is presented in µmoles of reducing units formed per min per mg protein, using glucose solutions for the calibration curve.

Also, amylase activity was determined with 1% dextrin β limit as substrate under the same conditions as with starch. The inhibitory influence of disodium ethylenediaminetetraacetate (EDTA), p-hydroxymercuribenzoate (PMB) and N-ethylmaleimide (NEM), as well as the protective effect of 2-mercaptoethanol (ME) were tested with the enzyme solution.

IDENTIFICATION OF AMYLASE ACTIVITY PRODUCTS. One ml enzyme solution was incubated at 37°C with 1 ml of 1% soluble starch in 0.1 M phosphate buffer, pH 6.6, containing 0.1% synthomyctin and 0.1% cycloheximide. Ten to 50 µl samples were removed from the reaction mixture at different times during 22 hr of incubation, and were chromatographed on Whatman No. 3 paper; 3 benzene: 3 butanol: 5 pyridine: 1 water (by vol) was the carrier solution, in a descending system. Reducing sugars were detected by AgNO₃ reagent as described by Trevelyan et al. (19). Starch solution with boiled enzyme solution, incubated for 22 hr, served as a blank.

Ethylene and carbon dioxide were determined by gas chromatography (10). A refractive index method (3) was used to determine oil content, and firmness was determined with a 'Chatillon' pressure tester (15).

STARCH DETERMINATION. A modification of Nielsen's method (13) for starch determination was employed. The precipitate that remained after exhaustive ethanolic extractions was dissolved in diluted perchloric acid (0.3 g in 5.7 ml). The solution was brought to a volume of 25 ml with water and a sample of 7 ml was mixed with iodate (mixture of 0.1% KI and 0.02% KIO_3). Optical density was determined at 675 nm. A calibration curve was made with potato starch to convert colorimeter readings to mg starch per g dry weight (DW).

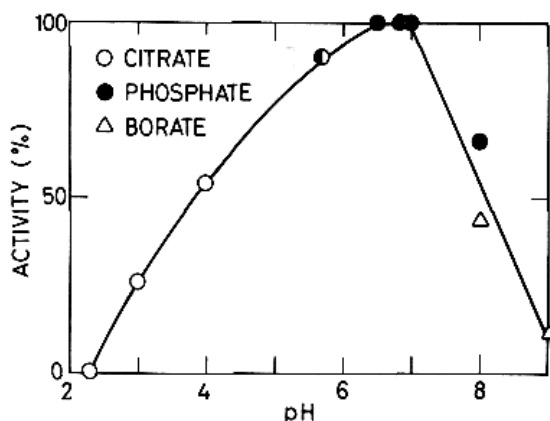


Fig. 1. Effect of pH on amylase activity.

ELECTRON MICROSCOPY. Tissue for electron microscopy was fixed from mature fruits (14.9% oil content, 11.2 kg firmness) on the day of harvest and after 10 days of storage in the dark at 20°C. Cubes (1 x 1 mm) from the pale green zone of the pericarp were fixed at room temp for 2 hr with 4% glutaraldehyde and 0.1 M phosphate buffer (pH 7.5). The tissue was rinsed in buffer and post-fixed in 2% OsO_4 in the same buffer for 1 hr at room temp. It was dehydrated in an alcohol series followed by propylene oxide and

embedded in Epon 812. Silver-grey sections were cut, mounted on copper grids, stained in uranyl acetate-lead citrate, and viewed in a Jeol electron microscope (JEM-T7).

Data represent at least 4 experiments which were carried out during 2 seasons; either averages or representative curves are presented.



Fig. 2. Descending paper chromatography of products of avocado fruit pulp amylase activity. (G = glucose standard; h = hours; 0 = zero time; M = maltose standard; B = blank.)

Results

The optimum pH for the amylolytic activity was between 6.5 and 7.0 (Fig. 1). The activity which was obtained at pH 5.8 with citrate buffer was the same as that with phosphate buffer. The enzymes were inactivated by a heat treatment (15 min at 70°C) and by a prolonged treatment at pH 3.3.

The thiol reagents did inhibit amylase activity: with NEM (5×10^{-4} M to 5×10^{-2} M) the inhibition was irreversible, while with PMB (10^{-3} M to 10^{-2} M) the inhibition could be

reversed by ME ($10^{-2}M$). On the other hand, EDTA at $10^{-3}M$ to $10^{-2}M$ also inhibited the activity. Soluble starch and β limit dextrin both served as substrates for the extracted enzyme solution. These data suggest the presence of both an α and a β amylase.

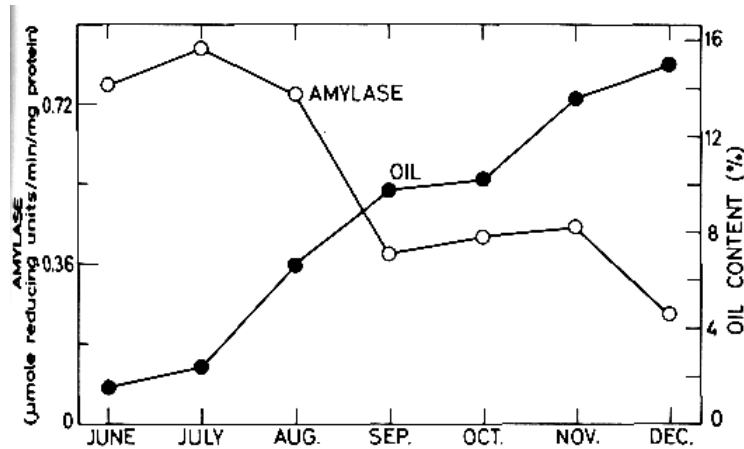


Fig. 3. Amylolytic activity and oil content in avocado fruit pulp during development.

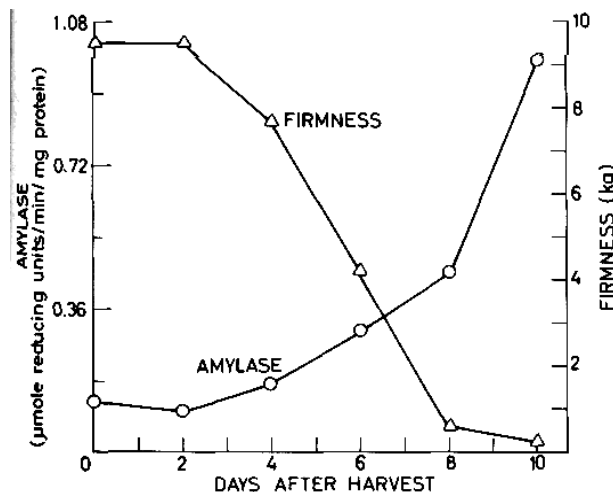
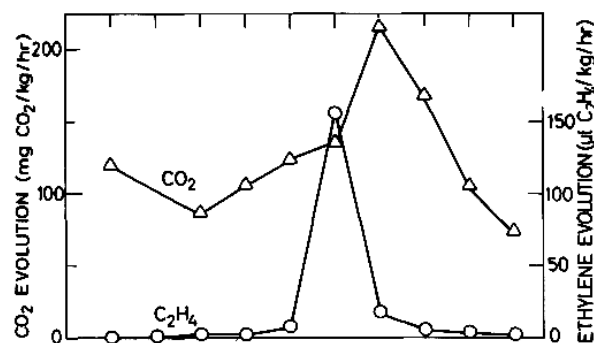


Fig. 4. Changes in amylase activity, firmness, carbon dioxide evolution and ethylene production during mature avocado fruit ripening at 20°C.

The main products of avocado amylase activity when soluble starch was used as substrate, were maltose, glucose, and various dextrans and oligosaccharides (Fig. 2). After 22 hr of incubation there was more glucose and maltose than after 3 hr. Neither sugar was detectable in the blank or at zero time of the enzymatic reaction.

The above-mentioned data do not justify the classification of avocado amylases. Therefore

the term amylase is used to signify the total amylolytic activity of avocado fruit pulp.

Amylolytic activity on the day of harvest was highest in young fruits and decreased as the fruit matured and oil content increased (Fig. 3). A distinct increase in amylase activity paralleled 3 well-known ripening phenomena (Fig. 4). This increase started, together with the onset of the respiratory climacteric and ethylene production, on the fourth day after harvest.

Along with the increase in amylase activity, there was a substantial reduction in the starch content of the fruit (Fig. 5). Ethylene treatment, which accelerated ripening and the increase in amylase activity, also enhanced starch disappearance at a similar rate. In addition, electron microscopic studies revealed amorphous starch grains in the plastids of mature hard avocado fruits (Fig. 6B). However, no starch was observed in electron micrographs of plastids of mature soft fruits (Fig. 6A).

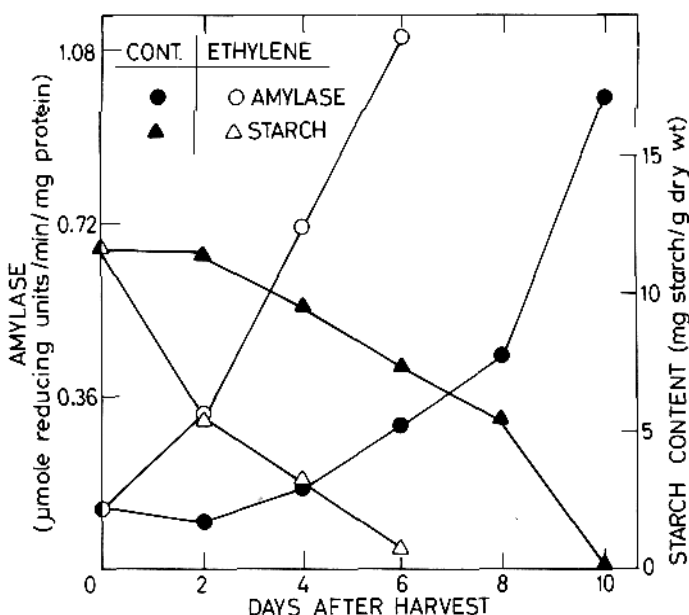


Fig. 5. Effect of postharvest ethylene treatment on amylase activity and starch content in mature avocado fruit pulp.

Discussion

Amylase activity extracted from avocado fruit pulp was found to have both α and β amylase reaction products; however, all our attempts to separate and characterize them failed. The presence of phosphorylase activity in our extracts was ruled out as at pH 5.8 (Fig. 1) the same activity was obtained with phosphate buffer as with citrate buffer, although the latter did not contain any phosphate ions which are required for phosphorylase activity (9). The inhibition by NEM, PMB, and heat inactivation favor the β amylase version while the pH optimum (pH 6.9), inhibition by

EDTA and the fact that dextrin β limit could serve as substrate, point to α amylase as the active enzyme. There may be only one enzyme, which is different from the known α and β amylases, and the type of amylase may not always be defined by the above-mentioned criteria (18). Drumm et al. (8) also could not separate α or β amylases from starch-degrading enzymes that were extracted from mustard seeds.

Amylases in fruits are involved in the transformation of starch to low molecular wt sugars (2, 11, 14), which in turn may be used during respiration and other energy-consuming processes. Therefore, it seems reasonable that amylase activity would be high during the early stages of fruit development until August (Fig. 3) and that it would be lower when the fruit reaches maturity in September and later.

The post harvest increase in amylase activity occurred together with the increase in various energy-consuming processes such as respiration and ethylene production.

Because it seems quite reasonable that starch serves as a substrate for energy in avocado fruit, we could expect to find a higher hydrolysis rate in the pulp during periods with higher energy consumption. Indeed, along with the increase in amylase activity after harvest, there was a decrease in the starch content of the pulp, and both phenomena were accelerated to the same extent by ethylene treatment of the fruit. The disappearance of starch during ripening was also demonstrated by electron microscopy (Fig. 6).

It is suggested that starch is a substrate for energy-consuming processes in avocado fruit pulp and that amylase activity is responsible for the hydrolysis of the starch. However the exact nature of the avocado pulp amylase(s) remains to be studied.

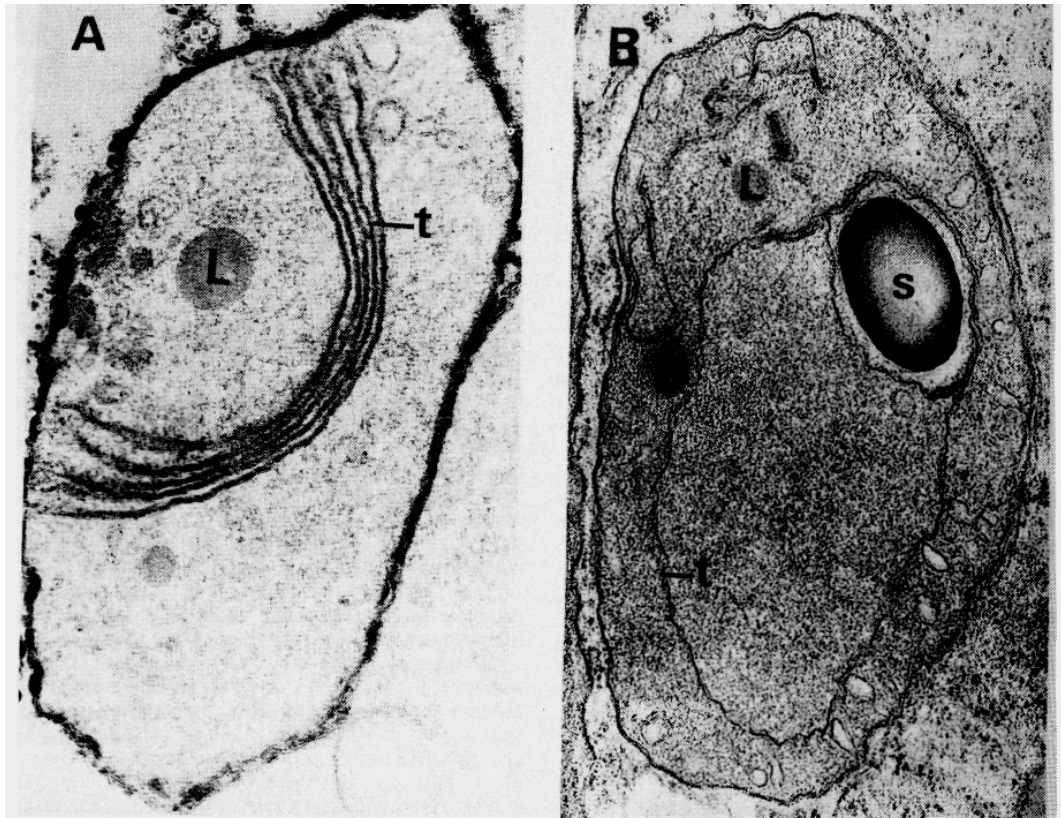


Fig. 6. Electron micrographs of plastids of mature avocado fruit. A. Soft fruit after 10 days at 20°C, $\times 50,400$. B. Hard fruit on day of harvest, $\times 29,400$. (L = lipid droplet; S = starch; T = thylakoids.)

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