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SOLUBILIZATION AND PROPERTIES OF THE ENZYME-CLEAVING 13-L-HYDROPEROXYLINOLENIC ACID IN TEA LEAVES

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Key Word Index—*Thea sinensis*; Theaceae; tea; hydroperoxide lyase; hexenals; 13-L-hydroperoxylinolenic acid.

Abstract—The membrane bound hydroperoxide lyase (E_2'') which catalyses the cleavage of 13-L-hydroperoxides (18:3-OOH and 18:2-OOH) of linolenic and linoleic acids to C_6-volatile aldehydes (hexenals and *n*-hexanal) was found to be localized in the chloroplast lamellae of tea leaves. It was selectively solubilized from the lamellae with 0.5% (w/v) Tween 20. The enzymatic cleavage of the hydroperoxides occurred even under anaerobic conditions. The optimal pH of E_2'' was 7–8. The common structural features shown by substrates of E_2'' were the presence of a L-hydroperoxy group at ω-6 with a conjugated *trans,cis*-diene at ω-7 and ω-9 in a C_{18}-fatty acid. E_2'' had an apparent K_m of 2.5 and 1.9 mM for 18:3-OOH and 18:2-OOH, respectively. No significant differences were found between chloroplast E_2'' and solubilized E_2''.

INTRODUCTION

The enzyme system (E_2)* producing C_6-aldehydes† from linolenic acid (18:3) and linoleic acid (18:2) is tightly bound to the chloroplast lamellae of tea leaves [2, 3]. It was demonstrated recently that 13-L-hydroperoxides of 18:3 and 18:2 fatty acids (18:3-OOH and 18:2-OOH) are detectable during C_6-aldehyde formation by tea chloroplasts [4]. Therefore it is presumed that C_6-aldehyde formation in tea chloroplasts consists of two enzymatic reactions catalysed by an oxygenase (E_2') and a hydroperoxide lyase (E_2'') [5, 6]. E_2'' catalyses the cleavage of 18:3-OOH and 18:2-OOH which are produced by a stereospecific addition of oxygen at C-13 of 18:3 and 18:2, respectively [4, 7]. In this investigation, we describe the solubilization and properties of the E_2'' from tea chloroplasts.

RESULTS AND DISCUSSION

Localization of E_2'' in tea leaves

The subcellular localization of E_2'' was investigated by using differential centrifugation. The greater part of the E_2'' activity was located in the 4000 *g* precipitate i.e. the chloroplast-rich fraction (Table 1). This fraction also contained the enzyme system (E_2) for C_6-aldehyde formation from 18:3 and 18:2 [2, 3]. Further fractionation of the chloroplasts indicated that the E_2'' activity was associated with the lamellae (Table 2).

Table 1. Localization of hydroperoxide lyase (E_2'') activity in tea leaves

	C_6-aldehydes formed (μmol/g fresh leaves)	
Fraction	18:2-OOH*	18:3-OOH*
4000 *g* precipitate	2.13	0.99
4000 *g* precipitate (boiled†)	0.01	0.01
25 000 *g* supernatant	0.04	0.11
25 000 *g* precipitate	0.01	0.09

* 6 μmol substrate/assay flask.
† 100°, 10 min.

Table 2. Distribution of hydroperoxide lyase (E_2'') in sub-fractions of tea chloroplasts

	C_6-aldehydes (μmol/g chloroplasts)	
Fraction	18:2-OOH*	18:3-OOH*
Chloroplasts	20.5	23.7
Lamellae	18.7	19.8
Stroma	0	0
Lamellae + stroma	16.8	21.5

* 6 μmol substrate/assay flask.

Solubilization of E_2'' from chloroplasts

Addition of Tween 20, up to 0.5% (w/v), to reaction mixtures containing chloroplasts had little effect on E_2'' activity, whereas the system for C_6-aldehyde formation (E_2) from 18:3 or 18:2 was markedly inhibited (Fig. 1). When the chloroplast suspension was treated with Tween

* Abbreviations: E_2, enzyme system producing C_6-aldehydes from 18:2 and 18:3 acids; E_2', oxygenase; E_2'', hydroperoxide lyase; 18:2-OOH, 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 18:3-OOH, 13-L-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid.

† Hexenals (*cis*-3-hexenal, *trans*-3-hexenal [1] and *trans*-2-hexenal) are formed from 18:3 or 18:3-OOH and *n*-hexanal from 18:2 or 18:2-OOH.

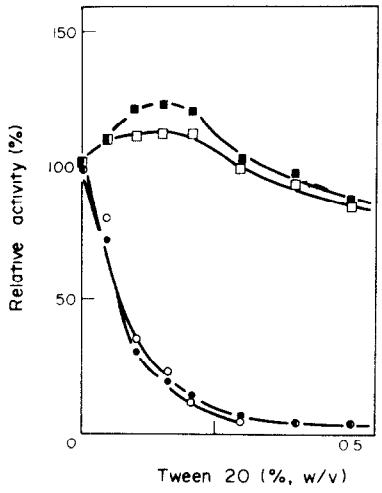


Fig. 1. Effect of Tween 20 on the activities for hydroperoxide lyase (E_2'') and C_6-aldehyde formation from 18:3 and 18:2 (E_2) in tea chloroplasts. Chloroplasts (0.1 g) were suspended in 10 ml of McIlvaine's buffer, pH 7.0 or 6.3, containing Tween 20 at the concentration indicated and assayed for E_2 and E_2'' activities. □–□, E_2'' at pH 7.0 with 18:3-OOH; ■–■, E_2'' at pH 7.0 with 18:2-OOH; ○–○, E_2 activity at pH 6.3 with 18:3; ●–●, E_2 activity at pH 6.3 with 18:2.

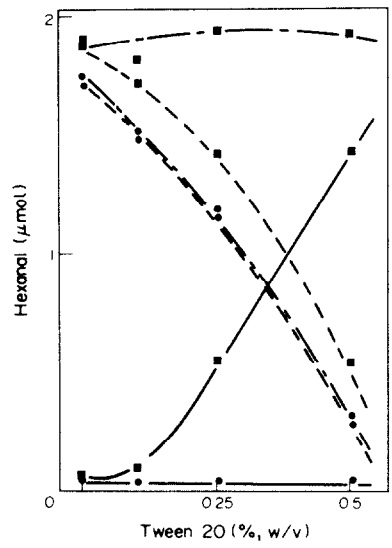


Fig. 2. Effect of Tween 20 on solubilization of the enzyme activities producing C_6-aldehydes in tea chloroplasts. Chloroplast pellet (2 g) was suspended in 20 ml of McIlvaine's buffer, pH 7.0, containing Tween 20 at the concentration indicated and homogenized in a chilled Teflon homogenizer. The homogenate was then centrifuged at 25 000 *g* for 20 min, and the ppt suspended in 20 ml of McIlvaine's buffer, pH 7.0. One ml of either supernatant or ppt suspension was made up to 10 ml with McIlvaine's buffer, pH 7.0 or pH 6.3, and assayed. ■–■, E_2'' activity (18:2-OOH, 6 μmol, pH 7.0) in supernatant; ■ ···■, E_2'' activity in precipitate; ●–●, E_2 activity (*n*-hexanal formation from 18:2 at pH 6.3) in supernatant; ● ···● , E_2 activity in precipitate. ■ · · ■ and ● · · ●, sums of E_2'' and E_2 activities, respectively recovered in supernatants and precipitates.

20, the E_2'' activity was transferred from a 25 000 *g* precipitable fraction to a supernatant fraction as the concentration of Tween 20 increased (Fig. 2). At 0.5% (w/v) Tween 20, 70–80% of the initial E_2'' activity was recovered in the supernatant fraction and 20–30% remained in the 25 000 *g* precipitate (Fig. 2 and Table 3). Most of the chlorophylls (>95%) remained in the precipitate. The system (E_2) for C_6-aldehyde formation from 18:2 was not found in the supernatant though the E_2 activity in the precipitate decreased similarly to the E_2'' activity (Fig. 2 and Table 3). Oxygen uptake with 18:3 or 18:2 and formation of 18:3-OOH and 18:2-OOH were not detected in the solubilized E_2'' preparation.

These findings indicate that the reduction in E_2 activity in the presence of Tween 20 is due to the sensitivity of E_2' to this detergent. When the light-yellow supernatant obtained by centrifugation at 25 000 *g* was subjected to centrifugation at 100 000 *g* for 60 min, the E_2'' activity was found in the 100 000 *g* supernatant (Table 3). Therefore, a 25 000 *g* supernatant was routinely used as the solubilized E_2''. The solubilized E_2'' preparation (25 000 *g*, supernatant) contained 12 protein bands on SDS disc electrophoresis. It was stable and retained 90% of original activity after 3 months at −20°. The enzyme system producing C_6-aldehydes from 18:3 and 18:2 was not reconstituted when the 25 000 *g* precipitate was added to solubilized E_2'' fraction.

pH *optimum for* E_2'' *activity*

A solubilized E_2'' preparation had its maximum activity in the range 7–8 as in particulate tea chloroplast

Table 3. Solubilization of hydroperoxide lyase (E_2'') from chloroplasts by Tween 20

	C_6-aldehydes (μmol)			
Fraction	18:2	18:2-OOH	18:3	18:3-OOH
Chloroplasts, untreated	2.06	3.73	3.39	4.40
25 000 *g* supernatant	0.01	2.42	0.03	3.67
25 000 *g* precipitate	0.34	1.60	0.93	1.83
100 000 *g* supernatant	0.01	2.48	tr	3.49
100 000 *g* precipitate	tr	0.15	tr	0.10

After the treatment of chloroplasts with 0.5% Tween 20, the mixture was fractionated by centrifugation, and the E_2'' activity in each fraction assayed with 6 μmol of substrate by the headspace method.

preparations [5]. On the other hand, the optimum for the formation of hydroperoxides and C_6-aldehydes from 18:3 and 18:2 was *ca* pH 6.3 [5].

Oxygen requirement for the enzymatic cleavage of 18:3-OOH *and* 18:2-OOH

C_6-aldehyde formation from 18:3-OOH and 18:2-OOH was shown to be oxygen-independent in chloroplasts and solubilized E_2'' by the demonstrations that cleavage of 18:3-OOH and 18:2-OOH was fully active under anaerobic conditions (N_2), whilst C_6-aldehyde formation from 18:3 and 18:2 was oxygen-dependent [2]. The oxygen-independency for cleavage of hydroperoxides of 18:3 and 18:2 was also reported in cucumber fruits [9].

Thermal stability of the enzyme system producing C_6-*aldehydes*

The membrane-bound enzyme of E_2'' in tea chloroplasts was more stable to heat-treatment than the solubilized E_2'' (Fig. 3). E_2 activity in chloroplasts was less stable than solubilized E_2'' activity. This result shows that E_2 is relatively heat-sensitive compared to E_2''.

Effect of inhibitors

The effects of inhibitors were examined with 18:2-OOH as substrate using solubilized E_2''. Significant inhibition was obtained with 1 mM 2-dimethylaminoethyl-2,2-diphenyl valerate (SKF 525-A) (74% inhibition), methylene blue (50%) and 2,6-dichlorophenol indophenol (65%). Unlike E_2'' activity in watermelon seedlings [8], the enzyme activity was not inhibited by 1 mM *p*-chloromercuribenzoate (*p*CMB). No inhibition was observed with dithiothreitol, glutathione, L-cysteine, EDTA, or *o*-phenanthroline at 1 mM. The metal ions Zn^{2+}, Al^{3+}, Fe^{3+}, Fe^{2+}, Cu^{2+}, Mn^{2+} and Ca^{2+} (each 1 mM) had no effect on E_2'' activity although it was inhibited by 1 mM Cu^{2+} [2].

Substrate specificity

The substrate specificity of solubilized E_2'' was examined for 13-L-,9-D-hydroperoxides of 18:3 and 18:2 and their analogues (Table 4).

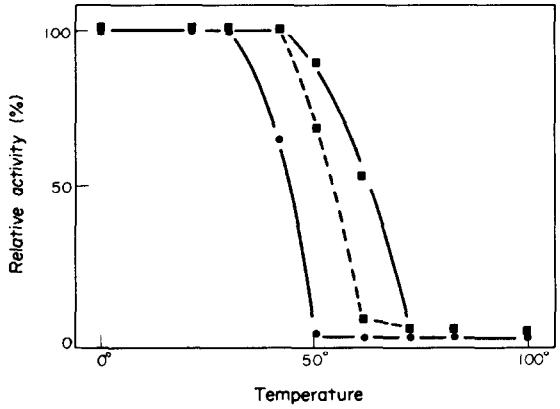


Fig. 3. Effect of temperature on enzyme stability. The enzyme solutions were incubated at various temperatures for 10 min and the enzyme activities determined by the headspace method. ●–●, E_2 activity of chloroplasts with 18:2; ■–■, E_2'' activity of chloroplasts; ■ –·–·–■, solubilized E_2''.

When the 13-L-hydroperoxides of 18:3 and 18:2 were used as substrates, hexenals and *n*-hexanal were produced in high yields (Table 4). Unlike cucumber E_2'' [9], no appreciable conversion of 9-D-hydroperoxides of 18:3 and 18:2 to C_6-aldehydes and no conversion to C_9-aldehydes was observed.

Hence, as in tomato fruits [10] and alfalfa seedlings [11], E_2'' from tea chloroplasts utilized 18:3-OOH and 18:2-OOH, but not 9-D-hydroperoxides of 18:3 and 18:2. The enzyme system (E_2) producing C_6-aldehydes in tea chloroplasts had an apparent K_m of 4.8×10^{-4} and 4.8×10^{-4} M for 18:3 and 18:2, respectively. The apparent K_m values for 18:3-OOH and 18:2-OOH with solubilized E_2'' were 2.5×10^{-3} and 1.88×10^{-3} M, respectively.

E_2'' formed *n*-hexanal from both the methyl ester and primary alcohol corresponding to 18:2-OOH (Table 4). Modification of the carboxyl group reduced the reactivities of the hydroperoxides as substrates for E_2''. The 13-L-hydroperoxide of γ-linolenic acid was converted to *n*-hexanal by solubilized E_2'' to the same extent as γ-linolenic acid was cleaved to *n*-hexanal by chloroplast E_2. However, the 15-L-hydroperoxide of arachidonic acid was not cleaved to *n*-hexanal. As described in a previous paper [5], chloroplasts are able to oxygenate arachidonic acid but it is not converted to C_6-aldehydes. Thus, the hydroperoxides of arachidonic acid appear to be formed and accumulated by the enzyme system (E_2) in tea chloroplasts. Any hydroxy compound derived from 18:3-OOH and 18:2-OOH did not act as a substrate for E_2''. α-Ketol which was proposed as a possible precursor of C_6-aldehydes by Jadhav *et al.* [12], could not act as a substrate for E_2'' (Table 4) [13].

The structural requirements of substrates for E_2'' are that they contain a *cis,trans*-conjugated diene system and that the *trans* double bond is adjacent to the L-hydroperoxide-bearing carbon atom. A further requirement is that the L-hydroperoxy group is at ω-6 in a C_{18}-fatty acid.

EXPERIMENTAL

Plant material. Fresh leaves of tea plant (*Thea sinensis* cv Yabukita) were harvested in August and used immediately. The chloroplast fraction was prepared as a 4000 *g* ppt. [2,3].

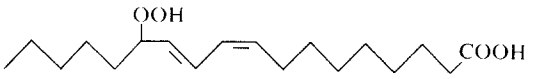
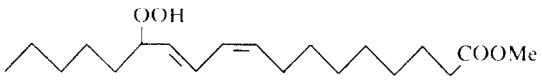
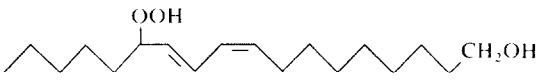
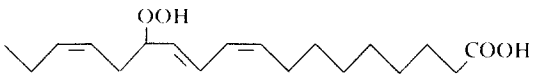
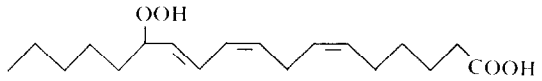
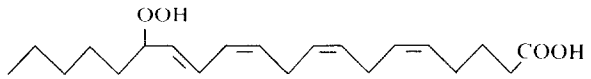
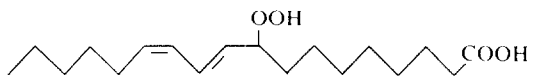
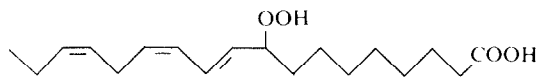
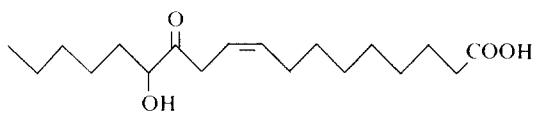
Fractionation of tea leaves. Tea leaves were homogenized with dil. McIlvaine's buffer (4 vol), pH 6.3, containing 0.4 M sucrose and fractionated according to the method in ref. [2].

Enzyme assays. (i) Hydroperoxide lyase (E_2'') was determined by measuring the formation of hexenals (*cis*-3-hexenal, *trans*-3-hexenal [1] and *trans*-2-hexenal) from 18:3-OOH or *n*-hexenal from 18:2-OOH (6 μmol/assay) at pH 7.0 by the headspace method with GLC [3]. (ii) Oxygen uptake with 18:3 or 18:2 were determined at pH 6.3 by the method described in ref. [3]. (iii) The enzyme system (E_2) producing C_6-aldehydes from 18:3 or 18:2 was determined by the formation of hexenals from 18:3 or *n*-hexanal from 18:2 (6 μmol/assay) at pH 6.3 by the headspace method with GLC [3].

Determination of hydroperoxide. Hydroperoxide formation from 18:3 or 18:2 was determined by HPLC [11].

Preparation of substrate. 9- and 13-hydroperoxides were prepared from 18:3 and 18:2 using potato lipoxygenase [14,15] and soybean lipoxygenase [4]. 13-L-Hydroperoxy-*cis*-9,*trans*-11-octadecadien-1-ol was prepared by soybean lipoxygenase from *cis*-9,*cis*-12-octadecadien-1-ol which was obtained by reduction of Me linoleate with $LiAlH_4$. The Me ester of 18:2-OOH was derived from 18:2-OOH by methylation with

Table 4. Substrate specificity of hydroperoxide lyase (E_2'')

Substrate	C_6-aldehyde formation*			
	Solubilized E_2''		Chloroplast E_2''	
	V_{max} (%)	K_m (mM)	V_{max} (%)	K_m (mM)
	100	1.88	100	2.00
	26	0.10	21	0.23
	55	0.37	49	0.38
	127	2.50	113	2.50
	18	0.48	28	0.56
	0	--	0	—
	0		0	--
	0	—	0	--
	0	--	0	—

* Incubation mixture (10 ml), containing a substrate and solubilized E_2'' corresponding to 0.1 g of chloroplasts, was incubated for 10 min at 35°. C_6-aldehydes were then determined by the headspace method. V_{max} is expressed relative to the value (moles/min) obtained with 18:2-OOH. K_m values were obtained from Lineweaver–Burk plots (0.5–12 μmol substrate).

CH_2N_2. Hydroperoxides of γ-linolenic acid and arachidonic acid were prepared by soybean lipoxygenase reaction [16]. A 13-hydroxide of each hydroperoxide was prepared by $NaBH_4$ reduction of the appropriate 13-hydroperoxides. α-Ketol, 13-hydroxy-12-oxo-octadeca-*cis*-9-enoic acid, was prepared by enzymatic isomerization of 18:2-OOH with an aq. extract from hexane-defatted corn germ flour [17]. The structures of the compounds prepared were confirmed by UV, IR and ^1H NMR spectra.

Solubilization of hydroperoxide lyase (E_2'') from tea chloroplasts. Chloroplast (2 g) was suspended in 20 ml of chilled 50 mM McIlvaine's buffer, pH 7.0, containing 0.5% (w/v) Tween 20 and homogenized in a chilled Teflon homogenizer (10 strokes). After centrifugation at 25 000 g for 20 min, the resultant supernatant was centrifuged at 100 000 g for 60 min. Routinely the 25 000 g supernatant was used as a solubilized hydroperoxide lyase (E_2'').

SDS disc electrophoresis was performed by the method of ref. [18].

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