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Browning phenomena in stored artichoke (*Cynara scolymus* L.) heads: enzymic or chemical reactions?

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Data concerning phenolics and polyphenol oxidase in artichoke heads are presented. Biochemical changes at different physiological stages and after mechanical damage or chilling injuries, together with the characteristics of iron/phenolic complexes and the subcellular localization of polyphenol oxidase, are considered. Results lead to a proposed mechanism of browning phenomena in cold-stored, non-mechanically damaged, artichoke heads.

INTRODUCTION

Many fruits and vegetables become brown or discoloured after mechanical or physiological injury suffered during harvesting or storage, and this reactivity raises an important economic question in the post-harvest physiology of plant commodities. During the processing and storage of fruits and vegetables the need to prevent colour changes or browning is considered to be a matter of great importance from the point of view of consumer acceptability and palatability as well as retention of the original quality of the food (Lattanzio, 1988; Lattanzio *et al.*, 1989; Mathew & Parpia, 1971). Discoloration is caused by the oxidation of certain phenolic compounds catalysed by specific enzymes and it is generally agreed that polyphenol oxidase (PPO) is the enzyme system mainly responsible for browning. However, the contribution of other enzyme systems (e.g. peroxidase) to total browning could also be relevant for some fruits and vegetables (Vamos-Vigyazo, 1981).

When the cellular compartmentalization is disrupted, plant phenols are involved in both enzymic and non-enzymic browning reactions. In the former case the oxidation of phenolic compounds is catalysed by a PPO activity and the quinones formed can take part in secondary reactions bringing about the formation of more intensely coloured secondary products (Loomis & Battaile, 1966; Mathew & Parpia, 1971; Pierpoint, 1983; Kahn, 1985). In the latter case the common cause of darkening is attributable to the interactions between phenolics and heavy metals, especially iron. It is generally accepted that a dark coloured complex of ferric iron and an ortho-dihydric phenol is responsible for discoloration. It has been suggested that the phenol involved is chlorogenic acid (5-*O*-caffeoylquinic acid) and that disruption of the cells during processing and/or storage allows the organic ligand to chelate the iron (Bate-Smith *et al.*, 1958; Hughes *et al.*, 1962; Hughes & Swain, 1962*a,b*; Price, 1968; Mathew & Parpia, 1971; Pollard & Timberlake, 1971; Rao & Rao, 1985). Since the metal is originally present in the reduced state, a colourless complex is first formed which, upon exposure to oxygen, is oxidized to yield a coloured compound.

Nevertheless, the results obtained in previous studies (Weurman & Swain; 1955; Lattanzio & Van Sumere, 1987) show that changes in the concentration of phenolic substrates during development and ripening do not run parallel to the changes in the actual browning. Therefore, it is clear that factors other than the amount of phenols alone are responsible for the intensity of browning. The in-situ enzyme activity can be considered an even more important factor contributing to browning than the concentration of phenolic substrates, but PPO activity does not change in a parallel way to the browning reaction (Lattanzio *et al.*, 1989).

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The effect of other cell constituents on the extent of enzymic browning *in situ* has been little studied, although many naturally occurring compounds (i.e. citric and ascorbic acid) have been used to control browning (Baruah & Swain, 1953; Lattanzio *et al.*, 1989). So far, no working strategies have been found to prevent the browning reaction. Additional information about the mechanism of this reaction and the role of environmental parameters (temperature-controlled or modified atmosphere) on this mechanism, will help resolve this problem.

In previous papers (Lattanzio & Van Sumere, 1987; Lattanzio *et al.*, 1989; Leoni *et al.*, 1990), changes in phenolic compounds, PPO and phenylalanine ammonia lyase (PAL) activities and the formation of iron/chlorogenic acid complexes occurring during the low temperature storage of fresh marketable artichoke (*Cynara scolymus L.*) heads have been reported. In addition, data concerning the effect of citric and ascorbic acid on the phenolic browning reaction were reported. It was hypothesized that browning phenomena that are not due to handling or other mechanical damage may be both of enzymic and non-enzymic nature, with probably the latter reaction being dominant.

This paper will give further information about the changes in phenolics and PPO activity in healthy and injured tissue of stored artichoke heads and the cellular localization of PPO activity. Results may aid understanding of the mechanism of browning. Knowledge about this mechanism can help prevent browning phenomena during cold storage of fruits and vegetables.

MATERIALS AND METHODS

Plant material

Artichoke heads (cv. Catanese) of marketable quality were harvested at different periods during the plant-growing season. Some of the heads were immediately analysed for phenolic compounds, while others were stored in closed polyethylene bags at 4°C. In this way (low temperature and modified atmosphere) most of the artichokes could be kept in good condition for 1–2 months. Fresh and stored artichoke heads were analysed for phenolic compounds, PPO and iron/phenolics complexes.

HPLC analysis of phenolic compounds

For qualitative and quantitative determination of the phenolics, the plant material was first homogenized with MeOH–EtOH (1:1) and then refluxed for 2 h under nitrogen. (Full details of this procedure can be found in the paper by Lattanzio & Van Sumere, 1987.)

HPLC analyses were performed with a Perkin-Elmer Series 4 liquid chromatograph, which was equipped with a 3600 Data Station, a fluorimetric detector LS-3 and a spectrophotometric photodiode array detector 1040-HP, coupled with a HP-85 computer and a HP-9121 disk drive. The pilot signal to the spectrophotometric detector was set at 325 nm and the fluorimetric detector at 265 nm (excitation) and 345 nm (emission).

An analytical Waters (Milford, Massachusetts, USA) column (300 mm x 4 mm i.d.) packed with μBondapak C_{18}, having an average particle size of 10 μm, was used throughout this work. The solvent system consisted of (A) MeOH and (B) acetic acid–water (5/95; v/v). The elution profile was as described by Lattanzio & Van Sumere (1987). The flow rate was 1 ml/min and at the start the column pressure was 15 MPa. Samples of 6 μl were applied to the column by means of a 6 μl loop valve.

Determination of enzyme activity

Plant material was macerated in a blender for 10 min with (2.5 ml/g) cold acetone (–15°C). The suspension was centrifuged and the residue was twice homogenized with cold acetone. The acetone powder was extracted for 80 min, in the presence of insoluble polyvinylpyrrolidone, with 20 mM K-acetate buffer at pH 5.7 (5 ml/g original tissue wt) (Leoni *et al.*, 1990). Enzyme activity was determined polarographically at 30°C by measuring the oxygen uptake with a Clark electrode connected to a Gilson Oxygraph (Medical Electronics, Middleton, Wisconsin, USA). HPLC assays of PPO activity were also carried out as previously described by Lattanzio *et al.* (1989).

Subcellular localization of PPO was performed by means of a discontinuous sucrose gradient (52% and 30%) and centrifugation at 80 000 g for 45 min at 4°C on a Beckman (Palo Alto, California, USA) L7-55 ultracentrifuge (Palmer, 1986). Soluble protein concentration was measured by the method described by Bradford (1976), using the Bio-Rad protein assay.

Iron-chelating activity of phenolics

Assays on the complexing activity of iron (ferrous and ferric ions) were carried out in 0.02 M K-acetate buffer at pH 6.0 as described by Lattanzio *et al.* (1989). HPLC analysis of the residual phenolic content in the test solution was carried out using a solvent system consisting of (A) MeOH and (B) 0.02 M K-acetate buffer at pH 5.7. The elution profile was identical to the one used for HPLC analysis of phenolics. The test solutions were also assayed colorimetrically at 580 nm by means of a Perkin-Elmer LC-55 spectrophotometer.

RESULTS

Table 1 shows the main phenolics in artichoke heads: their level changes depending on the physiological stage of the plant and/or the organ: the *o*-diphenol content falls gradually and continuously as the plant grows (Lattanzio & Morone, 1979; Lattanzio & Van Sumere, 1987). Furthermore, Fig. 1 shows that chlorogenic acid content may be inversely related to the whole plant or

Table 1. Mono- and di-caffeoylquinic acids in artichoke heads

Compound	Concentration (mg/100 g dry wt)
Monocaffeoylquinic acids:	
1-*O*-caffeoylquinic acid	38·1
3-*O*-caffeoylquinic acid	57·2
4-*O*-caffeoylquinic acid	267
5-*O*-caffeoylquinic acid	1 545
Dicaffeoylquinic acids:	
1,3-*O*-dicaffeoylquinic acid	61·2
1,4-*O*-dicaffeoylquinic acid	143
4,5-*O*-dicaffeoylquinic acid	225
3,5-*O*-dicaffeoylquinic acid	347
1,5-*O*-dicaffeoylquinic acid	837
3,4-*O*-dicaffeoylquinic acid	429

tissue development: the arrow on the *x*-axis in the figure indicates the ageing of plant tissues, inner bracts being younger than outer ones. These phenolics are good substrates for both enzymic and non-enzymic reactions (Lattanzio *et al.*, 1989; Leoni *et al.*, 1990). Ideally,it would be desirable to compare samples where the differences in the intensity of blackening were due solely to the variation of a single chemical factor, but, unfortunately, this is not the case for artichoke. Because of the localization of the blackening at the level of inner bracts, changes in chemical parameters between inner and outer bracts and between browned and healthy tissues of the same bract were observed.

Although iron could be directly involved in the formation of the coloured pigments, its concentration is of lesser importance than other factors in determining the distribution of blackening on individual heads, since it is almost constant in the different bracts. In fact, its concentration ranges from 8 to 10 mg/100 g dry wt (Lattanzio *et al.*, 1981). Also PPO activity (Fig. 2) was found not to be very different between inner and outer bracts, being slightly higher in the former where browning phenomena are observed. The highest activity values were found in the heart, which represents the younger tissue of artichoke heads. On the other hand, chlorogenic acid content (Fig. 1) rapidly decreases from

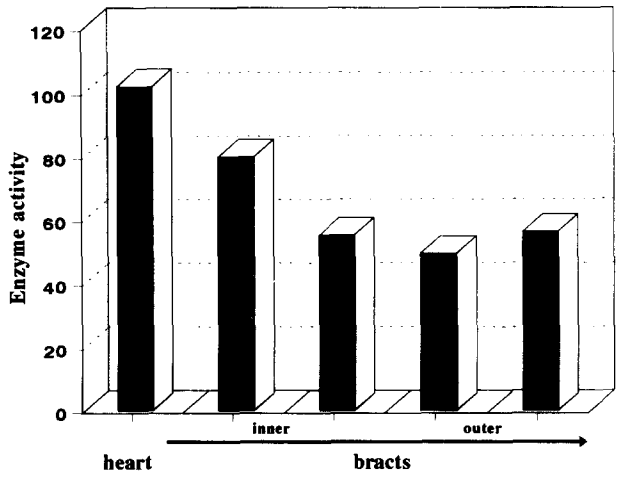

Fig. 2. PPO activity (μmol consumed oxygen/min/mg protein) in tissues of artichoke heads at different physiological stages.

the inner to the outer bracts, its level being 5–6 times higher in the former than in the latter. So, although a direct correlation was frequently observed between the concentration of chlorogenic acid and intensity of blackening, the interaction of one or more of the other factors is believed to be involved.

It has been shown that exposure of artichoke heads to low non-freezing temperature can stimulate phenolic metabolism. Low-temperature storage leads to a rise in caffeoylquinic acid derivatives, particularly chlorogenic acid, involving a cold-induced stimulation of PAL activity (Rhodes *et al.*, 1981; Lattanzio *et al.*, 1989). Both PAL activity and phenolic content in artichoke heads show a peak during the early stage of storage, followed by a gradual fall back to very low levels. Figure 3 shows changes in PPO activity during the storage at 4°C: the trend observed is a gradual increase in PPO activity, but this increase is not so relevant from a statistical viewpoint. This small activation of PPO could be induced by ageing phenomena in the tissues and/or by stress conditions during storage, which result in increased levels of enzyme, apparently due to release from membranes (Mayer, 1987). These data show that enzyme activity and the amount of

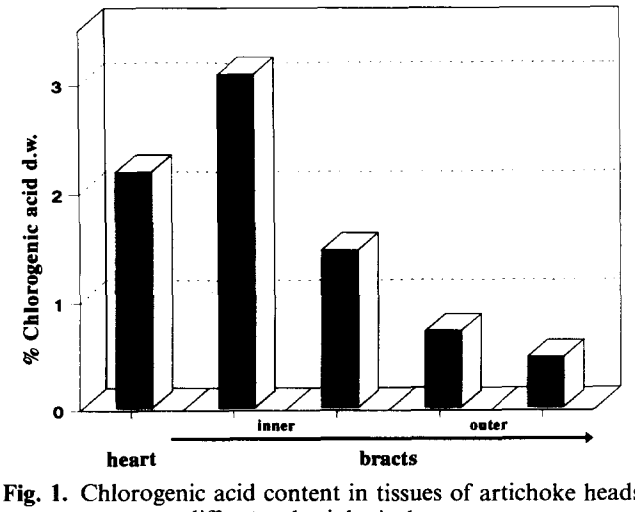

Fig. 1. Chlorogenic acid content in tissues of artichoke heads at different physiological stages.

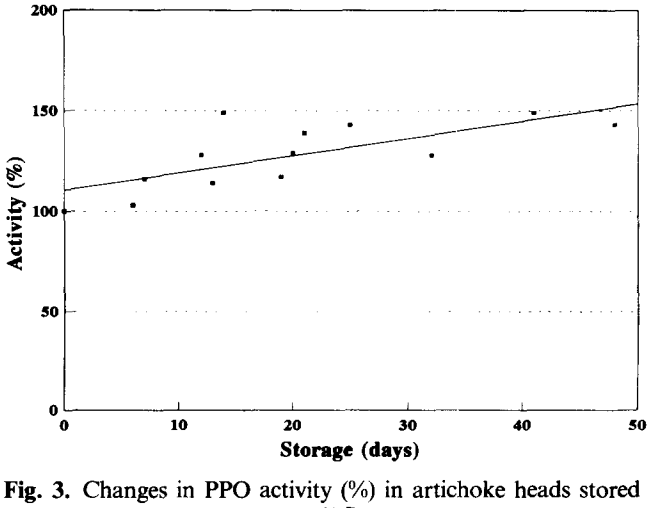

Fig. 3. Changes in PPO activity (%) in artichoke heads stored at 4°C.

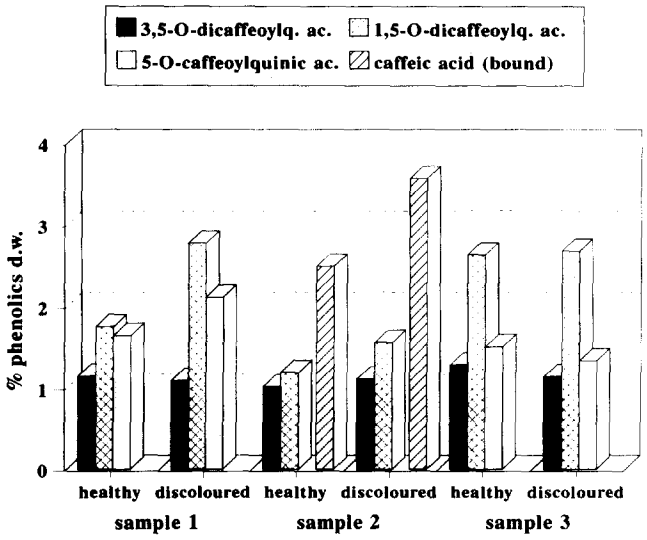

Fig. 4. Changes in phenolic compounds in browned artichoke tissues after cold storage (samples 1, 2 and 3 represent different harvesting data).

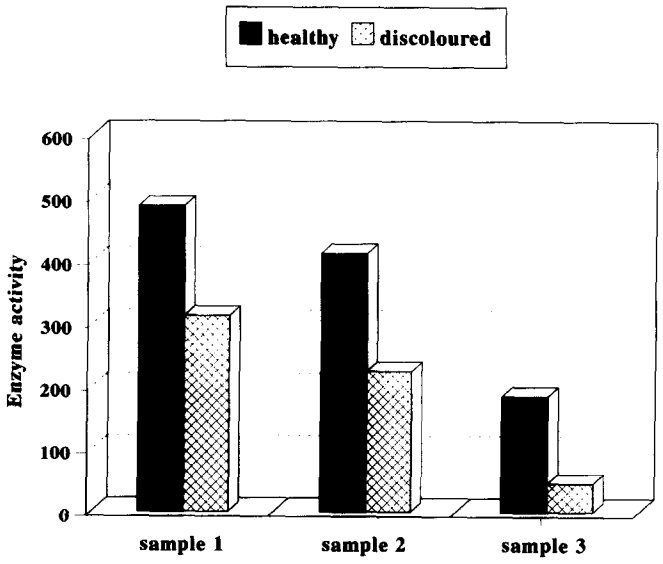

Fig. 6. PPO activity (μmol consumed oxygen/min/mg protein) in cold-stored artichoke tissues from samples with different harvesting data.

phenolic substrates do not change together. In addition, it is a characteristic of many plant tissues that phenolics which act as substrates for PPO are either sequestered in special cells, or in the vacuole, away from the enzyme (Mayer & Harel, 1979). The optimum pH of artichoke PPO usually lies between 5.0 and 8.0 for the different substrates, indicating that it is present in a cell compartment separated from the rather acid cell vacuole. Thus,it seems that PPO is not involved in the oxidation of the bulk of phenolics present in non-mechanically damaged tissues. It is possible that in intact tissues the normal substrate is only a fraction of the total located in the tissue so as to be available to the enzyme.

Because the pigment responsible for the post-harvest blackening could be also a phenolic/iron complex, interaction between chlorogenic acid or 1,5-*O*-dicaffeoylquinic acid and iron were studied. It is known that phenols with two free *o*-hydroxy groups give coloured complexes at pH 6.0–6.5 (the physiological pH of artichoke head tissues ranges between 6.0 and 6.2). Under these conditions chlorogenic acid and 1,5-*O*-dicaffeoylquinic acid form dark-coloured complexes with Fe^{3+}. Since these compounds are found in very high amounts in artichoke heads, their paramount importance in discoloration must be considered (Lattanzio *et al.*, 1989). It was observed that in the absence of oxygen these phenolics form colourless complexes with Fe^{2+}. After exposure to air the complexed Fe^{2+} was quickly oxidized to Fe^{3+} to give coloured compounds. Citric acid produced a 100% reduction in colour when an iron–citric acid ratio of 1:10 was used and the solution pH was kept unchanged. Citrate must be considered a potent sequestering agent for iron and this sequestering action was mainly responsible for its beneficial effects on artichoke head discoloration. HPLC analyses have also demonstrated that more than 85% of chlorogenic or

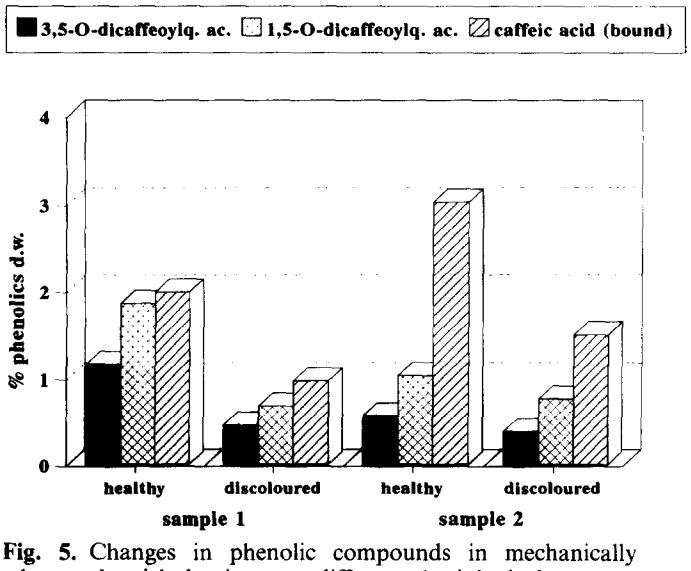

Fig. 5. Changes in phenolic compounds in mechanically damaged artichoke tissues at different physiological stages.

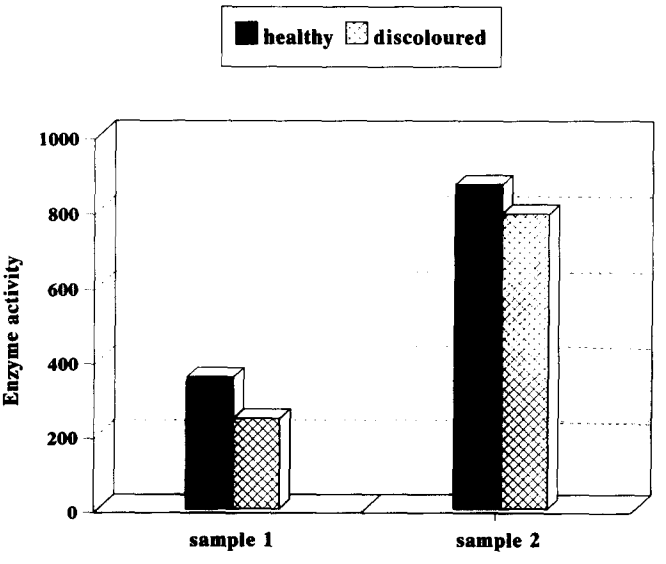

Fig. 7. PPO activity (μmol consumed oxygen/min/mg protein) in mechanically damaged artichoke tissues at different physiological stages.

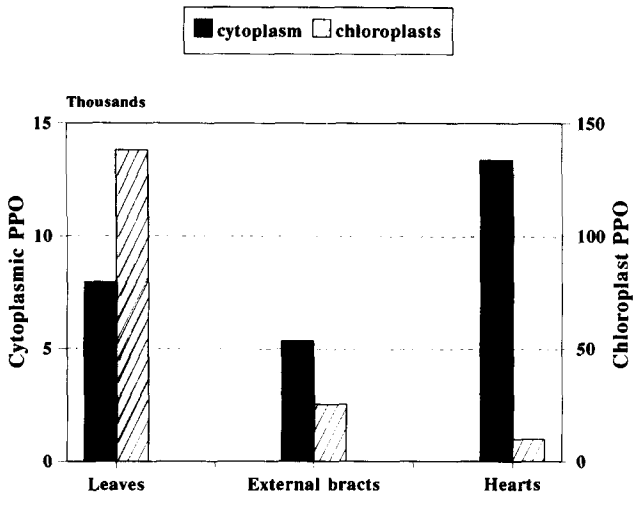

Fig. 8. PPO activity (μmol consumed oxygen/min/g fresh wt) in cytoplasm and chloroplasts of different artichoke tissues.

1,5-*O*-dicaffeoylquinic acid was released from the complex when the solution pH dropped from 6 to 3.

Additional information may be obtained when discoloured tissues are compared with healthy tissues from the same artichoke heads, and the subcellular localization of the PPO activity is considered. Figure 4 shows the phenolic content in discoloured tissues of stored artichoke heads.It is noticeable that phenolic content is generally higher than in healthy tissues of the same artichoke bracts, which does not agree with the hypothesis of enzymic browning. In fact, in this case a remarkable lowering of phenolic content, due to the oxidative phenomena, would have been expected.

Furthermore, when tissues suffered enzymic browning after mechanical damages and short exposure to air (Fig. 5), the phenolic content was much lower (= 50%) than that of intact tissues. It is very hard to understand the changes of PPO activity in the same samples. In browned stored artichoke tissues, PPO activity is about half of that in healthy tissues sampled from the same bract (Fig. 6). In mechanically damaged tissues the enzyme activity is not so different from healthy tissues: a small decrease could possibly be due to the inhibitory effect of the reaction products (Fig. 7).

As far as the subcellular localization of PPO (Fig. 8) is concerned, in all tissues analysed the enzyme activity was found mainly in the cytoplasm. In the internal tissues of artichoke heads, where the browning phenomena are localized, the cytoplasmic activity was about 1400 times higher than the chloroplast activity which, in turn, was very low.

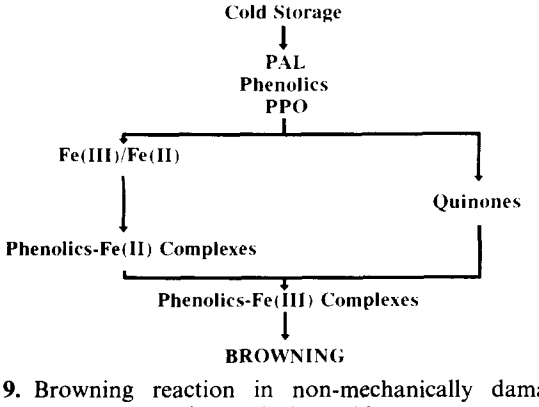

Fig. 9. Browning reaction in non-mechanically damaged artichoke tissues during cold storage.

DISCUSSION

Phenolics, PPO,PAL and iron play important roles in blackening reactions. Amongst phenols, chlorogenic acid, the best substrate of artichoke PPO, 1,5-*O*-dicaffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid, ligands for iron together with chlorogenic acid, being generally the most representative artichoke phenolics, must be considered. In addition, the cellular localization of these chemicals must be considered. Artichoke PPO is an intracellular enzyme located mainly in the cytoplasm while the phenolic substrates are located in special compartments within the cell. This results in the separation between the enzyme and the bulk of its phenolic substrates *in situ* (Mayer & Harel, 1979, 1981).

Biochemical and ultrastructural evidence suggests strict compartmentation in the synthesis and transport of phenolic compounds in the cell. Such compartmentation can be envisioned by pathways consisting of complexes composed of consecutively assembled, membrane-associated enzymes, where end products of synthesis are accumulated in a membrane enclosure. These vesicles could then be transported to the central vacuole for internal sequestration or to the plasma membrane for secretion. According to this mechanism, chloroplasts are involved in some steps of phenolic biosynthesis leading to the formation of cinnamic derivatives. It is likely that cinnamic units, formed at the level of PAL associated with endoplasmic reticulum, produced quinic esters in the chloroplasts (Alibert & Boudet, 1982; Boudet *et al.*, 1985; Hrazdina & Wagner, 1985).

Finally, as far as the localization of Fe is concerned (Hughes *et al.*,1962; Price, 1968; Bienfait & Van der Briel,1980), different studies all agree that plastids contain the bulk of the iron of the cell and that most of this iron is present in the ferric state. This is consistent with the fact that ferritin is the major eukaryotic iron-storage protein. In plants, ferritin is known to be present in chloroplasts and, especially, in plastids of non-photosynthesizing tissues, where the apoprotein forms a hollow sphere, which is made up of 24 similar subunits and pierced by six tiny channels. Ferritin serves to solubilize and sequester iron; the iron core of the protein is composed primarily of a ferric oxy-hydroxide phosphate polymer containing up to 4500 iron atoms. Good evidence exists that iron is delivered to ferritin as Fe^{2+}, deposited as Fe^{3+}, and released upon reduction back to Fe^{2+} (Jones *et al.*, 1978). Several chelating agents in the presence of a reducing agent are able to promote the release of ferritin iron (Jacobs *et al.*, 1989; Boyer *et al.*, 1990). It has been shown that plant phenols, including caffeic acid and chlorogenic

acid,can promote the reductive release of ferritin iron (Boyer *et al.*, 1988*a*, 1989): a direct correlation exists between oxidation–reduction potential and the rate of iron release. It has also been suggested that electrons are carried to the centre of the protein by ferrous ions produced in the entrance to a channel by interaction of labile Fe^{3+} with the reducing agents (Boyer *et al.*, 1988*b*). In addition, reductant access to the ferritin iron core, when molecules are relatively small, is also likely. All attempts to isolate or obtain evidence for the expected *o*-quinone product from the reductants have been unsuccessful, these products being highly reactive in aqueous solution (Pierpoint, 1969, 1983; Boyer *et al.*, 1990; Mathew & Parpia, 1971).

Data presented in this research and the results concerning the phenolic metabolism and changes in PPO and PAL activity during the cold storage of artichoke heads, reported in previous papers (Lattanzio & Van Sumere, 1987; Lattanzio *et al.*,1989), suggest a mechanism of browning reaction in non-mechanically damaged tissues presented in Fig. 9. During cold storage of artichoke heads, low-temperature induction of PAL activity caused a biosynthetic increase of phenolics, especially chlorogenic acid. On the other hand, PPO activity did not change significantly during the storage period. The increased content of phenolics provided an adequate substrate for the browning phenomena. These reactions started from the chloroplasts, the site of chlorogenic acid biosynthesis and where the iron is stored as ferritin. A release of ferritin iron, as Fe^{2+}, was induced by the chlorogenic acid,thus originating a colourless complex with the excess of chlorogenic acid (and/or other phenolic ligands). Oxidizing conditions (O_2 and/or quinones formed by cytoplasmic PPO, when a cold-induced membrane modification caused a partial cell decompartmentation) lead to the formation of a grey-blue chlorogenic acid/Fe^{3+} complex and then to the browning phenomena. This complexed phenolic substrate, removed from the normal metabolism occurring during storage, was released in the free form during the procedure of extraction and separation of plant phenolics, when acidic pH conditions caused the complex to break down.

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