(EC 1.1.1.49) by the method of ref. [14]. Total proteins were estimated by the method of ref. [15].

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BEHAVIOUR OF SOME MITOCHONDRIAL ENZYMES IN RIPENING TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; ripening process; mitochondrial enzymes.

Abstract—The activities of four mitochondrial enzymes were studied in four stages of ripening tomato fruit. The highest enzyme activity was recorded for malate dehydrogenase followed by cytochrome *c* oxidase. Succinate dehydrogenase and NADH oxidase levels were low and could only be determined in the green stage of the fruit. However, peaks of various enzyme activities coincided in identical mitochondrial fractions on the sucrose density gradient. Moreover, the levels of malate dehydrogenase and cytochrome *c* oxidase were constant during the ripening process while the other two enzymes, succinate dehydrogenase and NADH oxidase, declined. This might indicate that mitochondria retain some of their essential functions through the ripening process.

INTRODUCTION

The phenomenon of fruit ripening is quite complex [1, 2]. The production of ripening hormones and other endogenous factors is affected by the environmental conditions [3] and the presence of specific enzymes. Baqui *et al.* [4] have investigated some mitochondrial enzymes, particularly Krebs cycle enzymes, and have indicated a significant increase in the activities of these enzymes in the ripening mango fruit. On the other hand, total mitochondrial number and size are reported to diminish during the ripening process [5, 6]. The present investigation was undertaken to study the behaviour of some enzymes in the purified mitochondrial fraction of tomato fruit.

RESULTS AND DISCUSSION

The mitochondrial fraction was isolated from 4 stages of ripening tomato fruit and purified on a sucrose density gradient. Four enzymes were assayed in the mitochondrial fraction.

Protein distribution curve

Protein concentration and malate dehydrogenase activity emerged in one gradient fraction. This was considered to be an indicator of the mitochondrial band. Malate dehydrogenase can be used for identification of mitochondria [6, 7], although this enzyme is not specific for the mitochondrial fraction.

Malate dehydrogenase

There were two peaks of malate dehydrogenase activity from all 4 stages of ripening fruit. The larger peak was considered to correspond to the mitochondrial band because it coincided with the protein distribution peak, it fell in the range 1.35–1.39 M density of sucrose gradient and it overlapped with the peak activity of other characteristic mitochondrial enzymes mentioned later. The smaller peak was marked by the pigmented band present towards the top of the gradient (sucrose density 1.1 M), presumably containing broken cells, chloroplasts, etc. The units of enzyme activity (0.73×10^{-4}–1.22×10^{-4} kat/mg of mitochondrial protein) were constant throughout the ripening period.

Cytochrome c *oxidase*

Similarly, two peaks of cytochrome *c* oxidase activity were observed. It was 20–25-fold less than the level of malate dehydrogenase. As cytochrome *c* oxidase is strictly a mitochondrial enzyme, its activity in the smaller peak might be due to the association of a small amount of mitochondria with the debris (band towards the top of the gradient). The smaller peak was insignificant in the mitochondrial fraction isolated from green tomato and almost non-existent in 'B + 6d' fruit extract.

All the enzyme peaks obtained from different stages of ripening fruit overlapped in one fraction (sucrose density 1.35–1.39 M). Like malate dehydrogenase, the difference in the activity of cytochrome *c* oxidase (4.67×10^{-6}–5.00×10^{-6} kat/mg mitochondrial protein) during the ripening process was insignificant.

Succinate dehydrogenase and NADH oxidase

These two mitochondrial enzymes were only determined in the green stage of fruiting. The activity per mg of mitochondrial protein was 1.30×10^{-6} kat for succinate dehydrogenase and 1.06×10^{-6} kat for NADH oxidase. The enzyme activity, being small and requiring a relatively large quantity of mitochondria, could not be estimated in other stages of fruiting. Moreover, negligible activities of these two enzymes were observed towards the end of the ripening period. The major peaks of these enzymes also coincided with the main peaks of malate dehydrogenase and cytochrome *c* oxidase which reconfirmed the position of a single band of mitochondrial fraction on the sucrose density gradient.

The constancy in mitochondrial enzyme activities has been reported in many other plant tissues [8, 9]. Baqui *et al.* [4] observed an acceleration of Krebs cycle intermediates by isolated mango pulp mitochondria. On the contrary, Kane *et al.* [3] reported a decline in succinate oxidation capacity of ripening mango pulp mitochondria after an initial increase for *ca* 10 days. The mango fruits were ripened at 20°. Also, Alexandrescu *et al.* [10] noted either a drop or constant levels of activities of several enzymes in apple and pear saps.

Our study has confirmed that the activities of malate dehydrogenase and cytochrome *c* oxidase did exhibit constancy during the ripening process but the levels of succinate dehydrogenase and NADH oxidase declined sharply. It may be inferred from our observations that mitochondria retain at least some of their functions in ripening tomato fruit, and perhaps regulate the process of ripening and senescence.

EXPERIMENTAL

Tomato fruits (cv Manhatan) were picked at green and breaker (when fruits turned pink at the distal end) stages. The breaker fruits were artificially ripened at 19° for 3 days (B + 3d) and 6 days (B + 6d). The mitochondrial fractions were isolated from 4 stages of ripening fruit and were further purified on linear density sucrose gradient [11]. After centrifugation the gradient was fractionated into 16 × 0.5 ml and 4 × 1 ml fractions. All fractions were stored at 0–4° and were used within 1–15 hr for enzyme assays. All observations were made at least in triplicate.

Malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC: 1.1.1.37) activity was measured spectrophotometrically [12] with slight modifications. The reaction mixture contained 0.4 μmol NADH, 1.0 μmol oxaloacetate (pH 6–7), 0.3 mmol K-Pi buffer, pH 7.5 and 0.02 ml mitochondrial fraction in a final vol. of 3 ml. A decrease in *A* at 340 nm was recorded.

Cytochrome c *oxidase* (cytochrome *c*: O_2 oxidoreductase, EC: 1.9.3.1) activity was also assayed spectrophotometrically [13] with some modifications. The reaction mixture contained 0.8 mg reduced cytochrome *c*, 0.27 mmol K-Pi buffer, pH 7 and 0.1 ml mitochondrial fraction in a total vol. of 3 ml. Cytochrome *c* (oxidized) was reduced by Na ascorbate (1:20) for 20 min at room temp. Cytochrome *c* was separated from ascorbate by passing through a Sephadex G-50 column. The reduction of cytochrome *c* was very efficient. It exhibited an absorbancy ratio of 14:1 at 550 and 565 nm, respectively.

Succinic dehydrogenase (succinate: (acceptor) oxidoreductase, EC: 1.3.99.1) was determined according to a modified method [14]. To 0.2 ml mitochondrial fraction, 1.6 μmol $CaCl_2$ (0.8 ml) and 0.15 mmol K-Pi buffer, pH 7.2 was added and the mitochondria allowed to swell for 4 min. The remaining components, 0.25 μmol NaN_3, 1 mg BSA, 0.3 μmol 2,6-dichlorophenolindophenol, 5.4 μmol phenazine methosulphate and 30 μmol of neutralized succinate were then added. A decrease in *A* at 607 nm was recorded.

NADH *oxidase* ($NADH_2$: (acceptor) oxidoreductase, EC: 1.6.99.3) activity was measured by the method of Blair [15]. All enzyme assays were made at 24–25°. Mitochondrial protein was dissolved in 0.1 N NaOH at 50° for 1 hr and determined colorimetrically [16].

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PEROXIDASE CATALYSED OXIDATIVE DECARBOXYLATION OF VANILLIC ACID TO METHOXY-*p*-HYDROQUINONE

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Key Word Index—Peroxidase; vanillic acid; vanillin; vanillyl alcohol; syringic acid; methoxy-*p*-hydroquinone; methoxy-*p*-benzoquinone.

Abstract—Horseradish peroxidase catalysed the oxidative decarboxylation of vanillic acid to methoxy-*p*-hydroquinone and subsequent oxidation of the hydroquinone to methoxy-*p*-benzoquinone. Peroxidase also catalysed the oxidation of vanillyl alcohol to vanillin and vanillic acid; however, neither vanillyl alcohol nor vanillin appeared to give rise to methoxyhydroquinone directly. Correspondingly, peroxidase catalysed the oxidative decarboxylation of syringic acid to 2,6-dimethoxy-*p*-hydroquinone and subsequent oxidation of the hydroquinone to 2,6-dimethoxy-*p*-benzoquinone.

INTRODUCTION

Phenoloxidases are widely distributed among plants [1], and have been found as extracellular and intracellular enzymes [2] in white rot fungi where they have been implicated in lignin degradation [3, 4]. Evidence has accumulated that these enzymes catalyse the formation of a phenoxy radical as an initial step in the oxidation process [5, 6]. The unpaired electron is then stabilized by delocalization throughout the molecule. The radicals can then condense to form a polymer (lignification) or react with H_2O_2 or O_2 to form higher oxidation product(s). The importance of phenoloxidases in oxidative or dehydrogenative polymerization is well established [7]. Their role in the oxidative degradation of phenols, however, has been limited to syringyl compounds. 2,6-Dimethoxy-*p*-benzoquinone has been shown to be a phenoloxidase oxidation product of syringylglycerol-β-guaiacyl ether [5, 8], of syringic acid [9], of syringaldehyde and acetosyringone [5]. When similar studies were performed with guaiacylglycerol-β-guaiacyl ether [10] and other guaiacyl compounds [5], however, no methoxy-*p*-benzoquinone (MQ) was detected. Where fungal cultures were used [5], further degradation of MQ cannot be ruled out.

Since previous studies have indicated that vanillic acid is an intermediate in the fungal degradation of lignin [11], we were interested in examining the possible oxidative degradation of this compound by peroxidase. In this study we demonstrate that vanillic acid can be oxidatively decarboxylated to methoxy-*p*-hydroquinone (MHQ) by H_2O_2–peroxidase with further oxidation of MHQ to MQ. In bacteria specific monooxygenases have been found which oxidatively decarboxylate aromatic acids. Thus salicylate (2-hydroxybenzoic acid) hydroxylase [12] and anthranilate hydroxylase [13] catalyse the oxidative decarboxylation of salicylate and anthranilate to catechol.

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RESULTS

*Isolation and characterization of methoxy-*p*-hydroquinone*

After incubation of vanillic acid (1.2 mM) with peroxidase–H_2O_2 for 10 min at 38°, most of the vanillic acid remained unchanged (>95% recovery). A small amount (0.3%) of a new compound was produced, however, which after silanation co-chromatographed on the GC with authentic MHQ-TMSi. The simple mass spectrum of this compound *m/e* 284 (M^+), 269 (M − 15) and 254 (base, M − 30) and a few less intense peaks at lower mass was similar to that of authentic MHQ-TMSi. No MHQ was formed if the enzyme was boiled for 2 min prior to incubation. In addition no MHQ was formed if either H_2O_2 or the enzyme was omitted from the reaction mixture. Finally, although this experiment was routinely performed with Type II peroxidase, the more highly purified Type VI enzyme gave essentially identical results. All these results indicate that peroxidase–H_2O_2 is catalysing the reaction.

In an attempt to detect methoxy-*p*-benzoquinone in the reaction mixture, an underivatized sample was chromatographed at 100° in the isothermal mode. (MQ is unstable under the conditions used for silanation.) The retention times for MQ and MHQ were 4 and 12 min, respectively, under these conditions. GC analysis in-