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LIPOXYGENASE ACTIVITY IN APPLES IN RELATION TO STORAGE AND PHYSIOLOGICAL DISORDERS

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Key Word Index—*Malus pumila*; Rosaceae; apple; lipoxygenase activity; physiological disorders.

Abstract—Different methods for the preparation of active lipoxygenase (LOX) extracts from apples were compared. Highest activities were obtained using a 0.25 M phosphate buffer (pH 7) containing 1% Triton X-100 and 10^{-2} M metabisulphite as extraction solvent. LOX activity during storage was investigated in the core, flesh, and peel. Activity was always highest in the core and peel. On storage, activity was increased in each part of the fruit but especially in the core and peel. Increase in LOX preceded the browning of the core. LOX may be responsible for the browning and may be concerned in the induction of superficial scald.

INTRODUCTION

As part of a research program studying the biochemical origin of physiological disorders of fruits, previous work from this laboratory determined the changes in the fatty acid composition of apples during storage. It has been shown that there is a typical breakdown of linolenic acid, parallel with the browning of the core of the apple variety 'Schone van Boskoop' [1]. In this paper, we propose that lipoxygenase (LOX) (EC 1.13.11.12), that catalyses the hydroperoxidation of polyunsaturated fatty acids, may be responsible for the typical breakdown of linolenic acid in the core of the apple fruit. As was shown for apples by Galliard [2], the polyunsaturated acids are mainly present in the lamellar lipids. Disruption of the integrity of the lipoprotein membrane structures may cause a total disorganization in the cell, leading to the browning of the core.

LOX activity in apples during growth and storage has been hardly investigated. Meigh and Hulme [3] found an increase in LOX activity of the peel of apple fruit developing 'on' and 'off' the tree and suggested that LOX may be responsible for the decrease of linoleic and linolenic acids during ripening. This has been associated with the possible role of LOX in the biogenesis of ethylene [4]. Although LOX is found in the tissues of a wide variety of higher plants, there are many questions with regard to its function in plant lipid metabolism. Several authors [5–7] have reviewed extensively the current theories regarding the biological significance of this enzyme.

The survey published by Rhee and Watts [8] on LOX content of various fruits and vegetables showed that crude apple extracts (Golden and Red Delicious) contained relatively little activity of a fr. wt basis. On the other hand, one of the highest activities on the basis of protein was noted. A similar survey by Pinsky *et al.* [9] reported a relatively small activity on the basis of fr. wt of crude apple extracts in the presence of the non-ionic detergent Triton X-100. In a recent study, Kim and Grosch [10] carried out a partial purification of a LOX from apples (Golden Delicious) also using Triton X-100 in the extraction medium. Triton acts as a solubilizing agent, indicating that the LOX from apples may be associated with membranes. Efficient extraction of LOX from several other plant sources was only successful by using an extraction solvent containing Triton X-100 [9–13]. One of the most undesirable difficulties in isolating active enzyme preparations from apples is the high phenolic content [14]. This is clearly shown by the rapid browning of the cell-free extracts. The interference caused by the oxidized phenolic compounds is prevented mainly by adding polymers or reducing agents to the extraction solvent [15].

The present work presents evidence that LOX may be the immediate cause of the browning of the core. A method has been developed for the preparation of highly active crude LOX extracts from apples and the activity has been followed in different parts of the apple fruit during storage.

RESULTS AND DISCUSSION

Extraction procedure

When varying the Triton X-100 concentration, a 1% (v/v) yielded the highest activity. This corresponds with a 1% (vol./fr. wt apple) concentration in the first extraction step. This value is highly critical because half-maximal activities were noted at 0.5 and 2%. Grossman *et al.* [11] obtained highest LOX activities in extracts from alfalfa leaves by using a solution of 1% (v/v) Triton X-100 or 5% (v/w). Unlike these authors, we did not find any difference between Tween 80 and Triton X-100 as solubilizing agent. Kim and Grosch [10] extracted a LOX from apples (Golden Delicious) using a 0.2% (v/v) Triton X-100 medium or 0.12% (v/w).

$Na_2S_2O_5$ was added to the extract to prevent rapid darkening of the homogenates caused by oxidation of phenolics. Good results were obtained with this polyphenoloxidase inhibitor for the isolation of active fractions from potato-tuber tissue [16, 17]. It is advantageous that the concentration of metabisulphite is independent of the polyphenoloxidase activity and the concentration of endogeneous phenolics [15]. Optimal extraction occurred with 10^{-2} M $Na_2S_2O_5$ for the first extraction step and 10^{-4} M $Na_2S_2O_5$ for the second. This is *ca* 0.2% (wt/fr. wt apple), being the lowest concentration at which the extract remained colourless. Half-maximal activities were noted at 0.1 and 0.4%. The reducing agent did not interfere with LOX assay under the conditions used.

The optimal extraction procedure using $Na_2S_2O_5$ was compared with several procedures using various polymers. Highest activities with polymers were obtained using a 0.25 M phosphate buffer (pH 7) containing 1% (w/v) BSA, 1% (w/v) water insoluble PVP and 1% (v/v) Triton X-100. These extracts were fairly viscous and a relatively large quantity of polymer was necessary. Moreover, the procedure using $Na_2S_2O_5$ gave *ca* 6 times higher activities. A vacuum-infiltration and a highly buffered extracting-medium with high density containing PVP have been successfully used by Hulme *et al.* [18, 19] for the preparation of active enzyme fractions from apples. This extraction method, whether in the presence of 1% Triton or not, yielded only 17% of the activity of the method using $Na_2S_2O_5$.

Maximum activity was thus recovered using a solvent containing 1% (v/v) Triton X-100 and 10^{-2} M $Na_2S_2O_5$. A highly buffered medium was used to neutralize the high acidity of the tissue. The pH of the extraction medium was optimal at pH 7, with half-maximal activities for pH 6 and 7.5.

LOX activity in apples during storage

In these experiments, it is assumed that LOX activity is comparable in crude extracts from apple tissues of different morphological and physiological status. Fig. 1 shows that during the whole storage period, LOX activity is always markedly higher in the core than in the other parts of the apple fruit. However, comparing the outer flesh region with and without peel, it can be calculated that enzyme activity in the peel is as high as in the core. In the flesh region, there was a decrease in enzyme activity towards the outer parts of the fruit. LOX activity rises markedly, especially in the core during storage at 3.5°. But calculating the enzyme activity in the peel alone yielded activities higher than in the core. A temperature change from 3.5 to 20° stimulates the rise in activity to a high degree. Data on the sp. act. during storage showed a similar pattern. The sp. act. of the core after 28 weeks storage was 0.083 μmol O_2/min/mg protein at 3.5° and 0.2 μmol O_2/min/mg protein at 20°. However, after 21 weeks storage, there was a maximum in the sp. act. of the peel as well as in that of the core. A probable explanation is that many enzymes exhibit a greater activity during climacteric, mainly in the peel and in the core.

From the 20th week, a slight browning of the core was perceptible during storage at 3.5° and attained a relative score of 20% after 30 weeks. A change to room temperature accelerated the browning in such a way that a relative score of 80% was noted after 2 weeks. The relatively high LOX activity in the core may be responsible for the typical core browning during storage. This kind of physiological disorder may arise from the action of LOX on the polyunsaturated acids, destroying the integrity of the lipoprotein membrane structures. The resulting disorganization of the living cell might explain the typical browning reactions.

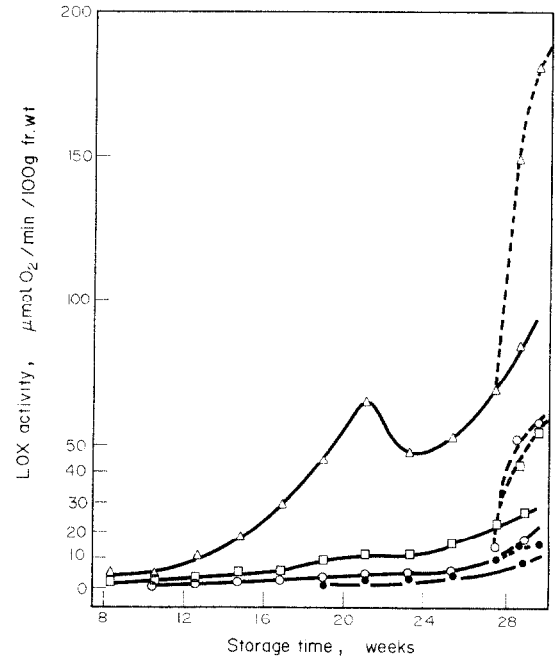

Fig. 1. LOX activity in crude apple extracts from different parts of the apple during storage at 3.5° (———) and at 20° (– – –): (△) core; (□) inner flesh region; (○) outer flesh region with peel; (●) outer flesh region without peel. Reaction medium: linoleate substrate 0.66 mM; 0.2 M Na–Pi buffer pH 7.

The relatively high level of LOX activity in the peel is also striking. According to Huelin and Coggiola [20, 21], superficial scald, a physiological disorder in the peel of stored apples, is caused by the autoxidation of α-farnesene (2,6,10-trimethyl-2,6,9,11-dodecatetraene). It is also possible, however, that the LOX oxidation of polyunsaturated acids is involved in the mechanism of scald induction.

There are undoubtedly unknown factors determining the interaction of LOX with endogenous lipid substrates. It must be assumed that, in the intact cells, enzymes and substrates are separated, or that the enzymes are present in inactive states. Another related problem is that the content of free fatty acids in plants is extremely low compared with the phospho- and galactolipids. It has been suggested [6, 22, 23] that LOX oxidation needs a preceding hydrolysing action, but it is questionable whether this is necessary *in vivo*.

EXPERIMENTAL

Materials. Apples 'Schone van Boskoop' were harvested on 5 October 1978 from commercial orchards in the region

of Haspengouw, Belgium. Fruit of dia. 70–75 mm was used and stored at 3.5° in air.

Substrate emulsion. Linoleic acid (0.1 ml) was dissolved in 10 ml 0.1 N NaOH. Unless otherwise stated, 150 μl Tween 20 was added and the soln was emulsified in an Ultra Turrax for 1 min. After diluting to 50 ml with H_2O, the substrate emulsion contained 6.64 mM linoleic acid, 0.3 vol.% Tween 20, 1/1.5 (v/v) linoleate/Tween 20. Control emulsion was prepared in the same way using oleic acid. Emulsions were stored under N_2 at 4° in dark for not longer than 10 days.

Assay of LOX activity. Activity was determined polarographically at 25° with a Clark O_2 electrode. Incubation mixtures contained 2 ml 0.2 M Na–Pi buffer pH 7, 0.3 ml substrate emulsion and 0.7 ml apple extract. Enzyme activities were calculated from initial rates of O_2 uptake. Initial dissolved O_2 concn was determined relative to pure H_2O. The O_2 concn of H_2O determined by the method of ref. [24] was 8.347 mg/l. Control assays were run with oleic acid as substrate. All data are the mean of at least 3 runs.

Preparation of crude apple extracts. All steps were performed below 5°. Diced apple (100 g) was mixed with a Sorvall mixer for 3 min in 100 ml 0.25 M Na–Pi buffer (pH 7), containing 1% (v/v) Triton X-100 and 10^{-2} M $Na_2S_2O_5$ (added each day). The slurry was homogenized with an Ultra Turrax for 2 min. The pH was adjusted to pH 7 with a few drops of 10 N NaOH. The homogenate was centrifuged at 8000 g for 20 min (supernatant 1). The pellet was suspended in 60 ml 0.25 M Na–Pi buffer pH 7 containing 10^{-4} M $Na_2S_2O_5$ and the same procedure for homogenizing and centrifuging was repeated (supernatant 2). Activity in the second pellet was negligible. Supernatants 1 and 2 were combined and used as the source of enzyme. LOX activity during storage was followed in the core, flesh and peel of the apples. Because it is difficult to investigate the peel by itself, the outer flesh region of the fruit with and without the peel was examined.

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