CHANGES IN PROTEIN COMPOSITION DURING STRAWBERRY (*Fragaria x ananassa* Duch.) FRUIT RIPENING[1]

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ABSTRACT

The changes in strawberry native proteins and polypeptides during ripening were studied. Analysis of protein extracts by nondenaturing electrophoresis showed the presence of a protein species only in the '25% Red' and later ripening stage. This 40 kD polypeptide was hardly detected in 'Large green' and 'White' fruit extracts. The polypeptide profile, obtained with SDS-PAGE, also changed during ripening. While most polypeptides were found in all ripening stages, the distribution of some of them varied during ripening.

Protein synthesis (measured by ^{35}S-methionine labelling) in strawberry was mainly directed towards the regeneration of previously existing proteins. As ripening proceeded, the synthesis of 67 and 63 kD polypeptides increased, while those of 82, 56 and 25 kD decreased. Three very-abundant polypeptides (36, 24 and 23 kD) were located in the achenes and were not labelled in any ripening stage.

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INTRODUCTION

The ripening of fruits is a complex physiological process, and the biochemical changes associated with it have been studied mainly in climacteric fruits, e.g., banana, avocado, mango, pear, apple and, especially, tomato (Christoffersen *et al.* 1982; Brady 1987; Gray *et al.* 1992; McGarvey *et al.* 1992; Grierson and Schuch 1993; Seymour and Tucker 1993; Seymour 1993; Lizada 1993; Knee 1993; Hobson and Grierson 1993). On the other hand, the ripening of nonclimacteric fruits is less studied, though some works have been reported for several fruits such as orange (Alonso *et al.* 1992), acid lime (Echeverría 1992), calamondin (Purvis 1980; Purvis and Barmore 1981), and strawberry. The latter fruit received more attention since Given *et al.* (1988a) revealed the fundamental role of the auxins in the regulation of the ripening process. Others have studied the activity of different enzymes during strawberry fruit ripening, such as phenylalanine-ammonium-lyase (PAL) (Given *et al.* 1988b; Given *et al.* 1988c; Cheng and Breen 1991), polyphenoloxidase (Wesche-Ebeling and Montgomery 1990a,b), polygalacturonase (Matsuhashi and Hatanaka 1991; Nogata *et al.* 1992), cellulase (Abeles and Takeda 1990), uridine diphosphate glucose:flavonoid O^3-glucosyltransferase (UDPGFT) (Given *et al.* 1988b), pectinesterase (Barnes and Patchett 1976) and invertase (Ranwala *et al.* 1992).

Veluthambi and Poovaiah (1984) studied the variation of polypeptide composition associated with the early development of the fruit, as well as the existing relationship between the level of some polypeptides and the presence of auxins. They followed the process over the first twenty days after the start of pollenation. In that work, the authors demonstrated that the polypeptide electrophoretic pattern changes significantly as the fruit develops, and that some polypeptides appear only in the presence of auxins. Manning (1994) showed that the population of messenger RNAs experience important modifications during strawberry ripening, detecting an increase of at least 44 messenger RNAs, and a decrease in 14 messenger RNAs as the fruit passes from green to ripe. Recently, Wilkinson *et al.* (1995) examined mRNA populations in ripening strawberry fruit using polymerase chain reaction (PCR) differential display. They identified five mRNAs with ripening-enhanced expression, three of which seem to be fruit-specific.

In the present work, we analyzed the electrophoretic profiles of strawberry extracts to identify proteins and polypeptides showing changes in their levels during ripening. The use of ^{35}S-methionine in tissue discs allowed us to compare the intensity of protein synthesis in the different ripening stages, as well as the composition of the polypeptides synthesized.

MATERIALS AND METHODS

Plant Material

Strawberries (*Fragaria* × *ananassa* Duch., cv. Selva) grown either in the field (October to March) or in greenhouses (April to September) were used in different experiments. In some experiments, field-grown fruits were used, while in others greenhouse-grown fruit were employed. Neither experiment was carried out with fruits of mixed origin. The fruits were harvested at different ripening stages and classified on the basis of their surface color and size as Small Green (SG), Large Green (LG), White (W), 25% Red, 50% Red, 75% Red, 100% Red and Over-Ripe (OR); the berries were then washed, the calix and peduncle removed and they were either immediately used or frozen at −80C until needed.

Preparation of Deachened Fruit

Achenes (seeds) of fruits collected at different ripening stages were removed by means of a thin spatula.

Extraction and Determination of Pigments

Chlorophylls. Strawberries frozen at −80C were crushed in a refrigerated mill (Tekmar, Model A-10) and the powder so obtained was poured in acetone previously cooled to 0C; after 10 min, the suspension was centrifuged at 5000 g for 5 min at 4C, and the supernatant was retained. The content of chlorophylls a and b was determined by the Bruinsma method (Bruinsma 1963).

Anthocyanins. In this case, the crushed fruits were poured in 1% HCl in methanol (1 mL HCl in 100 mL solution). After 10 min at 0C the mixture was centrifuged at 1500 g for 5 min at 4C, and the supernatant retained. The absorbance at 515 nm was then read, and the anthocyanin content, as pelargonidin-3-glucoside, was calculated by using $E_{abs.molar} = 36000$ $1.mol^{-1}.cm^{-1}$ (Woodward 1972).

Extraction and Characterization of Proteins

For these operations, several buffers noted as A, B, C, D and E were used, and the composition of each is described below:

Buffers.

A: 76 mM K_2HPO_4; 27 mM KH_2PO_4; 25 mM EDTA; 1 mM Cysteine; 50 g/L PVPP (polyvinylpolypyrrolidone); 1 mM PMSF (phenyl-methyl-sulfonyl fluoride); pH 7.3.

B: A + 2% (v/v) Triton X-100 + 2 M NaCl.

C: 60 mM Tris-base; 15% (w/v) Sucrose; 25 mM EDTA; 1 mM Cysteine; Bromophenol blue 0.002% (w/v); pH 6.8.

D: 50 mM Tris-base; 2% (w/v) SDS; 2% (v/v) Mercaptoethanol; 1 mM EDTA; 5% (w/v) Sucrose; 6 g/L PVPP; pH 7.0.

E: 62.5 mM Tris-base; 2% (w/v) SDS; 5% (v/v) Mercaptoethanol; 25 mM EDTA; 15% (w/v) Sucrose; pH 6.8.

Extraction of Native Proteins

Total Native Extract. The starting material consisted of fruit frozen at −80C which was crushed in a refrigerated mill (Tekmar, model A-10). The powder so prepared was extracted with three volumes of Buffer B at 4C for 90 min, under stirring. The mixture was centrifuged at 9200 g at 4C for 15 min, and the supernatant retained. It was then precipitated by adding solid $(NH_4)_2SO_4$ to reach 75% saturation. The suspension was centrifuged under the conditions described in the previous step, and the precipitate, which was the part retained, was suspended in buffer C and frozen at −80C until needed. All steps were performed at 4C.

Extracts of Either Soluble or Insoluble Native Proteins in Phosphate Buffer. Three volumes of Buffer A were added to the frozen and crushed fruit, and the resulting suspensions were stirred for 30 min at 4C. They were then centrifuged at 9200 g for 10 min at 4C, and the supernatant (S1) separated from the precipitate. The latter was extracted with 3 volumes of Buffer B for 60 min at 4C; the suspension was centrifuged and the supernatant (S2) separated. In turn, supernatants S1 and S2 were precipitated with $(NH_4)_2SO_4$ and treated as described for total extracts. By these procedures, we obtained either soluble or insoluble proteins in phosphate buffer.

Preparation of Acetone Powder

Fresh fruits were cut and immediately immersed in acetone precooled at −20C; they were then crushed in an Omnimixer at maximum speed for two to three minutes, and the suspension vacuum-filtered through filter paper. The residue was washed several times with cold acetone until the washing fluid was colorless, and then placed on a Petri dish in a desiccator, where it was vacuum-dried. The residue was finally stored at −80C until needed.

Extraction of Denatured Proteins

An amount of 0.5 g of acetone powder was stirred in 60 mL of Buffer D for 40 min at room temperature. The suspension was centrifuged at 9200 g for 10 min at 20C, and the supernatant separated; the precipitate so obtained was extracted again with 40 mL of Buffer D at the same conditions. The extracts were pooled and heated at the boiling temperature for 3 min to inactivate enzymes. We added solid trichloroacetic acid (TCA) to this crude extract to obtain a 10% (w/v) concentration. The mixture was shaken and left overnight at 0C. The precipitate was washed twice with 80% (v/v) acetone to remove the remaining acid. The acetone was eliminated by vacuum-evaporation and the resultant pellet dissolved in 500 μL of Buffer E and left overnight at 0C. The mixture was then centrifuged, and the supernatant so obtained was stored at −80C.

Electrophoresis under Nondenaturing Conditions (PAGE)

We used the buffer system described by Ornstein (1964) and Davis (1964), and prepared gels with a linear gradient of acrylamide concentration (4–12%). The electrophoresis were done at a constant current intensity, of 20 mA/plate in the stacking gel, and 40 mA/plate in the separating gel. The gels were stained with silver salts (Blum *et al.* 1987).

Electrophoresis under Denaturing Conditions (SDS-PAGE)

SDS-polyacrylamide gels were prepared according to the discontinuous buffer system of Laemmli (1970). The gels were prepared in a linear gradient of acrylamide concentration (10–18%) and the electrophoresis performed at constant intensity (15 mA/plate in the stacking gel and 25 mA/plate in the separating gel). After the electrophoresis, the gels were stained with 0.1% (w/v) Coomasie-Blue R-250. As the samples were dissolved in buffer E, the exact protein content could not be determined owing to interferences caused by the high concentrations of SDS and mercaptoethanol (Lowry *et al.* 1951; Bradford 1976). Therefore, we used the criterion described by Veluthambi and Poovaiah (1984), and performed trial runs to determine the sample volumes providing comparable intensities for common polypeptide bands.

Bidimensional Electrophoresis

The first and second dimensions corresponded to nondenaturing and denaturing conditions, respectively.

The first dimension was carried out by placing the sample in a 0.75 mm thickness, nondenaturing acrylamide gradient (4–12% acrylamide) gel. Then, the

selected part of the gel was cut and frozen at −20C in a 10% (w/v) aqueous glycerol solution, until used.

A denaturing acrylamide gradient gel (10–16%) of 1.0 mm thickness was prepared with continuous and reference lanes. A strip of the first dimension gel was thawed and incubated at 55C during 10 min two times with pre-treatment buffer (62.5 mM Tris-base; 1% w/v SDS; 10% w/v sucrose; pH 6.8). The strip was then placed on the plate, and sealed at 70C with a solution of agarose (1% w/v agarose in pretreatment buffer containing bromophenol blue). The electrophoresis was performed at constant current intensity (30 mA until the dye entered, and 50 mA thereafter). The gel was developed with silver salts.

^{35}S-methionine Labelling

Fruits were cut transversely at mid-length into 1–2 mm thick tissue slabs or discs. They were incubated for 30 min at 20C in a pH 7.0 pretreatment buffer of the following composition: 60 mM K_2HPO_4; 36 mM KH_2PO_4; 5 mM Dithiothreitol (DTT); 0.6 M Glycerol. The buffer was then removed and a solution of ^{35}S-methionine (specific activity > 600 Ci/mmol) was added. The volume and concentration of the solution were selected to deposit 10 μCi at ^{35}S-methionine in each disc. In other experiments, the ratio of the quantity of radioactive species to tissue mass was kept constant at 15 μCi/g. The incubation with radioactive methionine was maintained for 4–6 h after which the discs were rinsed and then crushed with a mortar. With this material, denatured proteins were extracted with 20 mL of buffer D, according to the above-described method. Two aliquots of 1 mL were taken from the extract and poured on 10 mL of 10% (w/v) TCA to precipitate the proteins. The system was allowed to rest overnight at 0C, then filtered through 0.45 μm filters, and the quantity of radioactivity incorporated into the proteins was determined by a scintillation counter (Rackbetta 1214, Pharmacia).

The remaining part of the extract was precipitated as usual with solid TCA and then analyzed by denaturing electrophoresis and subsequent fluorography.

The incorporation of ^{35}S-methionine was measured in achenes from white fruits. To this end, we incubated 110 mg of achenes in radioactive solution (40 μCi) for 5 h at 20C, and the protein extract was prepared as previously described.

Electrophoresis and Fluorography

On 5–10 μL of the protein extracts, the quantity of the radioactive species was measured by a scintillation counter (Rackbetta 1214, Pharmacia). The extracts were analyzed by electrophoresis (SDS-PAGE). For this stage, various amounts of the different extracts were seeded to have the same radioactivity (usually 2,000

to 15,000 cpm) in each lane. The gel so obtained was fixed with TCA 10% (w/v), and a fluorography profile was prepared according to the method described by Bonner and Laskey (1974). Once treated, the gel was wrapped with a water-permeable plastic film, dried at 37C and put in contact with a Kodak film X-OMAT-XK-1 for 2-3 weeks at −60C.

Experimental Design

All experiments were repeated at least three times. Pigment determinations were carried out in triplicate; the results were analyzed by ANOVA and the means compared by the LSD test.

RESULTS AND DISCUSSION

Variations of Pigment Level During Ripening

Levels of total chlorophyll, chlorophylls *a* and *b*, and anthocyanins were analyzed in strawberries, to characterize the different ripening stages. The results obtained are shown in Fig. 1.

As can be noticed in Fig. 1(a), the total chlorophyll level, expressed as micrograms of chlorophyll per g of fruit, decreases gradually along the course of ripening. Besides, it can be observed that the level of chlorophyll *a* is higher than that of chlorophyll *b* throughout the different stages, except in Over-ripe, where the levels of both chlorophylls are very low. Figure 1(B) shows the anthocyanin levels determined at the different ripening stages. A large increase of these pigments is observed, in accordance with the color variation exhibited by the fruit as it ripes. The anthocyanin levels are low in the early stages (7.0, 8.3, 9.3 and 11.1 nmol/g at stages SG, LG, W and 25% R, respectively), but show a sudden four fold increase at the 50% R stage (41.3 nmol/g), attaining high levels at the late stages 100% R and OR (137.3 and 434.6 nmol/g, respectively). The levels of chlorophylls and anthocyanins of the strawberries used here (cv. Selva) were similar to those reported for other strawberry varieties such as Tillikum (Cheng and Breen 1991) and Brighton (Given *et al.* 1988a).

NonDenaturing Electrophoresis of Native Protein Extracts

Total extracts in phosphate buffer of either soluble or insoluble native proteins, were prepared from fresh fruits. These extracts were analyzed by nondenaturing electrophoresis, as described under Materials and Methods. The electrophoretic analysis of strawberry native proteins had not been previously described. The preparation of native protein extracts is troublesome in strawberry owing to in-

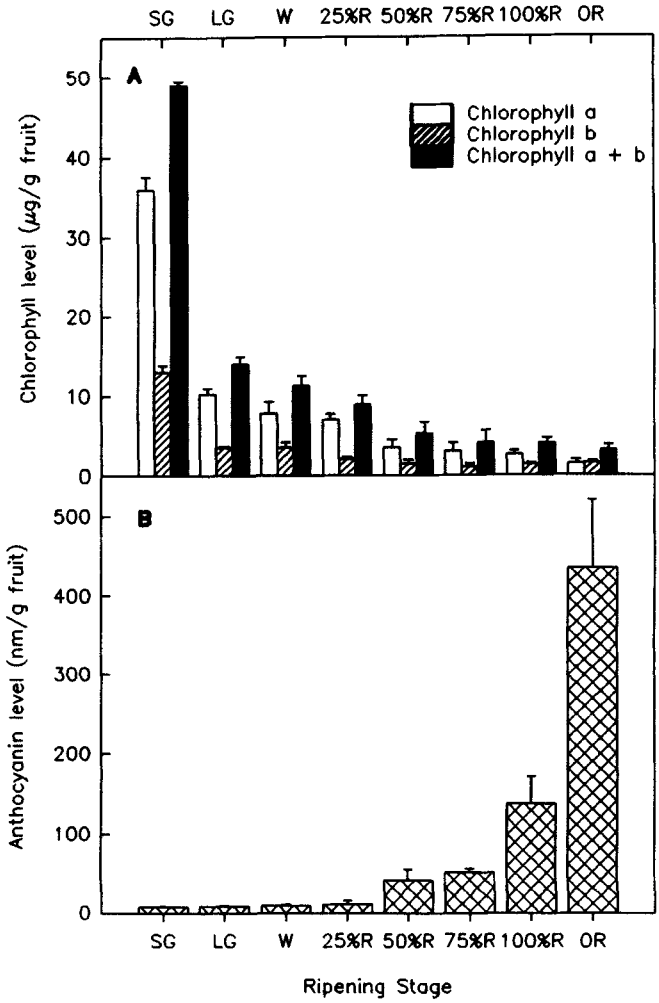

FIG. 1. PIGMENT LEVELS IN THE DIFFERENT RIPENING STAGES. (A) TOTAL CHLOROPHYLL, AND CHLOROPHYLLS 'a' AND 'b'. (B) ANTHOCYANINS
Ripening stages were noted as follows: Small Green (SG); Large Green (LG); White (W); Red (R); Over-ripe (OR).
All determinations were performed at least in triplicate. Bars indicate average values and lines represent standard deviation. $LSD_{0.05}$ in total chlorophyll = 1.86; $LSD_{0.05}$ in chlorophyll a = 1.36; $LSD_{0.05}$ in chlorophyll b = 0.87; $LSD_{0.05}$ in anthocyanin = 35.4.

terferences of phenols and to the high pectin content of the fruit (Wesche-Ebeling and Montgomery 1990b; Veluthambi and Poovaiah 1984). The extraction carried out in the conditions described in Materials and Methods, followed by

precipitation with $(NH_4)_2SO_4$ allow us to obtain extracts with adequate viscosity for their analysis by nondenaturing electrophoresis. The low protein content of the extracts so obtained required the use of silver salts staining to visualize protein bands after electrophoresis. It must be mentioned that the preliminary attempts for concentrating the proteins by precipitation with acetone failed owing to formation of insoluble gels.

Figure 2(A) shows the electrophoresis profiles of total native protein extracts corresponding to the stages LG, 50% R and OR. It can be noticed that the gel bands are grouped mainly in three zones: upper, middle and lower. The middle zone is rather blurred, possibly indicating several proteins of similar mobility. The lower zone shows several well-defined bands, one of them exhibiting differences for the different ripening stages. The analysis of profiles allowed us to observe the presence of a band (indicated with the arrow) which, as the fruit ripens, increases its intensity. This species is hardly detected in LG and W fruits,

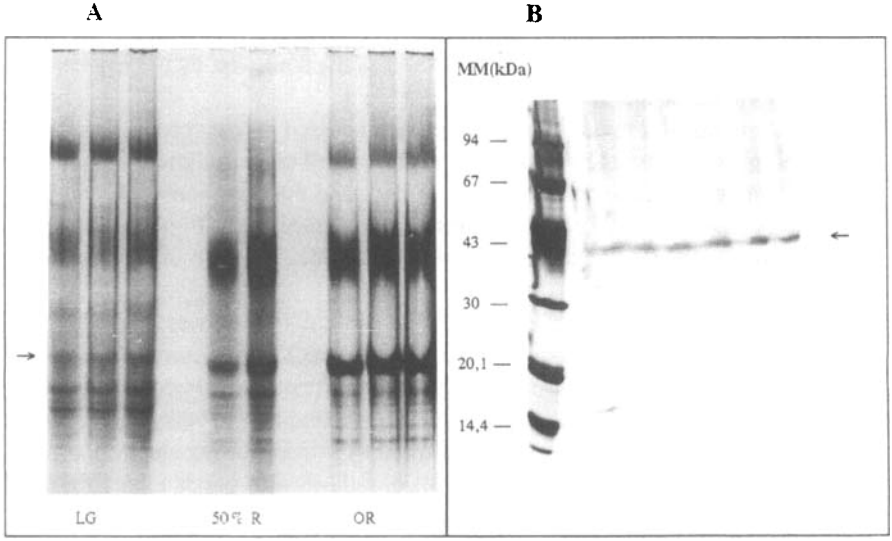

FIG. 2. (A) NONDENATURING ELECTROPHORESIS (PAGE) OF FRUIT EXTRACTS FOR DIFFERENT RIPENING STAGES

Acrylamide gradient gel (4–12%). Ripening stages: Large Green (LG); 50% Red (50% R); Overripe (OR). In each case (LG, 50% R and OR) several volumes of extract were applied, and the gel was stained with silver salts. The arrow shows the protein species of interest.

(B) TWO-DIMENSIONAL ELECTROPHORESIS OF THE PROTEIN SPECIES UNDER STUDY

A native extract from 100% Red fruit was first analyzed by nondenaturing electrophoresis (PAGE, 4–12% gel). Then, the selected part of the gel (signaled with an arrow in Fig. 2.A) was cut and analyzed by denaturing electrophoresis (SDS-PAGE, 10–16% gel). Molecular mass standards (Pharmacia) were run, and gel was stained with silver salts. The arrow shows the resultant band so obtained.

its level increases noticeably at the 25% R stage, and keeps on the increase in the later stages (data not shown for W and 25% R). These results were confirmed by using either acetone powder, ammonium sulfate precipitates, or crude extracts dialyzed and freeze-dried (without precipitation). By analyzing either soluble or insoluble native protein extracts in phosphate buffer, it was possible to determine that the aforementioned protein species is present both in the 'soluble' and 'insoluble' fractions of fruit extracts in the 25% R ripening stage or later, whereas it is absent in similar fractions corresponding to LG and W fruits (results not shown).

The possible effect of the presence of achenes (seeds) on the production of the protein species under study were also analyzed along the course of ripening. To this end we harvested white fruits, removed the achenes, and left the deachened fruits for three days, until they attained a 100% R ripening stage. White fruits with achenes were allowed to ripe for three days, they were then deachened and used as controls. The color development was faster in deachened fruits than it was in the respective controls, in agreement with the observations of previous authors (Given *et al.* 1988a; Kano and Asahira 1978). After doing the extraction and electrophoresis stages as described under Materials and Methods, the presence of the above-mentioned protein was detected in all fruits, so its presence is independent of the achenes.

Then, the molecular mass of the polypeptide included in that species was determined by means of bidimensional electrophoresis of protein extracts from ripe fruits. A native first dimension was performed, and the band including the protein under study was used for the denaturing second dimension. Figure 2(B) shows that the protein species includes only one polypeptide, its molecular mass being 40 kD.

To determine whether the increase of this protein species was caused by de nova synthesis, we did experiments in which a radioactive amino acid (^{35}S-methionine) was incorporated into synthesized protein. To this end, fruit discs at the stage 75% Red were incubated with the labelled amino acid, and proteins were extracted in native form and then analyzed by nondenaturing electrophoresis. Autoradiography of the gel allowed a series of radiolabeled-bands to be detected, however none of them corresponded with the position of the protein series in question (results not shown). This indicates that the increase observed during ripening is not due to an increase of its synthesis, but possibly caused by a protein degradation process.

Analysis of Protein Extracts by SDS-PAGE

Fruits at ripening stages from Small Green to Over-Ripe were used to prepare protein extracts under denaturing conditions. They were analyzed by electrophoresis in gels with polyacrylamide gradient, in the presence of SDS (Fig. 3).

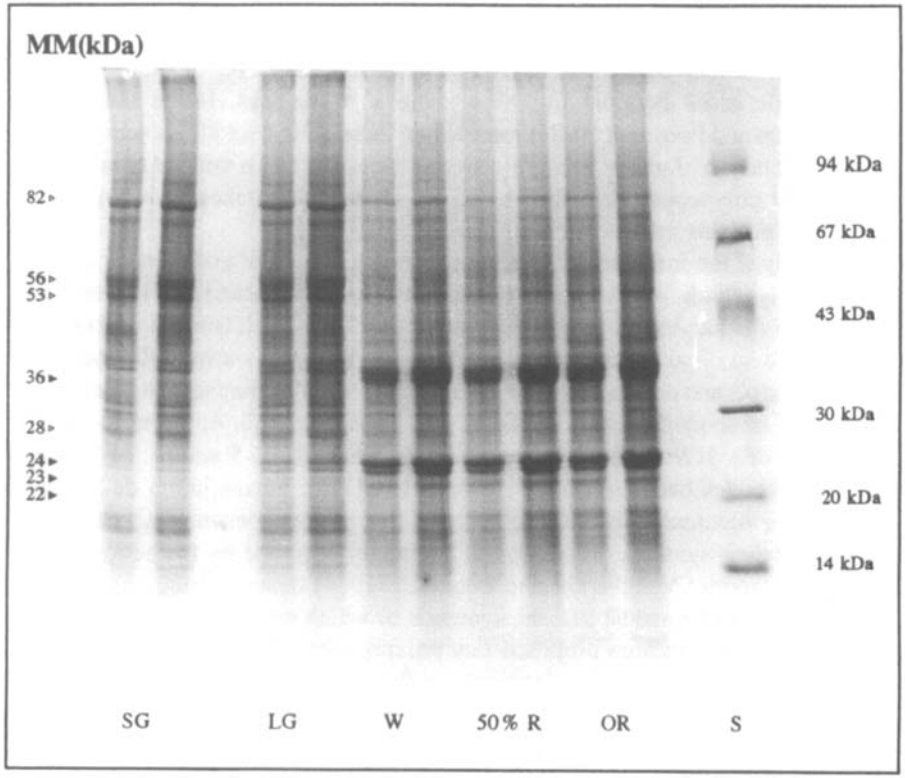

FIG. 3. DENATURING ELECTROPHORESIS (SDS-PAGE) OF STRAWBERRY EXTRACTS IN DIFFERENT RIPENING STAGES

Acrylamide gradient gel (10–16%). Ripening stages: Small Green (SG), Large Green (LG); White (W); 50% Red (50% R); Over-ripe (OR). Two extract volumes were analyzed in each stage. Molecular mass standards (S) (94, 67, 43, 30, 20.1 and 14.4 kD) were run. Gels were stained with Coomasie-Blue R-250.

Symbols: △ indicates the position of bands whose intensity decrease during ripening
▲ indicates the position of bands whose intensity increase during ripening

The electrophoretic profiles of the different ripening stages show numerous polypeptides with molecular weights ranging from 10 to 95 kD. Even when most polypeptides are present in all ripening stages, the distribution of some of them varies. For instance, a polypeptide of 36 kD, absent in stages Small Green and Large Green, appears in large amounts from the stage White on. In a similar fashion, two polypeptides of 24 and 23 kD, almost absent in the stage SG, are detected in the stage LG and later. In a lesser extent, a 22 kD polypeptide is detected from the stage W onwards. On the other hand, the level of polypeptides

with molecular weights of 82, 56, 53 and 28 kD decreased as the fruit ripened. The 82 kD polypeptide is probably the same described by Veluthambi and Poovaiah (1984) with a molecular mass of 81 kD. They found a lower amount of 81 kD polypeptide in 20 days old fruit than in those of 10 days. In the same study Veluthambi and Poovaiah (1984) reported an increase in a 37 kD protein 10 days after pollenation. As they used de-achened fruits and, as it will be seen below, the 36 kD polypeptide described by us is located in the achenes, it is unlikely that both proteins are the same.

To analyze the influence of achenes, a known source of endogenous auxins, on the polypeptide profile, fruits SG and W were de-achened in the plant, left for 4 days and harvested together with the controls (i.e., fruit containing achenes). The results so obtained are shown in Fig. 4A. The graph shows differences between controls and de-achened fruits. This is evidenced by the fact that de-achened fruits do not show the bands at 36, 24 and 23 kD (lanes 3, 4, 7 and 8), which are noticeable in fruits keeping their achenes (lanes 1, 2, 5 and 6). Moreover, the polypeptides bands of 56, 53, 40 and 35 kD are intensified in de-achened fruits. The accumulation of these polypeptides in the absence of achenes suggests that their synthesis is directly or indirectly inhibited by the action of endogenous auxins. Veluthambi and Poovaiah (1984) described the existence of polypeptides of 57 and 52 kD, the synthesis of which are inhibited by the action of auxins. These authors proposed that polypeptides of 57 and 52 kD have an inhibitory effect on fruit development. In this regard, it is considered that the polypeptides found in our study on strawberries cv. Selva (56 and 53 kD) are possibly the same as those described by Veluthambi and Poovaiah (57 and 52 kD) for strawberries cv. Ozark Beauty.

To complement the latter experiment, we analyzed, in separate form, the profile of polypeptides of de-achened fruits and that of the achenes. To this end, the achenes of fruits in the White stage were eliminated, and extracts were prepared in denaturing conditions both from the de-achened fruits, and from the achenes, separately. The extracts were analyzed by SDS-PAGE and the results are shown in Fig. 4B. In the photograph, it is observed that the bands of 36, 24 and 23 kD are found exclusively in achenes. A similar result was obtained when using ripe fruits.

Protein Synthesis at Different Ripening Stages

In different climacteric fruits (apple, pear, tomato, banana, avocado), it has been observed that the synthesis of proteins remains active over the whole ripening process. Its intensity increases during the climacteric rise, and then decreases (Brady 1987; Rhodes 1980). The synthesis process mostly replaces previously-

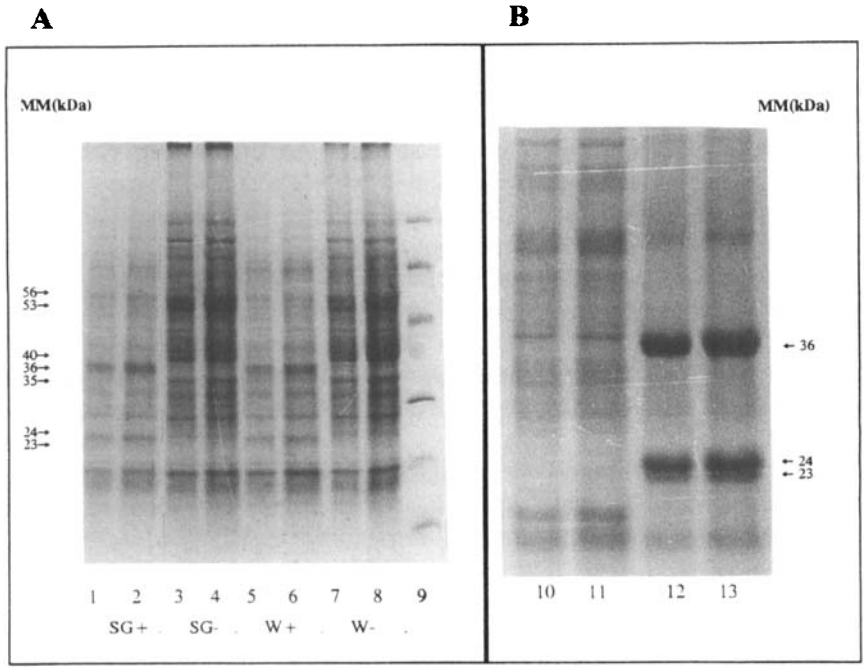

FIG. 4. (A) INFLUENCE OF THE ACHENES ON THE POLYPEPTIDE PROFILE. DENATURING ELECTROPHORESIS (SDS-PAGE)

Lanes 1–2 correspond to: fruit at Small Green (SG) stage with achenes (control), harvested in the same day as the de-achened fruits; 3–4 fruit in the stage SG, de-achened in the plant and harvested four days later; 5–6: fruit at White (W) stage with achenes (control), harvested in the same day as the de-achened fruits; 7–8: fruit in the stage W, de-achened in the plant and harvested four days later; 9: Molecular mass standards (94, 67, 43, 30, 20.1 and 14.4 kD). The control extracts (lanes 1–2 and 5–6 included achenes. The symbol '+' corresponds to achened fruits, and the symbol '−' corresponds to de-achened fruits.

(B) POLYPEPTIDE PROFILE OF BOTH DE-ACHENED FRUITS AND ACHENES

Denaturing electrophoresis (SDS-PAGE). Lanes 10–11 correspond to: fruits in the White (W) stage de-achened just before extraction; 12–13: achenes from White fruits.

Gels of acrylamide gradient (10–18%) were used both in experiments A and B. Gels were stained with Coomasie-Blue R-250.

existing proteins. Only a small proportion of protein synthesis leads to new protein species (Brady 1987; Rhodes 1980).

In comparison, the synthesis of proteins in nonclimacteric fruits have been less studied, so the literature on the subject is scarce. In our work, we analyzed the characteristics of this process during strawberry ripening by measuring the quantity of ^{35}S-methionine incorporated in the TCA-precipitable fraction. Besides, the composition of synthesized polypeptides was analyzed by fluorography.

Radioactive Incorporation Kinetics. Experiments were done to analyze the kinetics of the incorporation of ^{35}S-methionine into proteins, and to select an adequate time for incorporation of radioactive compounds in tissue discs. To this end, the amount of radioactivity per gram of White fruits was measured at different times. The results so obtained are shown in Fig. 5. It is observed that the quantity of labelled amino acid incorporated into the proteins increases linearly after 30 min of incubation. Shorter incubation times are not convenient, since they show a low radioactive incorporation, possibly because the amino acid cannot reach the inner part of the disc. Therefore, to ensure a good radioactive incorporation, incubation times were set for 4 and 6 h.

Protein Synthesis in Fruit Discs at Different Ripening Stages. The intensity of protein synthesis in tissue discs was evaluated at the different ripening stages (SG, W, 50% R, OR), by measuring the incorporation of ^{35}S-methionine into the protein fraction. As the disc size enlarges noticeably with the progress of ripening, we added variable quantities of radioactivity so as to keep the same amount of ^{35}S-methionine per gram of fruit in all ripening stages. Then, we observed

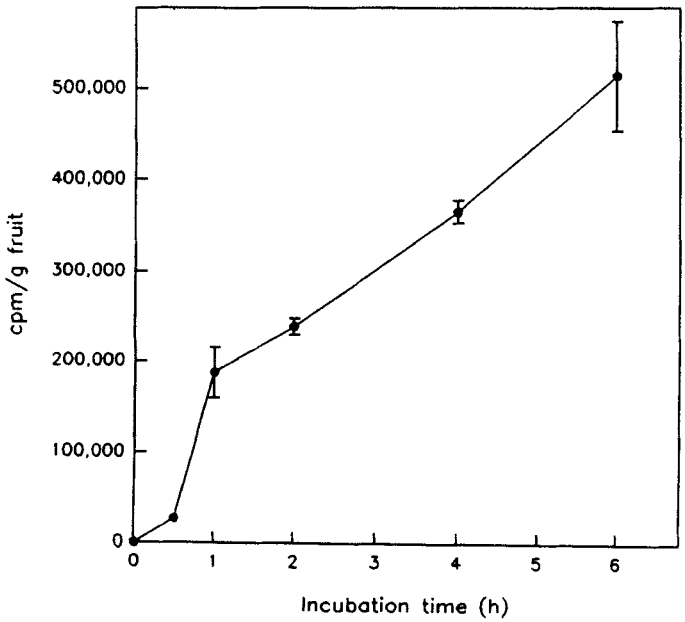

FIG. 5. KINETICS OF ^{35}S-METHIONINE INCORPORATION INTO PROTEINS OF TISSUE DISCS FROM FRUITS IN THE WHITE STAGE

The level of ^{35}S-methionine supplied was equal to 13 μCi per gram of tissue. Bars represent the standard deviation.

that the incorporation of ^{35}S-methionine to proteins (nearly 2.5×10^5 cpm ^{35}S-methionine per gram of tissue) was similar in all the ripening stages under analysis. As in the climacteric fruits, strawberries conserve their capacity to synthesize proteins up to the end of ripening. However, unlike such fruits, we did not detect any increase of synthesis intensity during strawberry ripening. These results coincide with those of Manning (1994), who observed that the total RNA and total protein content keeps high over the first ten days after anthesis, then gradually decrease up to, approximately, the 19th day (equivalent to fruit SG-LG), to keep constant from then to the end of ripening.

In achenes taken from White fruits incubated with radioactive methionine, the experiments revealed that an intense protein synthesis takes place in them as well (4.2×10^6 cpm incorporated per gram).

Electrophoretic Profile of Polypeptides Synthesized at Different Ripening Stages. Extracts prepared from fruits in different ripening stages were analyzed by fluorography, and the results are exhibited in Fig. 6. Although most polypeptides incorporated similar amounts of radioactivity in all the stages analyzed, some bands differed. The synthesis of some polypeptides (67 and 63 kD) increases, while in some others (82, 56 and 25 kD) it decreases as the fruit ripens. Moreover, some very abundant protein species (36, 24 and 23 kD), as observed with the Coomasie-Blue dye, were not labelled in any ripening stage. As previously described, these three polypeptides were found only in the achenes. In separate ^{35}S-methionine labelling experiments with achenes from white fruits, we observed several radiolabeled polypeptides, however, none of them corresponded to those at 36, 24 or 23 kD. Therefore, it can be concluded that such polypeptides are not synthesized in any stage after the Small Green. The nature and function of such achene-proteins is unknown, though, given their abundance, they may be storage proteins of seed.

The results obtained in strawberry coincide with those observed for climacteric fruits with respect to the destination of the incorporated amino acid, and thus with that of the protein synthesis in the different ripening stages. In both cases, the synthesis is mainly directed to the regeneration of previously-existing proteins and, therefore, there are few species synthesized 'de novo.' Such species are, for example, polygalacturonase, pectinesterase and ACC-synthase in tomato (Gray *et al.* 1992; Hobson and Grierson 1993), and cellulase in avocado (Christoffersen *et al.* 1984).

CONCLUSIONS

The composition of strawberry proteins changes during the ripening period, from the Small Green to the Red and Over-Ripe stage. The electrophoretic pro-

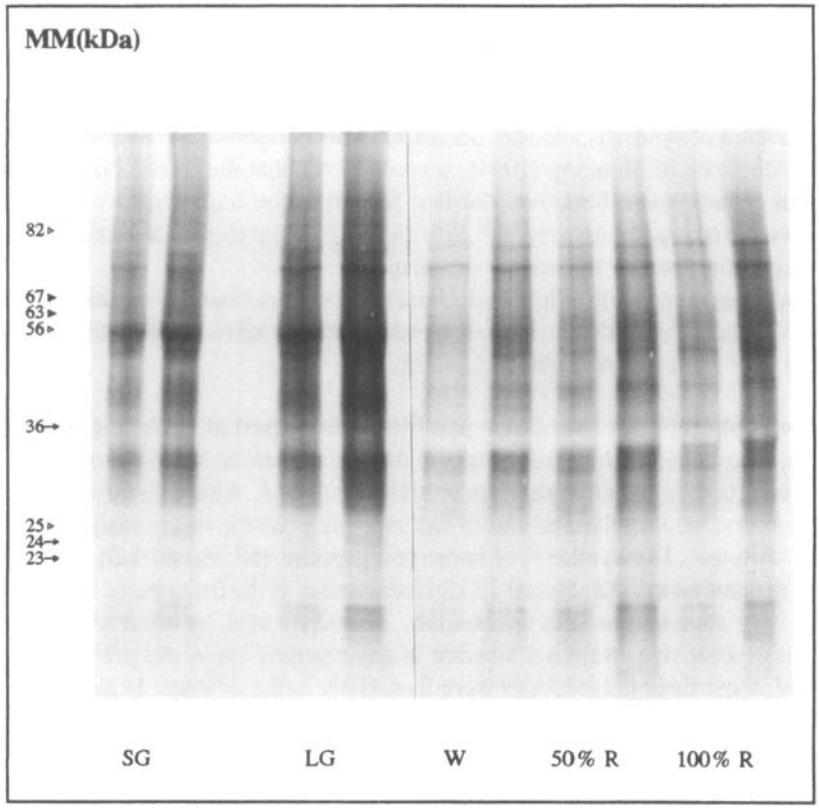

FIG. 6. FLUOROGRAPHY

Analysis of polypeptides labelled with ^{35}S-methionine, synthesized in the following ripening stages: Small Green (SG); Large Green (LG); White (W); 50% Red (50% R); 100% Red (100% R). Two extract amounts were analyzed in each stage.

Symbols: △ indicates the position of polypeptides whose synthesis decrease during ripening
▲ indicates the position of polypeptides whose synthesis increase during ripening
→ indicates the position of nonlabelled polypeptides discussed in the text

file of native proteins from fruits in the ripening stage 25% Red, or later, reveals a species that is absent in stages Green or White. The amount of this new protein species increases during the course of ripening. In a similar fashion, modifications were observed in the polypeptide profile, since species that are absent in stages Small Green or Large Green are then clearly detectable from the stage White on. On the other hand, it is observed that the amount of polypeptides of 82, 56, 53 and 28 kD decreases over the course of ripening.

The protein synthesis of this nonclimacteric fruit continues over the whole ripening process, and its intensity remains rather constant from the stage Small Green

on. In turn, the achenes of the fruit perform an active protein synthesis, at least in the stage White. The synthesis in the fruit is mainly to replace previously-existing proteins, whereas few polypeptides show changes in their levels. As ripening proceeds in the fruit, the synthesis of some polypeptides (67 and 63 kD) increases while, in some others (82, 56 and 25 kD) it decreases.

Moreover some very-abundant protein species located in the achenes of the fruit (36, 24 and 23 kD) are not synthesized from the Small Green to the Over-Ripe Stages.

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