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Chilling temperature storage induces changes in protein patterns and protease activity in cherimoya fruit

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Abstract

Storage at 6°C inhibited the ripening process and caused severe damage in cherimoya fruit (*Annona cherimola* Mill. cv. 'Fino de Jete'). In the present study, we analyzed the modifications in protein pattern, free amino acid content and protease activity of cherimoyas during storage at this chilling temperature. SDS-PAGE analysis revealed non-accumulation of some polypeptides related to the ripening process due to storage at 6°C, and two-dimensional electrophoresis confirmed the appearance of specific low-temperature polypeptides. While many polypeptides observed in freshly harvested fruit persisted during storage, several acid polypeptides were detected only during the first few days of storage at 6°C. A substrate-dependent change in protease activity was also found in fruit under chilling temperature storage, as compared to ripening fruit. After a decrease to barely detectable levels during the early phase of cold storage, the proteolytic activity then increased, mainly hydrolizing endogenous proteins to free amino acid components.

Keywords: Cherimoya fruit; Chilling temperature; Polypeptides; Proteasic activity; Free amino acids

1. Introduction

The shelf-life of tropical and subtropical fruits is seriously limited by damage occurring during long-term storage, below a particular critical temperature, usually between 0 and 10°C (An and Paull, 1990). As a result of chilling injury, a number of reactions associated with different metabolic processes are altered (Wang, 1982) Several studies have been performed on the physiological response of cherimoya to cold storage (Lahoz et al., 1993; Palma et al., 1993). In most cases, however,

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the role that a particular metabolic alteration plays in the damage process remains unclear. Both primary and long-term or secondary events have been defined in the chilling injury process in terms of the variability in the time course of these alterations (Raison and Orr, 1990). Primary events may lead to irreversible fruit damage depending on temperature, exposure time and tissue sensitivity. Changes in cell membrane lipids and in the conformation of cell structure have been proposed as primary effects of low temperatures in plant cells (Markhart, 1986). Other studies have demonstrated that plant cells rapidly initiate modifications in enzyme activities and protein content (Graham and Patterson, 1982). With respect to chilling damage, changes in protein synthesis have been identified (Watkins et al., 1990). Although most of these changes correspond to ripening-specific mRNAs (Dopico et al., 1993; Zamorano et al., 1995), some cold-induced mRNAs have also been identified. In tomatoes incubated at 4°C a mRNA encoding a thiol protease was accumulated but was not detected during ripening at ambient temperatures (Schaffer and Fischer, 1988). However, no correlation to increased proteolytic activity has yet been established in fruit subjected to chilling temperature treatment. In general, increased sensitivity to proteolytic digestion under stress conditions has been reported (Vierstra, 1993) and an increase in free amino acid content has been mentioned particularly for fruit (Walter et al., 1990).

The aim of this study was to investigate the alterations in the protein profile and protease activity during storage at 6°C. The relationship between these changes and the timing of induction of chilling damage was discussed.

2. Material and methods

Plant material and storage conditions

Cherimoya fruit (*Annona cherimola* Mill. cv. 'Fino dė Jete') harvested from a commercial orchard at Almuñecar (Granada, Spain) was transported to our laboratory in Madrid and stored at 6 and 20°C and a relative humidity of 80%. Ethylene production and respiration rate were measured daily by gas chromatography on ten cherimoyas enclosed in a continuous flow system. Ripening parameters such as firmness, titratable acidity, °Brix, and external and internal symptoms were assessed, in accordance with standard methods, in five fruits collected periodically during storage. Another three fruits were peeled, minced and frozen in liquid nitrogen, as were samples of unripe freshly harvested cherimoyas.

Protein extraction

Frozen tissue (20 g) was ground in a precooled coffee grinder and homogenized in 60 ml of ice-cold extraction buffer [50 mM Tris-HCl (pH 7.4); 400 mM NaCl; 20 mM $NaHCO_3$; 20 mM $MgSO_4$; 10 mM EDTA-Na_2; 10 mM cysteine hydrochloride; 5 mM 2-mercaptoethanol; 0.5 mM PMSF; 0.01 mM leupeptin hemisulfate; 10% (v/v) glycerol; 1% (w/v) PVP-40; and 1% (v/v) Triton X-100]. The homogenate was centrifuged at 20,000 *g* for 20 min at 4°C, and the supernatant was vigorously shaken with an equal volume of water-saturated phenol containing 20 mM 2-mercaptoethanol and 0.1% (w/v) 8-hydroxyquinoline. The phenol phase was sepa-

rated by centrifugation at 8000 *g* for 10 min at 18°C and re-extracted four times with an equal volume of extraction buffer. Five volumes of 0.1 M ammonium acetate in methanol were added to the final phenol phase, and proteins were allowed to precipitate overnight at −20°C and then centrifuged at 20,000 *g* for 20 min at 4°C. The resulting pellet was washed three times with 0.1 M ammonium acetate in methanol, twice with ice-cold 80% (v/v) acetone, dried and dissolved in either an SDS-PAGE sample buffer [63 mM Tris-HCl (pH 6.8); 10% (v/v) glycerol; 5% (v/v) 2-mercaptoethanol; and 2% (w/v) SDS], or in a 2D-PAGE sample solution [9 M urea; 5% (v/v) 2-mercaptoethanol; and 2% (w/v) Triton X-100]. Protein concentrations were measured using the Bradford (1976) method.

Electrophoretic analyses

Total soluble proteins (60 μg) were separated by SDS-PAGE on a 12% (w/v) polyacrylamide gels essentially as described by Laemmli (1970). For the IEF, proteins (40 μg) were separated on polyacrylamide tube gels (70 × 1 mm) following O'Farrell's (1975) method with minor changes. Ampholytes extended over pH's ranging from 5 to 8 and from 3.5 to 10. For two-dimensional electrophoresis, the focused gels were thawed, equilibrated for 15 min in O'Farrell's (1975) SDS-PAGE sample buffer and subjected to electrophoresis on 12% (w/v) polyacrylamide gels. After separation, the gels were shaken for 30 min in fixing solution, stained for 1 h in 0.04% (w/v) Coomassie brilliant blue G-250 in a fixing solution, and destained in 5% (v/v) acetic acid and 10% (v/v) methanol. Standard pI markers, ranging from 3.5 to 9.30, and molecular weight markers, ranging from 14,000 to 94,000, were used to estimate the pI's and molecular weights of the separated polypeptides, respectively.

Proteolytic activity of cherimoya extracts

Frozen tissue (2 g) was ground in a precooled coffee grinder and homogenized in 4 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 2% (v/v) β-mercaptoethanol. The homogenate was centrifuged at 27,000 *g* for 20 min at 4°C and the supernatant was used for the protease assay. Crude enzyme solution was incubated for 5 min at 25°C with 2.5 μl β-mercaptoethanol (BME) prior to reaction. In order to monitor endogenous proteolysis, aliquots (500 μl) of activated cherimoya extracts were added to 250 μl of 0.1 M sodium phosphate buffer (pH 7.0). To determine proteolytic activity against casein, 250 μl of activated extracts were added to 250 μl of 1% (w/v) casein (Hammerstein grade) and dissolved in 250 μl of 0.1 M sodium phosphate buffer (pH 7.0). After 90 min at 40°C, the reaction was stopped by adding 0.2 ml of 30% (w/v) trichloroacetic acid (TCA) solution. Insoluble protein was removed by centrifugation and protein content was measured using the Lowry et al. (1951) method. Amino acid released by hydrolysis was determined in the supernatant at 340 nm using the method of Church et al. (1983) based on the reaction between α-amino groups and *o*-phthaldialdehyde (OPA) and β-mercaptoethanol, using glycine as standard. One unit of specific protease activity is defined as 1 μmol of glycine equivalent liberated in 1 h per mg of soluble protein. A papain assay (Papainase; EC 3.4.22.2, Sigma) with whole casein or cherimoya extracts was used as standard.

3. Results

Protein pattern detected by SDS-PAGE and 2-D analyses

In order to determine changes in the protein pattern of cherimoya fruit due to storage at chilling temperature, electrophoretic analyses run on proteins from freshly harvested fruit (0 days) and fruit stored for 1, 3, 6, and 19 days at 6°C were analyzed (Fig. 1). Even after 19 days of cold storage, ethylene production was always below 1 $\mu l\ kg^{-1}\ h^{-1}$ and fruits failed to attain the soft stage (18 N). Ripening protein pattern was obtained at the fourth day from fruits stored at 20°C (soft stage, and maximum values in ethylene production, titratable acidity and soluble solid content).

SDS-PAGE analysis showed that many polypeptides in prestored fruit persisted during storage at 6°C. The preferential accumulation of polypeptides with molecular weights (MW) of 21, 23, and 31 kDa in ripening fruits kept at 20°C were absent during storage at 6°C. These above-mentioned polypeptides accumulated also in fruits ripening at 10°C.

Although most of the polypeptides resolved by 2D-PAGE appear to be constitutive, some relevant differences were observed (Fig. 2). Thus, two specific

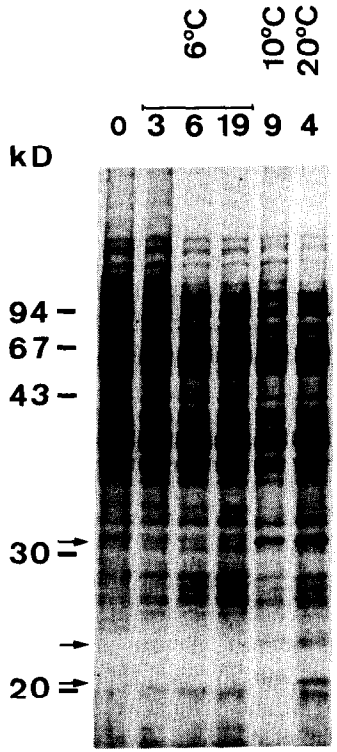

Fig. 1. SDS-PAGE protein patterns of cherimoya fruit stored for periods (days) and at temperatures indicated. Major ripening-related polypeptides differences are indicated by arrows. The molecular weights of standard proteins are expressed in kilodaltons (kDa).

polypeptides — 45 kDa (pI 4.65) and 50 kD (pI 4.75) — accumulated in fruit stored for 1 day at 6°C. Other polypeptides — 31.5 kDa (pI 7.75); 40 kDa (pI 6.40); 43 kDa (pI 4.85); and 46 kDa (pI 4.15) — were present in fruit stored for 1 and 3 days at 6°C. Two of these polypeptides — MW 31.5 kDa (pI 7.75) and MW 40 kDa (pI 6.40) — were not found consistently from experiment to experiment. Although additional work is needed to verify this peculiar finding, similar behaviour in specific proteins during low-temperature treatment has been observed by Faw and Jung (1972). Other polypeptides with molecular weights ranging from 30 to 50 kDa also tend to accumulate during storage at 6°C. So, polypeptides — 35 kDa (pI 7.40) and 39 kDa (pI 8.65) — were clearly observed in fruit stored for 6 and 19 days at 6°C.

None of these specific low-temperature polypeptides were present in unripe freshly harvested fruit or ripe fruit at 20°C (Fig. 3).

Proteolytic activity

The extent of hydrolysis shown by cherimoya extracts during ripening at 20°C is shown in Fig. 4A. Except for a decrease in the first day on endogenous proteins, the activity of "neutral" proteases at the end of the ripening process was similar to that observed at the beginning of the process.

The pattern of hydrolysis of cherimoya extracts during 19 days of storage at 6°C is shown in Fig. 4B. On day 3, a sharp decrease was observed in protease activity both in the absence and in the presence of casein. After an upsurge in protease activity, a clearly dependence of substrate was annotated. So, on day 13 hydrolysis of cherimoya endogenous proteins was observed to prevail.

Soluble protein and free amino acids content

Soluble protein and free amino acids content evolution during ripening at 20°C are shown in Fig. 5A. The trend of free amino acids was parallel to that of proteolytic activity against endogenous cherimoya extracts, just the reverse of the trend observed for protein content. Soluble protein and free amino acids content evolution during storage at 6°C are given in Fig. 5B. A point of inflection was also observed on the third day of storage, when protein and amino acids declined 2.2 fold and 1.6 fold, respectively, with respect to the initial content in fruit just after harvest. A steady increase in soluble proteins and amino acids content was observed throughout storage. This increase was parallel to the rise in proteolytic activity on the endogenous proteins.

4. Discussion

Cherimoya fruit is sensitive to cold storage and develops chilling injury if kept at temperatures below 6°C for long periods of time. In previous work (Alique et al., 1994), we observed that fruit kept for more than 5 days at 6°C lost its ability to ripen when rewarmed at 20°C. The reduction and/or inhibition of ripening processes at this temperature, according to physiological results, were in line with those observed by electrophoretical analyses. Thus, SDS-PAGE protein profiles for fruit stored at 10 and 20°C compared to those for fruit stored at 6°C, clearly revealed

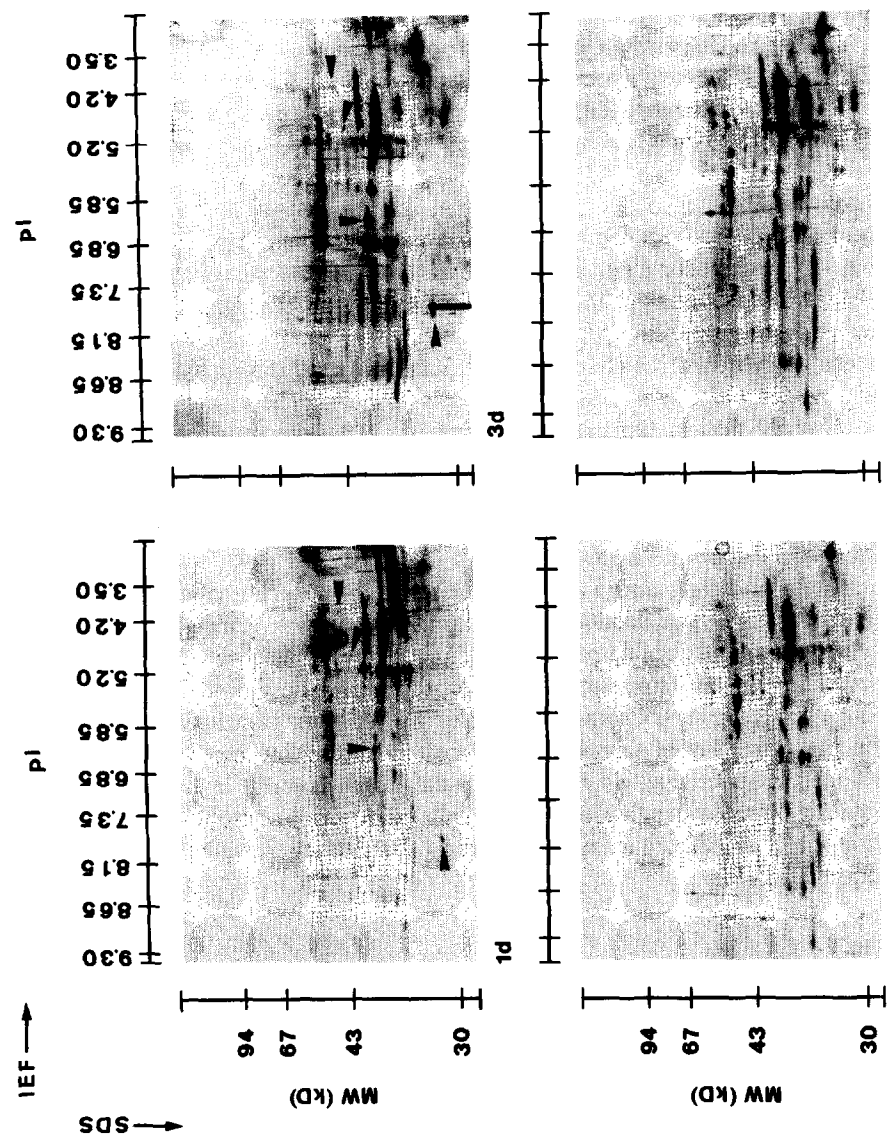

Fig. 2. Two-dimensional protein patterns of cherimoya fruit during storage at 6°C. Protein samples analyzed were extracted from fruit collected on the 1st, 3rd, 6th and 19th day of storage. Proteins separated by electrophoresis (40 μg) were visualized by silver staining. The specific low-temperature polypeptides at day 1 are marked by circles; at days 1 and 3 marked by arrows.

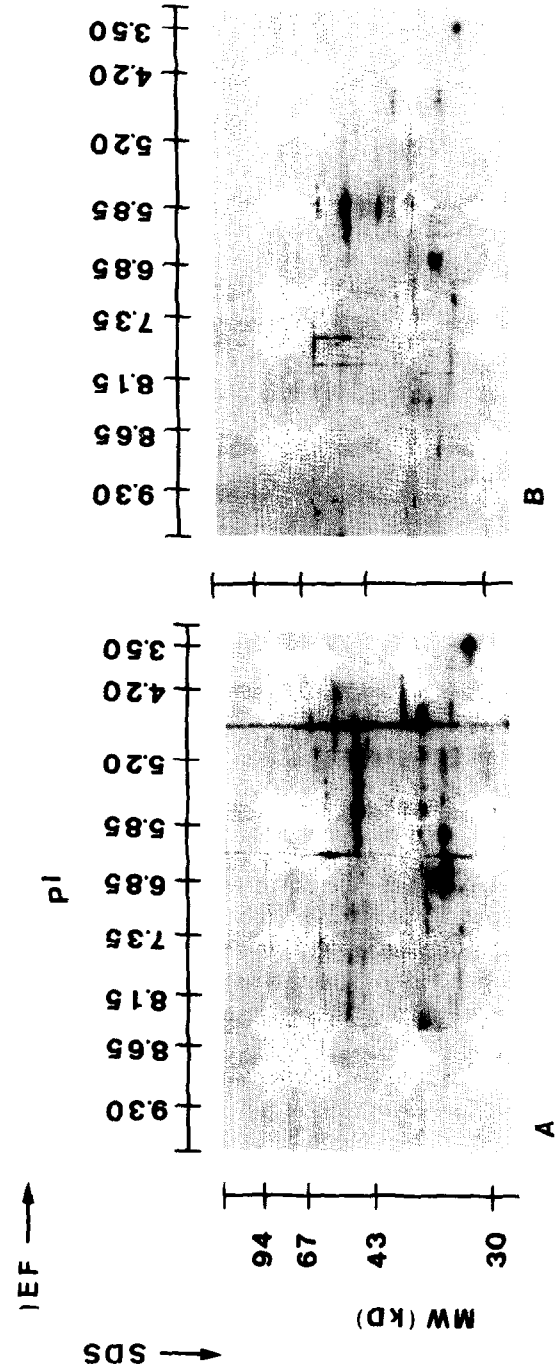

Fig. 3. Two-dimensional protein patterns of (A) unripe freshly harvested cherimoya fruit; and (B) ripe cherimoya fruit at 20°C.

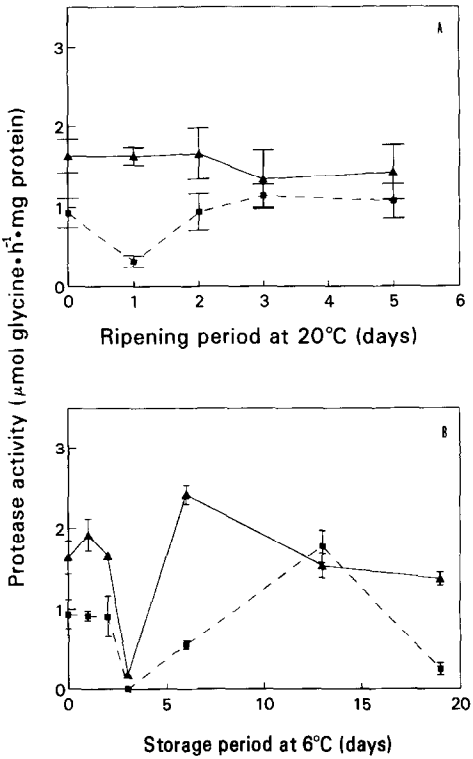

Fig. 4. Protease activity of cherimoya extracts on endogenous proteins (■) and casein (▼). (A) During ripening period at 20°C; and (B) during storage period at 6°C. Each point represents a mean at least of three replicates ± SD (vertical bars)

non-accumulation of polypeptides related to the ripening process due to storage at the chilling temperature. Moreover, specific low-temperature polypeptides were detected by two-dimensional electrophoresis analysis early during storage at 6°C. Other polypeptides accumulated to detectable levels only after long exposure to the chilling temperature. Comparison of these protein profiles to those for fruit kept at 20°C revealed that none of the polypeptides are related to the ripening process.

The early accumulation of specific polypeptides during low-temperature storage may be associated with de novo synthesis of polypeptides to withstand cold shock. It has been shown that several new proteins are synthesized rapidly when plants react to different kinds of stress (Van Loon, 1985; Cramer et al., 1986).

According to the results, cherimoya fruit, after 3 days at 6°C seems to enter into a second phase of the chilling process. Rapid and delayed genetic responses were also observed in fruit at low temperatures by Schaffer and Fischer (1988). Although most of the genetic responses to low-temperature storage are related to delays or alterations in cellular metabolism (Dopico et al., 1993), increases in some mRNAs suggest that prolonged cold storage stress may induce the synthesis of specific

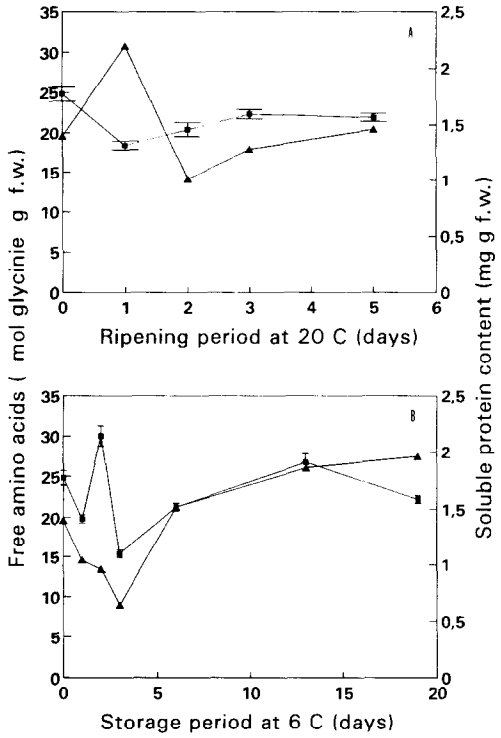

Fig. 5. Free amino acids (■) and soluble protein content (▲) in cherimoya fruit. (A) During ripening period at 20°C; and (B) during storage period at 6°C. Each point represents a mean of three replicates ± SD (vertical bars).

proteins in fruit (Watkins et al., 1990). Accumulation of mRNA encoding a thiol protease of the papain family has been observed (Schaffer and Fischer, 1990). The same authors proposed that a cold-inducible protease might degrade polypeptides damaged or denatured by exposure to low temperatures. In this work, we noted a sharp change in the trend of proteolytic activity, affecting mainly the endogenous proteins which after prolonged storage at chilling temperature seems to be especially sensitive to proteolysis. In other systems, activation of specific genes in response to large amounts of abnormal proteins was detected (Ananthan et al., 1986).

In conclusion, the present study shows that chilling temperature not only induced the appearance of specific polypeptides, but also modified the proteolytic activity of cherimoya extracts.

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