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Mango Starch Degradation. II. The Binding of α-Amylase and β-Amylase to the Starch Granule

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During mango ripening, soluble sugars that account for mango sweetening are accumulated through carbon supplied by both photosynthesis and starch degradation. The cultivar Keitt has a characteristic dependence on sugar accumulation during starch degradation, which takes place during ripening, only a few days after detachment from the tree. Most knowledge about starch degradation is based on seeds and leaves currently used as models. However, information about the mango fruit is scarce. This work presents the evaluation of α- and β-amylases in the starch granule surface during fruit development and ripening. Extractable proteins were assayed for amylase activity and detected by immunofluorescence microscopy and correlated to gene expression. The results suggest that both amylases are involved in starch degradation during mango ripening, probably under the dependence of another signal triggered by the detachment from the mother-plant.

KEYWORDS: Mango; ripening; starch granule; α-amylase; β-amylase immunolocalization

INTRODUCTION

The ripening process of mango fruit involves several biochemical changes, causing alterations in color, flavor, texture, and taste. Soluble sugars, which account for mango sweetening, are accumulated through carbon supplied by both photosynthesis and starch degradation during development and ripening of most cultivars. The occurrence of superimposed events, sugar accumulation and starch degradation, during mango development and ripening, make the study of starch degradation a difficult task. Among the commercially relevant cultivars, Keitt mango has a peculiar behavior regarding starch metabolism. During fruit development, starch accumulates to up 8% of the pulp fresh weight, but a low amount of soluble sugars is detected. However, when ripening takes place, a few days after detachment from the tree, the accumulated starch is degraded and accounts for the synthesis and accumulation of soluble sugars, which could reach levels as high as 10% of the pulp fresh weight (*1*).

Starch is mostly composed of two polymers of glucose, amylose and amylopectin, organized in semicrystalline and insoluble granules, with an internal lamellar structure. Amylopectin, the largest molecule with a branched structure is the major component in starches, responsible for their granular nature (*2, 3*).

The majority of the information about starch degradation comes from studies in two current models reported in literature: the cereal seed germination and the mobilization of transitory starch in leaves during the night (*4, 5*). In the endosperm (nonliving cells) of cereals, α-amylase secreted by the alleurone layer and the scutellum (living cells that surround the endosperm) initiates the starch granule degradation, releasing a mixture of both linear and branched soluble glucans, acting as substrates for other enzymes involved in starch degradation, such as β-amylase and α-glycosidase. In the case of transitory starch present in leaves, the degradation process and the role of α-amylase is not so clear (*6*). The currently proposed pathway is that the amylopectin phosphorylation by a glucan, water dikinase (GWD), is the first step for starch degradation by β-amylase and isoamylases. However, participation of α-amylase or another unknown hydrolase has not been discarded in this process yet (*5, 7*).

Even though almost all known enzymes involved in starch degradation had their activities detected during the development and ripening of mangoes, their real importance has not been established yet (*5*). On Keitt mangoes, α-amylase activity increased in parallel to the starch content, while β-amylase activity was detected only during ripening. However, higher levels of starch-phosphorylase activity were seen when fruits

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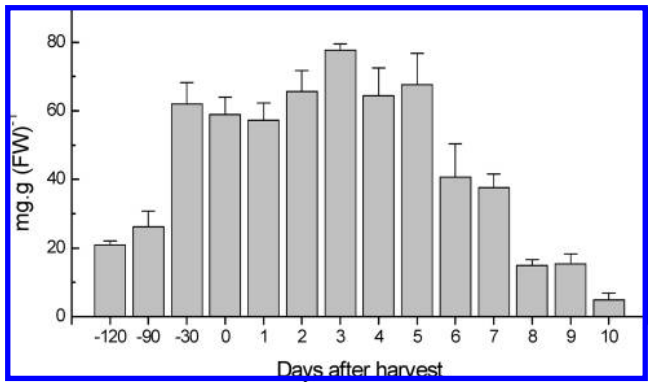

Figure 1. Starch profile during the development and ripening of Keitt mangoes.

were still accumulating starch, while isoamylases peaked both at fruit development and ripening (*1*).

In this work, the presence of α and β-amylases adhered to the granule surface during starch breakdown was checked. In order to accomplish that, extractable proteins linked to the surface of the granules (insoluble proteins) had their activities tested using specific substrates for α-amylases and β-amylases. Additional information was obtained by immunolocalization of these enzymes at the surface of the granules isolated from representative samples. The transcription profiles of both enzymes were also assessed in order to check the correlation of gene expression to the enzymatic activity and the main changes in starch content.

MATERIAL AND METHODS

Materials. Mango fruit (*Mangifera indica* Linn. cv. Keitt) was harvested in a plantation located in Joanópolis (São Paulo State, Brazil). The fruits were collected during development at 90, 120, and 180 days after anthesis (daa), immediately frozen in liquid N_2, and stored at −80 °C. Mangoes with approximately 207 daa and corresponding to the physiological maturity were stored at 20 °C temperature and 90% moisture under control, and then sampled on a daily basis. The samples were peeled, sliced, and immediately frozen in liquid N_2, and stored at −80 °C. As samples were being analyzed, they were thoroughly homogenized by powdering in liquid nitrogen. The degree of ripening was monitored through both CO_2 and ethylene levels. Each sample constituted of 3 mangoes, at least, at the same level of respiration and ethylene production.

Carbohydrate. Starch content was determined enzymatically as described by Cordenunsi and Lajolo (*8*).

Isolation of Starch Granules and Extraction of Starch Granule Associated Proteins (SGAPs). Starches granules were isolated from the samples representative of mango development (180 and 207 daa) and ripening (3, 5, and 8 days after harvest (dah)). The isolation was performed according to Ritte et al. (*9*), except for the volume of buffer which was larger (10-fold) in the step related to tissue homogenization to isolate the granules. Total protein, granule-enclosed proteins, and proteins bound to the granule surface were extracted as described by Ritte et al. (*10*), with modifications as follows. Proteins extracts obtained were concentrated with acetone (1:1) for 1 h in a freezer at −20 °C and centrifugated at 11,000*g* for 30 min. The remaining pellets were washed with acetone/ether (1:1) and centrifugated again, and the supernatants were discarded. The pellets obtained from these three extractions were suspended in denaturing buffer (30 μL buffer per mg dry weight) according to Laemmli (*11*) and heated for 5 min at 95 °C. After a 2-min centrifugation at 11,000*g*, the supernatant was applied to 7.5% SDS−PAGE and calibrated with molecular markers ranging from 20 to 97 kDa. After electrophoresis at 200 V under denaturing conditions, the gel was stained with silver, by using the Bio-Rad Laboratories Silver Stain Plus Kit (Bio-Rad, CA, U.S.A.) according to the manufacturer's instructions. The reaction was stopped with 5% acetic acid solution (v/v).

Extraction of Proteins Bound to the Starch Granule Surface and Determination of α- and β-Amylase Activities. Enzymes bound to the granules of starch were extracted according to Ritte et al. (*10*) by adding of 10 mL of buffer and 50 mM HEPES-KOH, pH 7.0, containing 1 mM $CaCL_2$ and 1 mM benzamidine in 200 mg of starch isolated as above. The suspension was mixed for 1 h and 30min at 37 °C and centrifuged for 3 min at 6,000*g*. The supernatant was concentrated in Centriprep-10 and Centricon-10 according to manufacturer's manual (Millipore Corporation, Bedford, MA 01730 U.S.A., 2000). The activity *in vitro* was determinated after incubation of the extracts obtained with a specific substrate of α-amylase (BPNPG7-*Ceralpha* - Megazyme) and β-amylase (PNPG5-Betamyl) in a microplate. The developed color was read at 410 nm, as described by Bassinello et al. (*12*). Native PAGE (7.5%) added with 0.1% amylopectin was used to detect starch-hydrolyzing enzymes according to Zeeman et al. (*13*). After electrophoresis at 4 °C, the gel obtained was washed twice with 100 mM Tris, pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM DTT for 15 min, then incubated overnight at 20 °C in the same buffer. The gel was stained with 0.67% I_2 (w/v) and 3.33% KI (w/v).

Enzymatic Extraction and α-Amylase and β-Amylase Activities in Mango Tissue. Approximately 0.5 g of mango pulp was ground in a mortar cooled with liquid nitrogen. The protein from the frozen powder was extracted in 2.5 mL of 50 mM Hepes-KOH buffer (pH 8.0) containing 1 mM benzamidine, 1% polyvinylpyrrolidone, MW 40,000 (PVP-40), and 20 mM cysteine. The supernatant obtained after centrifugation at 10,500*g* for 30 min was considered the crude extract. Total protein was quantified by the Bradford method (*14*). α-Amylase and β-amylase activities *in vitro* were determined as described above (*12*). The activities were expressed as μmol of *p*-nitrofenol released per milligram of protein per minute.

Immunofluorescence Microscopy. Immunolocalization was done according to Rocha et al. (*15*), with modifications. Starch granules were blocked with phosphate buffered saline, pH 7.2 (PBS), and 3% BSA (w/v) for 2 h at room temperature, subsequently incubated with antibodies against α-amylase or β-amylase, and diluted (1:10) in PBS

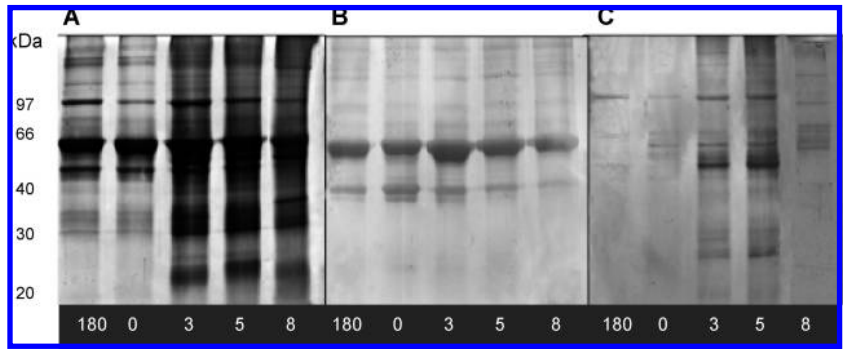

Figure 2. SDS−PAGE (7.5%) of (**A**) total protein of mango starch granule, (**B**) granule-enclosed proteins, and (**C**) proteins bound to the granule surface. The gels were stained with silver reagent. MW: molecular-weight markers from 20 to 97 kDa. Isolated starches: 180 days after anthesis (daa), zero, 3, 5, and 8 days after harvest (dah). Electrophoresis realized at 200 V.

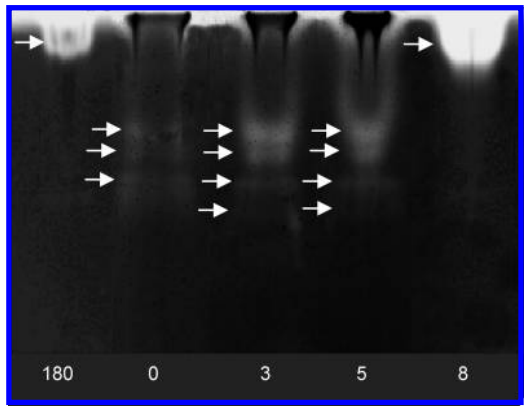

Figure 3. Native−PAGE (7.5%) obtained with 0.1% amylopectin to detect starch-hydrolyzing enzymes. After electrophoresis at 4 °C and 100 V, the gel was washed twice with 100 mM Tris, pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM DTT for 15 min, then incubated overnight at 20 °C in the same buffer. The gel was stained with 0.67% I_2 (w/v) and 3.33% KI (w/v) solution. Isolated starches: 180 days after anthesis (daa), zero, 3, 5, and 8 days after harvest (dah). Arrows indicate the bands.

and 3% BSA (w/v) overnight at 4 °C. Then, starch granules were washed three times with PBS/BSA for 30 min each and incubated for 6 h at room temperature with secondary antibody and fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (Sigma) (diluted 1:50). Starch granules were washed again with PBS buffer at pH 7.2. Labeled grains were viewed by using a Zeiss Axioskop 2 epifluorescence microscope and a JVC/TK-1270 color video camera.

Extraction of RNA and Reverse Northern Blot. Reamplified PCR fragments from α-amylase, *β*-amylase, and Actin cDNAs in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate) were denatured at 95 °C for 5 min and transferred to a nylon membrane (HybondN$^+$, Amersham Biosciences) by using the Bio Dot microfiltration system (Bio-Rad Laboratories). The blotted membrane was rinsed in denatured solution (50 mM NaOH, 0.5 M NaCl) for 5 min and transferred to a neutralization solution (100 mM Tris-HCl, pH 7.0, 0.5 M NaCl), air-dried, and fixed with an ultraviolet cross-linker (Hoefer, Inc.). The membrane was prehybridized for 2 h at 65 °C with 10 mL of PerfectHyb block solution (Sigma) and hybridized for 16 h at 65 °C in the same solution added with the denatured probe from the cDNA of mango fruits. The probes were synthesized for 1 h at 42 °C by reverse transcription of 10 *μ*g of total RNA from each sample using 50 *μ*Ci [α-^{32}P]-dCTP, 2 *μ*L of oligo $(dT)_{18}$ (1 *μ*g/*μ*L), 10 *μ*L of Superscript 5X buffer, 0.5 *μ*L of RNAsin (Promega), 5 *μ*L of DTT, 5 *μ*L of 10 mM d(AGT), and 1 *μ*L of reverse transcriptase Superscript II. After 15 min of incubation at 42 °C, 25 mM dCTP (2 *μ*L) samples were added into reaction and incubated for 45 min at 42 °C. The radioactive cDNA probes were purified in a Sephadex G50 Column (Microspin Columns, Armersham Biosciences). After the hybridization, the membrane was washed twice with 2X SSC/0.1% SDS and exposed to an X-ray film at −80 °C (*16*). After that, bands obtained in the film were digitalized in a GS700 densitometer (Bio-Rad Laboratories), and the densitometric analysis was made with the Quantity One (v. 5.2) software (Bio-Rad Laboratories), and the blots were normalized with the Actin bands.

RESULTS

Starch amount in the pulp of Keitt mangoes changed in accordance with the pattern previously observed (*1*), as indicated in **Figure 1**. Starch accumulated during fruit development and then degraded at ripening, after fruit detachment from the tree. In order to characterize the changes in proteins bound to the granules, starch was isolated from developing fruits (180 days after anthesis) and also from fruit sampled during ripening, at zero, 3, 5, and 8 days after harvest.

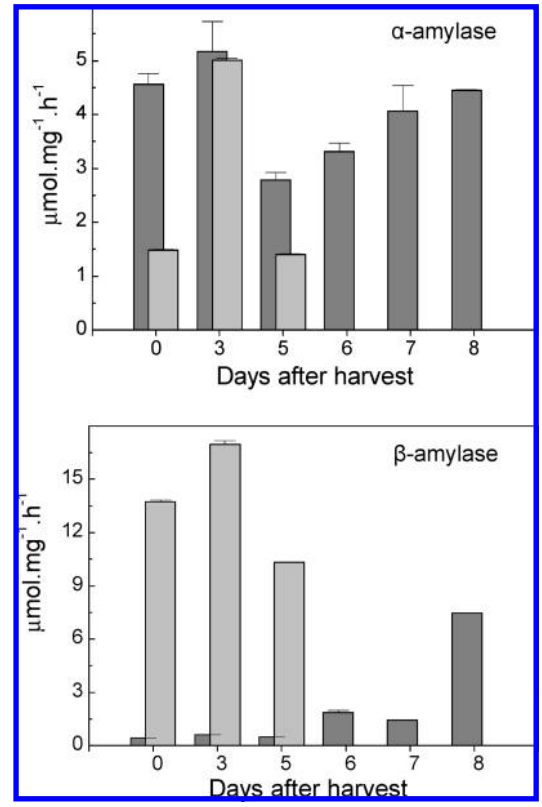

Figure 4. Activity *in vitro* profile of α-amylase and *β*-amylase enzymes bound on mango starch granules (light gray) and soluble on crude extract (dark gray), at different periods of ripening. Isolated starches: zero, 3 and 5 days after harvest (dah). Mango crude extracts: zero, 3, 5, 6, 7, and 8 days after harvest (dah).

Although protein quantification did not reveal any significant difference in the amount of total protein in the pulp fruit during ripening, the amount and diversity of proteins bound to the starch granules increased as the starch was degraded (from 3 days after harvest). Since the profile for proteins from the inside did not change significantly (**Figure 2B** and **A**), it seems that the proteins bound to the surface of the granule accounts for this difference (**Figure 2C**). The major changes observed in electrophoresis (**Figure 2A** and **C**) were the appearance of a large protein band of ∼120 kDa, and some others between 50 kDa, 60 kDa, and around 30 kDa.

When the proteins bound to the surface of the granules were solubilized and loaded on nondissociating PAGE−amylopectin, the hydrolytic activity of some of these proteins showed up (**Figure 3**). The in-gel activity profiles for starch-granule-surface-proteins obtained from fruit at development (180 daa) and at late ripening (8 dah) were quite similar, with amylolytic activity placed at the top of the gel. A very contrasting profile was observed for the extracts obtained from fruit sampled when the starch degradation was already in process. At least three bands of amylolytic activity could be clearly seen in the extract from fruit at the harvesting point (zero dah), which became stronger in the extracts from fruit sampled at 3 and 5 dah.

The amylolytic activity of starch granule surface proteins was also tested using specific substrates to detect α-amylase and *β*-amylase (**Figure 4**). The soluble α-amylase activity was much higher than the activity bound to the starch granules during all the period covered by the experiment. The activity associated with the starch increased only on the third day after harvest, reaching a peak, coincident with the onset of starch degradation, then, decreased to the lowest levels. In relation to *β*-amylase,

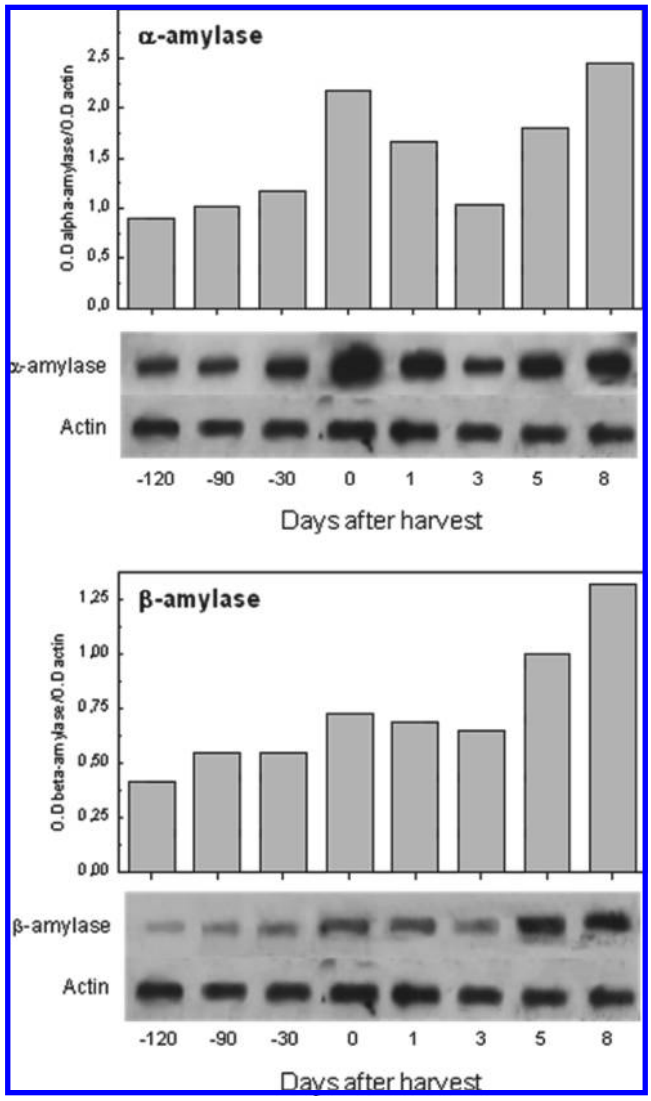

Figure 5. Normalized densitometric analysis (top) and reverse Northern blotting (bottom) of α-amylase and β-amylase from total RNA extracted from mango pulp during development (−120, −90, and −30 daa) and ripening (zero, 1, 3, 5, and 8 dah) of fruits. The Actin bands were used for normalization of the α- and β-amylase ones.

the activity assay showed that the enzyme was already bound to the granule surface in the following day after harvest. The activity of β-amylase bound to the granules was much higher than the soluble activity, and it peaked at day 3 after harvest, when starch had been degraded. As ripening proceeded, and the starch amount in the fruit pulp was reduced, the activity of soluble β-amylase continued to increase.

The expression analysis by Northern blotting (**Figure 5**) revealed a significant increase in α-amylase mRNA during development, peaking at the harvest day. Despite some minor changes after harvest, the transcript level remained high and correlated to the soluble activity of α-amylase. However, the activity of α-amylase bound to the starch granule showed an inverse correlation to the transcript level. In relation to β-amylase, the transcription level was more correlated to the profile of the soluble activity (**Figure 5**). The amount of the β-amylase mRNA was low during fruit development and increased gradually throughout ripening. However, the amount of the β-amylase transcript showed a 2-fold increment by day 5, followed by an increase in soluble β-amylase activity.

In order to confirm the binding of amylases to the mango starch granules, the particles were submitted to immunofluorescence microscopy using banana α-amylase and β-amylase antiserum. The results presented in **Figure 6** confirmed the presence through the immunolocalization of both enzymes in the surface of the granules isolated from mangoes at zero and 5 days after harvest (α-amylase, **Figure 6A−H** and β-amylase, **Figure 6I−P**).

DISCUSSION

The onset of starch degradation in Keitt mangoes presented a remarkable difference compared to that of the other mango cultivars (Tommy, Van Dyke, Haden and Palmer (*17*)) since it seems to be triggered by fruit harvest. In this way, the events related to the initiation of starch degradation and sugar accumulation could be more easily investigated. To check some biochemical and molecular changes that could be associated with the degradation of the granules, starch was isolated from fruit sampled at representative points of development and ripening. Although ripening could be followed during additional days, those samples were not analyzed because besides the low yield in isolated starch granules, the large amount of coextracted cell wall polysaccharides made the isolation process a difficult task. This observation also addresses the importance of avoiding the spurious binding of protein during the extraction procedure. On the basis of the methodology employed for starch isolation and the controls effectuated during the protein extraction procedures, it is possible to consider that appropriate conditions were achieved.

The electrophoretic profiles revealed that the number and amount of proteins attached to the surface of the granules increased during ripening. This observation is in agreement with the idea of exocorrosion of the granules by amylolytic enzymes. In fact, the size of these new proteins appearing at the granule surface is on the average of those supposed to be linked to the degradation process, which are mostly between 40 and 85 kDa. The abundant protein seen at 62 kDa is probably a starch synthase (*18*). Although this protein is located inside the granule, it would become more apparent as the particles eroded.

The amylolytic profile for the in-gel assays showed a clear difference at the early ripening stages. The appearance of additional starch-degrading protein bands were in agreement with the previous observation (**Figure 2**) in the number and amount of proteins attached to the granule surface. On the basis of the results of the in-gel assay, it is very likely that those bands would correspond to amylases. In a previous work, Bernardes-Silva et al. (*1*) observed that the increase in soluble α-amylase activity during fruit development was parallel to the starch content, achieving high values at the physiological maturity and being decreased at late stages of ripening. However, β-amylase activity, which was barely detected during fruit development, presented a sharp increase during ripening.

The specific activity assay for the amylases attached to the granules revealed that there was a discrete increase for α-amylase and a significant one for β-amylase at the early stages of ripening. An explanation for the remarkable increase in soluble β-amylase activity at late ripening could be the releasing of enzyme from the degraded starch granules. The occurrence of the amylases at the granule surface was confirmed by immunofluorescence microscopy, which revealed that in spite of the fact that the proteins could already have been detected at harvest time, the amounts of the proteins related to amylases could have been detected afterward when the starch degradation process was taking place.

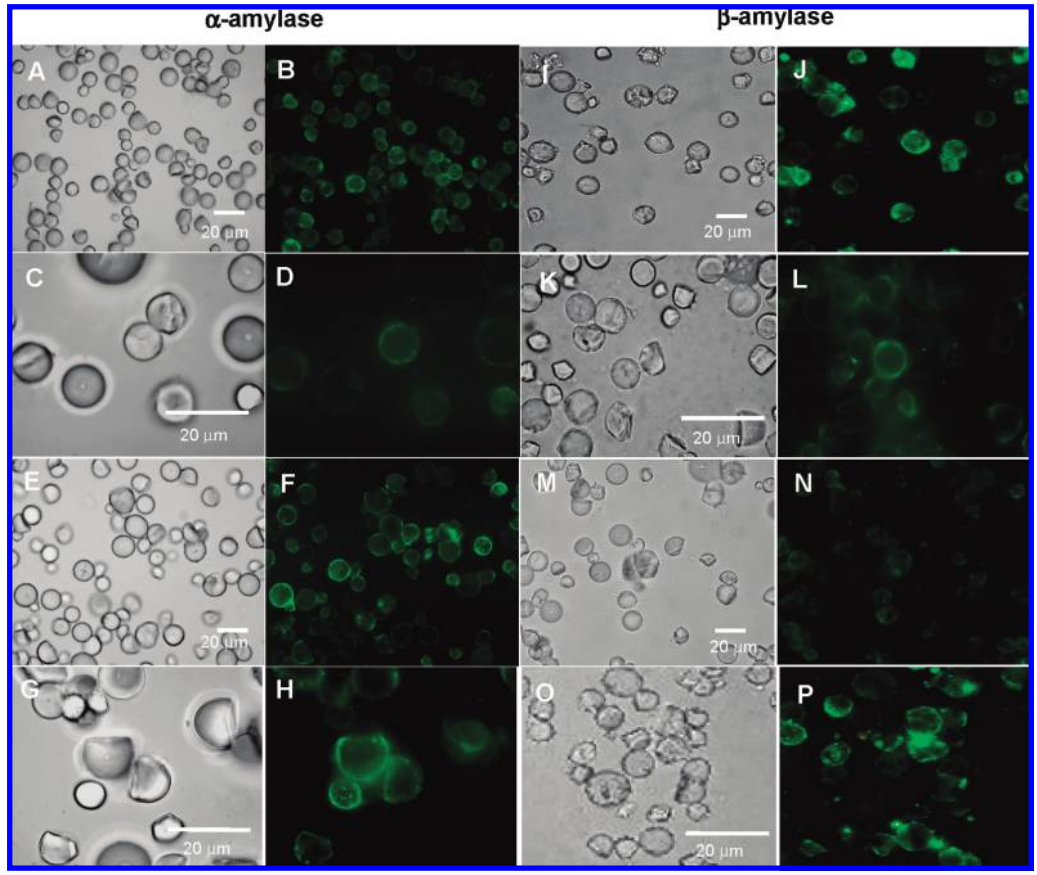

Figure 6. Optical microscopy (gray images) and immunofluorescence microscopy (green images) on starch granule surface utilizing primary antibodies against α-amylase (**A**−**H**) and β-amylase (**I**−**P**) and secondary antibody FITC-conjugated antirabbit IgG. Starches: zero day after harvest (dah), **A**−**D** and **I**−**L**; 5 days after harvest (dah), **E**−**H** and **M**−**P**. Magnitude 40×: **A**, **B**, **E**, **F**, **I**, **J**, **M**, and **N**. Magnitude 100×: **C**, **D**, **G**, **H**, **K**, **L**, **O**, and **P**.

The estimation of mRNA levels for α-amylase and β-amylase indicated that gene expression contributes to the increase in amylases during fruit ripening. The changes in mRNA and the activity of α-amylase soluble and bound to starch granules suggest a relevant role of transcription as a regulation mechanism of α-amylase activity by keeping significant amounts of the enzyme available to attach to the starch granule surface. Although the transcription of β-amylase seemed to be more correlated to the profile of the soluble enzyme activity, it is possible to consider that transcription also has an important role in regulating the levels of β-amylase activity. The high activity of the enzyme bound to the granule surface at the initial phase of mango ripening, when the transcript level was apparently low, indicates a high affinity of this protein to particles. In this way, the stimulation in transcription would account for the increased demand of β-amylase protein during starch degradation.

Although the increased demand for these degrading enzymes during ripening and the stimulation in gene transcription accounts for this need, a significant amount of the enzymes could already have been detected in the granule surface by the time of fruit harvesting. Since only two degrading enzymes were investigated, the dependence on other enzymes or proteins can not be ruled out. In fact, there are clear differences in the initiation of the starch degradation process depending on the plant tissue. α-Amylase is required for the initiation of starch breakdown that takes place during cereal germination, but it is not required for the degradation of transitory starch in *Arabidopsis* leaves. In this case, another mechanism has been proposed to explain the initial degradation of the starch: the prior phosphorylation of the granule by GWD and PWD exposes access points of the amylopectin chains, which would allow β-amylase action without previous initiation by α-amylase (*5*). Currently, a new pathway is emerging from the studies in other tissues such as turions, a perennation vegetative organ from *Spirodela polyrhiza* (*19*). These authors proposed that, in turions, the starch phosphorylation by GWD enhances the association of starch-degrading enzymes to the granule, including α-amylase, which is accompanied by changes in the starch degradation level.

The results presented here suggest that α-amylase and β-amylase are involved in starch degradation during mango ripening, which is clearly triggered by detachment from the mother-plant. Since the enzymes are adhered to the surface of the starch granule in the fresh harvested samples (zero day), without detectable starch degradation, it is likely that the initiation of starch degradation would be a response to an unknown signal occurring between days 3 and 5 after harvest. If this signal is related to starch phosphorylation, as seems to occur in turions, it remains to be established.

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