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Differential Protein Accumulation in Banana Fruit during Ripening[1]

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ABSTRACT

Banana (*Musa acuminata*, cv Dwarf Cavendish) proteins were extracted from pulp tissue at different stages of ripening and analyzed by two-dimensional electrophoresis. The results provide evidence of differential protein accumulation during ripening. Two sets of polypeptides have been detected that increase substantially in ripe fruit. These polypeptides were characterized as glycoproteins by western blotting and concanavalin A binding assays. Antibodies againts tomato polygalacturonase cross-react with one of these sets of proteins.

Ripening involves a series of changes during the early stages of senescence. After a phase of active cell division and further cell expansion, fruit growth rate declines and ripening usually begins. The physiological, ultrastructural, and biochemical changes that occur at this time are highly coordinated. There is increasing evidence that the expression of specific genes is required for normal ripening (1). An important turnover of preexisting proteins and the *de novo* synthesis of RNAs (6, 9, 10, 15) and proteins (5, 11) seems to be essential for ripening (2, 3). One of these proteins is polygalacturonase (22). It has been widely studied as one of the specific proteins that accumulate during ripening. The *de novo* synthesis of polygalacturonase occurs after ethylene production in climacteric fruits (11). There are three isoenzymes of polygalacturonase isolated from tomato pericarp (24, 26) that are encoded by a single gene (4).

The banana is a climacteric fruit like the apple, pear, peach, tomato, avocado, and others. This class of fruits is characterized by a large increase in ethylene synthesis at the onset of ripening. It is believed that ethylene regulates the expression of the genes involved in ripening. Brady and O'Connell (2) demonstrated an increase in the rates of turnover of proteins in banana pulp tissue. However, in banana fruits, little is known about the synthesis of particular proteins not previously present in preclimacteric stages. Recent studies on ethylene and O_2 effects in bananas suggest that the increase in enzyme activity observed during ripening corresponds to *de novo* protein synthesis (12). The present article reports the isolation of total proteins from banana fruits. Accumulation of specific proteins during banana ripening has been observed. One of this proteins immunoreacts with an antiserum against tomato polygalacturonase. The presence of this enzyme in banana ripe fruit is discussed.

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MATERIALS AND METHODS

Plant Material

Immature fruits of banana (*Musa acuminata*, cv Dwarf Cavendish) were obtained from Tenerife (Canary Islands). Fruits were dipped in fungicide solution (1‰ benlate, 3‰ dithane, w/v) and placed in ventilated jars with a known flow of humidified air at 22°C. The respiration rate of the fruit tissue, determined as CO_2 production, was measured by connecting the effluent air from each respiration jar to an infrared gas analyzer. Ethylene measurements were made with a gas chromatograph fitted with a flame ionization detector. Fruit samples at determinate ripening stages were immediately frozen in liquid nitrogen and stored at −80°C.

Preparation of Protein Extracts

To extract total protein, frozen pulp tissues at specific ripening stages were ground in a mortar under liquid N_2. The powder was delipidized in an acetone:hexane mixture (51:49, v/v) and centrifuged at 6000*g* for 20 min at 4°C. The final pellet was vacuum dried until a fine flour was obtained, then stored at −40°C.

Because the main difficulty in obtaining protein extracts from banana fruit is to get rid of other interfering compounds, we applied the following extraction procedure: equally weighted flour samples were first extracted with buffer: 0.25 M Tris-HCl, pH 8.4, 0.2 M glycine, 0.4% (w/v) SDS, and 10% (v/v) 2-mercaptoethanol. The supernatants obtained after centrifugation (12,000*g* for 20 min at 4°C) were immediately treated with an equal volume of phenol (previously equilibrated with 1 M Tris, pH 8). Both the phenolic phase and the interphase were recovered and washed four times with 10 mM Tris, 1 mM EDTA (pH 7.5). The proteins in the phenolic phase were precipitated with four volumes of methanol/0.1 M ammonium acetate at −20°C overnight. The pellet was then dried and dissolved in a sample buffer: 60 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v)

2-mercaptoethanol for one-dimensional SDS-PAGE, or in a lysis buffer (9.5 M urea, 2% [v/v] Nonidet P-40, and 2% [v/v] of a mixture of ampholines [Pharmacia], pH range 3–10 and 5–8) for two-dimensional electrophoresis. Protein concentrations were measured by the Lowry method as modified by Peterson (21) using BSA as a standard.

Electrophoresis and Immunoblot

Total proteins in the pulp extracts were separated by SDS-PAGE on a 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide slab gel (1.5 × 160 × 200 mm) according to the Laemmli method (14). An equal amount of protein (80 μg) was loaded in each lane. Gels were fixed in 50% (w/v) TCA and stained with Coomassie blue. In two-dimensional electrophoresis (NEPHGE[2] × SDS-PAGE), the first dimension, NEPHGE was performed according to the method of O'Farrell *et al.* (20) as modified by Meyer and Chartier (18). Equal protein quantities (160 μg) were loaded in cylindrical gels (length 7 cm, internal diameter 1.5 mm) containing 3.7% acrylamide, 0.21% bisacrylamide, 9.5 M urea, 2% (v/v) Nonidet P-40, and 0.8% (v/v) ampholines (Pharmacia, 3–10 and 5–8 pH range). Samples were placed on the acid end of the gel and covered with an overlay solution (8 M urea, 5% [v/v] Nonidet P-40, 5% [v/v] 2-mercaptoethanol, and 1% [v/v] ampholines [3–10 and 5–8 pH range]) and run for 30 min at 100 V, 45 min at 200 V, 75 min at 300 V, and 60 min at 500 V. Gels were equilibrated in sample buffer (60 mM Tris HCl [pH 6.8], 10% [v/v] glycerol, 2% [w/v] SDS, and 5% [v/v] 2-mercaptoethanol) for 20 min. In the second dimension, SDS-PAGE-equilibrated gels (1.5 × 7.3 × 10 cm) were run on 15% polyacrylamide slab gels for 3 h at 120 V. Standard molecular mass markers (from Sigma) were as follows: BSA (68 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21 kD), and lysozyme (14 kD).

Immunoblotting of two-dimensional electrophoresed proteins was performed as described (16). Antiserum to tomato polygalacturonase was used at a 1:1000 dilution. Immune complexes were detected using peroxidase-conjugated antirabbit immunoglobulin G and 4-chloro-1-naphtol as substrate. Preimmune rabbit antiserum was used as a control.

Detection of Glycoproteins

The proteins resolved in two-dimensional electrophoresis (NEPHGE × SDS-PAGE) were transferred to nitrocellulose sheets (0.45 μm, Schleicher and Schuell) as described by Towbin *et al.* (23). Transferred glycoproteins were localized on the blot after the procedure described by Clegg (7) to locate glycoproteins that bind concanavalin A. Essentially, the sheets were first incubated with concanavalin A (Pharmacia) and then with the enzyme glycoprotein, horseradish peroxidase (Sigma), and developed with *O*-dianisidine as a substrate.

Duplicate sheets were incubated only with peroxidase, leaving out the concanavalin A incubation to detect possible concanavalin A proteins in the extracts. To discard endogenous peroxidases, a second control was made, exposing nitrocellulose membranes to the peroxidase substrate.

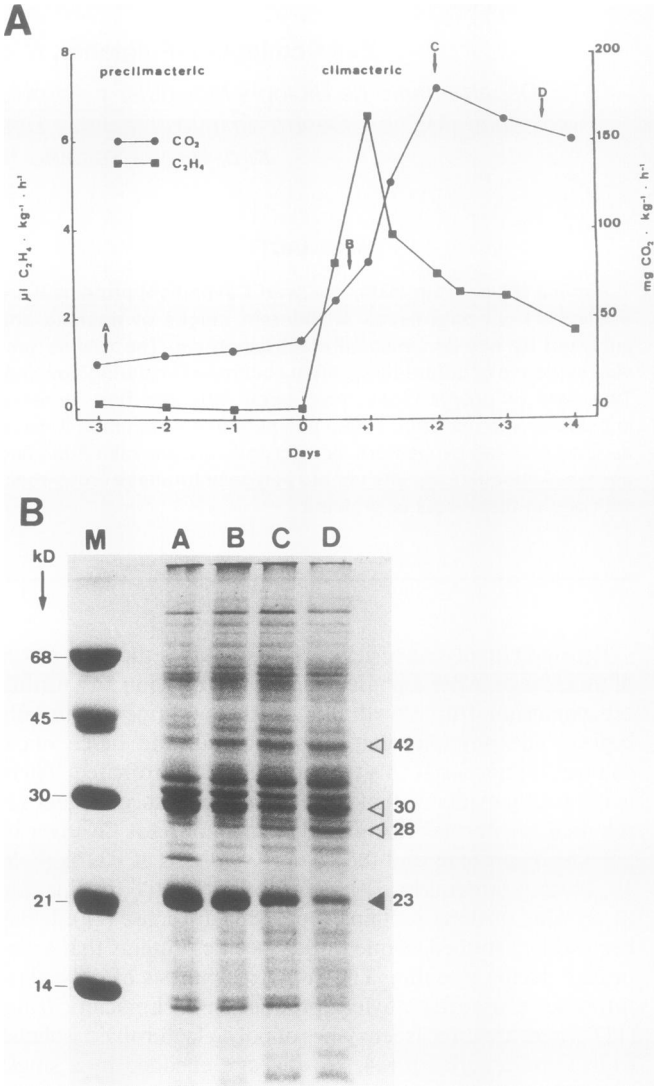

Figure 1. A, Respiration rates and ethylene production of whole banana fruit. Data represent the average of four fruits. Days were referred from the start of the respiratory climacteric. Arrows indicate the four stages at which samples were collected. A, B, C, and D correspond to the preclimacteric, early climacteric, climacteric, and postclimateric, respectively. B, SDS-PAGE of total proteins extracted from banana pulp. Lanes A, B, C, and D correspond to samples at different stages of ripening as shown in A. Eighty micrograms protein were applied to each lane. The molecular mass markers are in lane M. Black arrows show polypeptides in kD that decrease during ripening, and white arrows the polypeptides in kD that increase during this process. Gels were stained with Coomassie blue.

[2] Abbreviation: NEPHGE, nonequilibrium pH gradient electrophoresis.

RESULTS

Protein Analysis in Banana Pulp

As shown in Figure 1A, the ripening process in banana begins with an increase in respiration and ethylene production. The CO_2 production peak appears about 1 d after the ethylene peak. It subsequently declines as the fruit enter the postclimacteric period.

To determine the changes in protein content and/or accumulation during ripening, total proteins were extracted at different stages of the process. The arrows in Figure 1A correspond to preclimacteric (A), early climacteric (B), climacteric (C), and postclimacteric (D) stages. Samples of identical dry weight from fruits at these four stages were collected and processed for protein extraction. Many classic total protein extraction methods were used. However, due to the special characteristics of the banana fruit tissue (high content of polyphenols and polysaccharides), the use of the Tris-SDS/phenol extraction method improved the recovery of total proteins. The absolute protein values obtained from each sample and expressed in mg/g fresh weight were 3.7, 3.25, 2.15, and 1.40; these values correspond respectively to the stages indicated above (A, B, C, and D).

To examine the differential accumulation of banana proteins during ripening, samples of proteins extracted were subjected to SDS-PAGE analysis (Fig. 1B). As the fruit ripened, many changes were observed. Some polypeptides, for example, one of 23 kD that was prominent in immature fruit, decreased with ripening. Others, like relatively abundant polypeptides (28, 30, and 42 kD) increased from the preclimacteric to the postclimacteric stage. The 30 kD polypeptide appeared to have reached its lowest concentration during the climacteric stage, but a slight increase was observed at the ripened stage.

Two-dimensional electrophoresis (NEPHGE/SDS-PAGE) protein analysis improved the resolution of major polypeptides. Figure 2 shows that the main proteins remain present in the four stages. However, there are significant changes in the relative concentration of some polypeptides. Thus, the protein present at the preclimacteric stage (spot 12) increased in the course of ripening, but another (spot 10) decreased. The levels of polypeptides 9 and 11 fluctuate. Protein accumulation was observed at the preclimacteric stage, decreasing

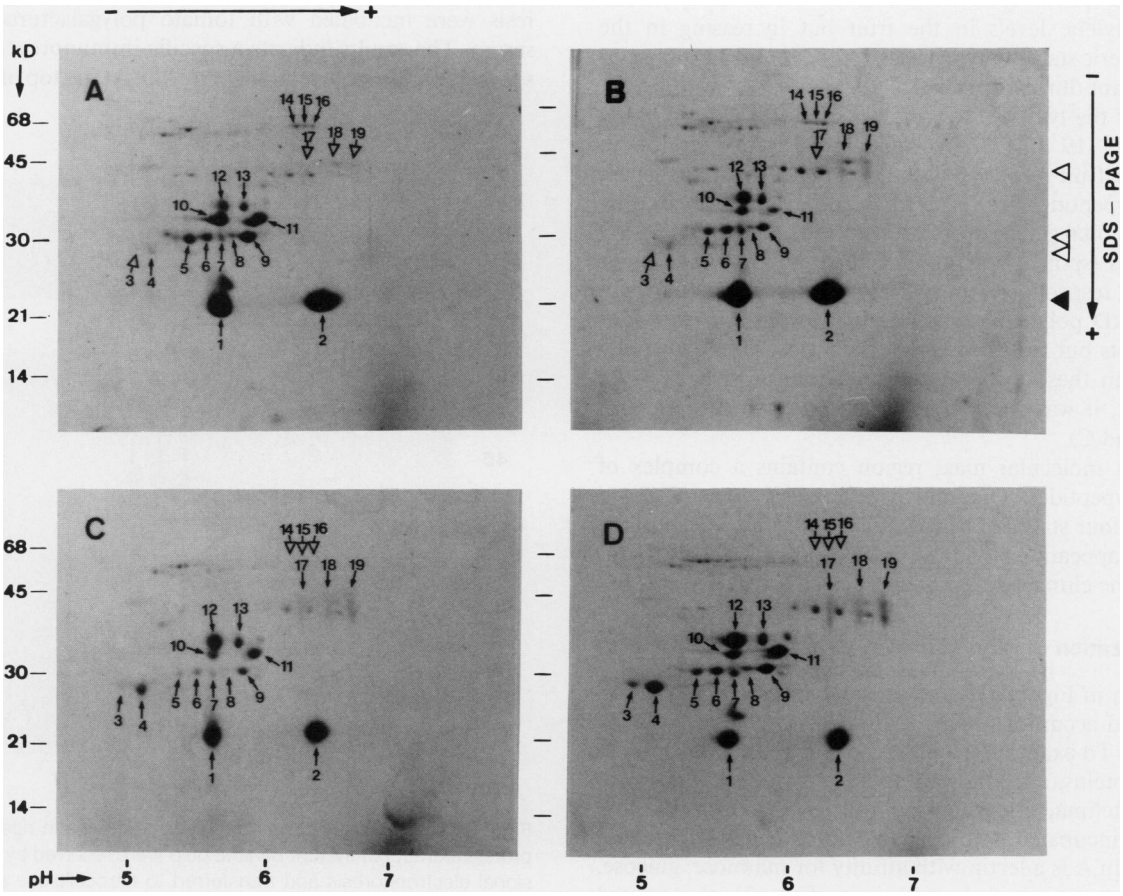

Figure 2. Two-dimensional electrophoretic patterns of total proteins during banana ripening. Figure 2A through D corresponds to samples at different stages of ripening as shown in Figure 1A and are the same used in Figure 1B. NEPHGE was carried out in the first dimension (3–10 pH range ampholites) and SDS-PAGE in the second dimension (15% acrylamide). The molecular mass markers are indicated on the right in kD. Lateral arrows (black and white) correspond to polypeptides that increase and decrease, respectively. White arrows indicate polypeptides lacking in comparison with other gels in the figure. Apparent isoelectric points are indicated on the bottom. Numbered spots are referred to in the text.

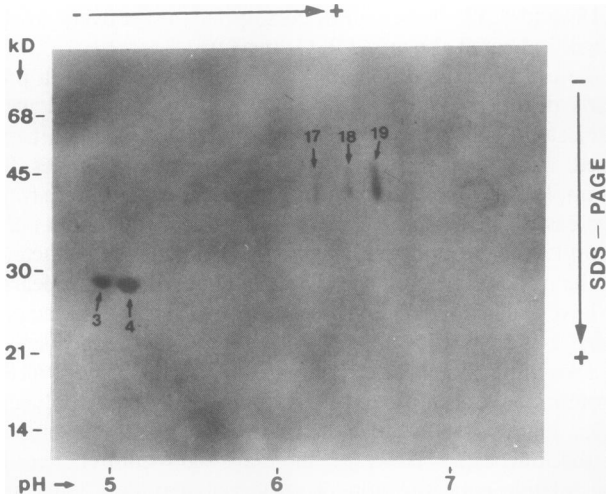

Figure 3. Detection of specific glycoproteins in postclimacteric banana fruit. Two-dimensional gel electrophoresis (see Fig. 2D) transferred to nitrocellulose sheet and visualized with the concanavalin A-peroxidase method.

at high ethylene levels in the fruit but increasing in the postclimacteric stage. Polypeptides 5, 6, 7, 8, and 13 remained fairly constant during ripening.

Note that the two sets of proteins (spots 3 and 4 of 28 kD and spots 17–19 of 42 kD) whose levels were very low in nonripening fruit increased with the onset of ripening. These sets of polypeptides are resolved in more than one spot by two-dimensional gel electrophoresis. In the case of the 28 kD polypeptides (spots 3 and 4) with apparent isoelectric point 4.9 and 5.2, respectively, spot 4 was more abundant than spot 3. The 42 kD polypeptides (spots 17–19) did not resolve as defined spots but as broad bands. The most abundant polypeptide from these three proteins was the most basic (spot 19). In fact, it was the first to be observed in the previous stages (B and C).

The high molecular mass region contains a complex of minor polypeptides. Qualitative differences were observed among the four stages of ripening. The most evident of these was the disappearance of the triplet of polypeptides 14, 15, and 16 in the climacteric peak and postclimacteric stages.

Characterization of High Mannose Glycoproteins

As shown in Figures 1B and 2, some specific polypeptides increase and accumulate during ripening (see spots 3, 4, 12, 13, 17–19). To examine the presence of carbohydrate moiety in these proteins, a lectin-binding assay was used. Total proteins of postclimacteric fruit were transferred to nitrocellulose sheets and incubated with concanavalin A (Fig. 3). Because concanavalin A is a lectin with affinity for mannose, glucose, *N*-acetylglucosamine, and sorbose residues (7), the stained polypeptides could contain one of these glycoconjugates. Thus, the three 42 kD polypeptides resolved as broad spots are glycoproteins. From one-dimensional SDS-PAGE-concanavalin A binding protein assays, it had previously been observed that the 42 kD band was a doublet (data not shown). A comparison of Figures 2 and 3 shows that these three proteins are located alternatively between other spots stained only by Coomassie blue. Unfortunately, we were unable to distinguish in two-dimensional electrophoresis the doublet previously observed in one-dimensional electrophoresis. These three glycoproteins could correspond to glycosylated forms of only one protein presenting charge heterogeneity. However, a single polypeptide could always be resolved in more than one spot due to different glycosylation contents. The acidic 28 kD proteins were also highly glycosylated, as was observed by concanavalin A binding experiments. Endogenous peroxidases or concanavalin A proteins that could interfere in the assay were not detected.

Accumulation of a Polygalacturonase-Related Protein in Ripe Banana Fruits

One of the ripening events that occurs in climacteric fruits after the synthesis of ethylene is polygalacturonase production (11). To determine whether one of the glycoproteins characterized in ripe fruits was banana polygalacturonase, an immunoblotting assay was carried out on total proteins. Results are shown in Figure 4. Total banana proteins from the postclimacteric stage resolved by two-dimensional electrophoresis were incubated with tomato polygalacturonase antiserum. The results indicate a specific immunoreaction of the serum with five polypeptides of 42 kD. At the top of the figure

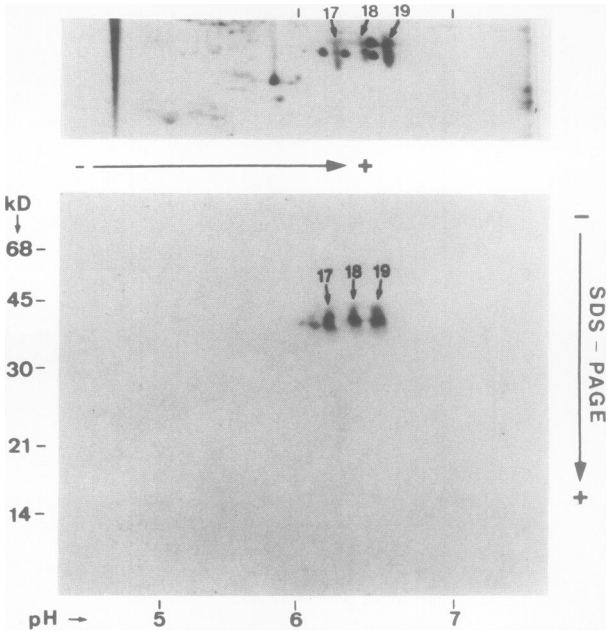

Figure 4. Immunodetection of polygalacturonase in ripe fruit. Total protein extracts of mature banana pulp were resolved by two-dimensional electrophoresis and transferred to nitrocellulose sheets. Blot was incubated with anti-polygalacturonase serum (dilution 1:1000) and immunoreactive bands labeled with peroxidase conjugate. Molecular mass markers are indicated on the left. Inset at the top of the figure shows the Coomassie blue-stained gel. Arrows show the spots that correspond to 17, 18, and 19 spots, positively reacted with the anti-polygalacturonase serum.

(see inset corresponding to Coomassie blue staining of the same gel transferred), it can be seen that the immunoreactive polypeptides were the glycoproteins 17, 18, and 19, plus two more acidic polypeptides of identical mol wt. However, we could not distinguish at this resolution level whether each spot was a doublet. The results suggest that the broad spots observed in Figures 2 and 3 could, in fact, be more than one protein. No cross-reaction against other polypeptides was observed with this immune serum. No reaction with preimmune serum was detected.

DISCUSSION

Ripening of fruits is associated with novel translational and/or transcriptional events. In bananas, there have been few reports about protein changes associated with fruit ripening. Brady and O'Connell (2) reported that most of the increment in protein synthesis early in the climacteric rise resulted in an increase in the turnover and the replacement of preexisting species of protein. On the basis of accumulation and/or activity changes of different cell wall enzymes, De Leo and Sacher (8) correlated increasing activities of acid phosphatase with synthesis *de novo*. Our present results indicate that there is a differential protein accumulation during banana ripening and some specific proteins of ripe fruit have been detected. The analysis of these differences was especially difficult in banana because of the presence of large amounts of starch (about 90% of the dry matter), polyphenolics in green fruit tissue, and soluble sugars in the ripe fruit (17). The main proteins of the four stages studied in the ripening process were present in all the stages. The qualitative differences observed may be related to the synthesis and hydrolysis of proteins involved in the ripening process (3). Most of them could be constitutive, structural, or storage proteins. However, our data provide evidence of stage-specific accumulations of particular polypeptides. The most significant changes were detected in the last two stages (C and D). Two polypeptides of 28 and 42 kD were present in the early climacteric stage but increased dramatically after the peak of ethylene production. The main reports of protein induced by ripening are focused in cell wall hydrolytic enzymes (12, 25). Recently, it has been shown that cellulase (5) and polygalacturonase (13) increase when ripening of avocado begins.

We have observed that one of the stage-specific polypeptides (42 kD) cross-reacts with tomato polygalacturonase antibodies (kindly given by Dr. G.A. Tucker). The 42 kD proteins are resolved in seven spots by two-dimensional gel electrophoresis. However, the antibody against tomato polygalacturonase recognizes only five of them. In lectin-binding experiments, we have observed that three of the five cross-reacting polypeptides bind concanavalin A, suggesting that these proteins contain a high mannose glycan moiety. These kinds of glycans are characteristic of N-glycosylation modification of proteins in the endoplasmic reticulum. The heterogeneity of charge observed both in immunoblots and in lectin-binding experiments could indicate the presence of different glycoproteins immunorelated or the presence of only one polypeptide with different levels of glycosylation.

The data presented here suggest that the 42 kD polypeptides could be polygalacturonase-related proteins. The immunological properties of these polypeptides indicate common antigenic determinants with tomato polygalacturonase. It should be noted that no other cross-reaction was observed in the total ripe banana extracts. In addition, the presence of neutral amino sugars was previously described in tomato polygalacturonase. The carbohydrate portion of the tomato enzyme contains mannose, fucose, xylose, and *N*-acetyl-glucosamine (19). However, the analysis of an enzymatic activity related to these polypeptides would be the approach to use to demonstrate the presence of polygalacturonase in ripe banana fruits.

It was interesting to note that another ripened stage-specific protein (28 kD) accumulated in large amounts in postclimacteric fruit. Recent experiments *in vivo* carried out in our laboratory indicate that this protein was not present in early climacteric stages, but that it was highly synthesized *de novo* in ripe fruit. However, the function of this polypeptide is unknown and no equivalent product was detected in other fruits. Antibodies raised against this protein will provide us with a tool to characterize it.

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