Effect of Lipolytic Acyl Hydrolase on the Activity for Six Carbon Aldehyde Formation in Tea Chloroplasts

The treatment of isolated tea chloroplasts with potato lipolytic acyl hydrolase (LAHase) caused reduction of the activity for C_6 aldehyde (1-hexanal, *cis*-3-hexenal, and *trans*-2-hexenal) formation from linoleic acid and linolenic acid. The activity of hydroperoxide cleavage enzyme was maintained even after potato LAHase treatment, whereas the activity for the overall reaction from C_{18} fatty acids to C_6 aldehydes was decreased. These indicate that the activity of an oxygenation step, rather than a cleavage step, of C_6 aldehyde formation was influenced by the treatment with potato LAHase.

When plant tissues are disrupted or injured, a sequential degradation of lipids leads to formation of volatile C_6 and C_9 aldehydes which are important flavor components in plants and plant products (Galliard, 1978). Glycerolipids are hydrolyzed by lipolytic acyl hydrolase (LAHase) at an initial step of degradation, and then released C_{18} unsaturated fatty acids with *cis*-1,*cis*-4-pentadiene moiety such as linoleic acid (LA) and linolenic acid (LNA) are oxygenated and cleaved into C_6 aldehydes and a counterpart, C_{12} oxo acid or C_9 aldehydes and C_9 oxo acid. We have demonstrated that volatile C_6 aldehydes (1-hexanal, *cis*-3-hexenal, *trans*-3-hexenal, and *trans*-2-hexenal) were produced from LA or LNA by the enzyme(s) bound to chloroplast lamellae in a case of tea leaves (Hatanaka and Harada, 1973; Sekiya et al., 1976 Hatanaka and Kajiwara, 1981). Recently, two enzymes were reported to be involved in the C_6 aldehyde forming enzyme system in isolated tea chloroplasts (Hatanaka et al., 1981). On the other hand, when chloroplasts were treated with potato LAHase, the C_6 aldehyde forming activity was reduced (Hatanaka et al., 1979). We have attempted to investigate changes in properties of the C_6 aldehyde forming enzymes when isolated tea chloroplasts were treated with potato LAHase.

EXPERIMENTAL SECTION

Fresh leaves of tea (*Thea sinensis* cv. Yabukita) were obtained from a local commercial tea garden. Chloroplasts were prepared as a 4000-g pellet from fresh tea leaves harvested in August and stored at −20 °C after being suspended in 2.1 M sucrose solution (Sekiya et al., 1976). This chloroplast pellet contained about 5 mg of chlorophyll/g of wet chloroplast pellet. The 13-hydroperoxide of linoleic acid (LA-13-HPO) was prepared according to the method described by Sekiya et al. (1976).

An overall activity of the C_6 aldehyde forming enzyme system was determined from 1-hexanal formation from LA by headspace vapor analysis with GLC (Hatanaka et al., 1979). Oxygen consumption with LA was measured by a Clark type of oxygen electrode (Hatanaka et al., 1979). An activity for cleavage of fatty acid hydroperoxide was determined from 1-hexanal formation from LA-13-HPO by headspace vapor analysis (Hatanaka et al., 1981).

Chloroplasts (0.1 g) were incubated for 10 min at 25 °C with potato LAHase (10 units) in 10 mL of McIlvaine's buffer (pH 6.3) as described previously (Hatanaka et al., 1979) or with snake venom phospholipase A_2 (20 units; Boehringer Manheim GmbH) in 10 mL of 50 mM Tris-HCl buffer (pH 8.5) containing 1 μmol of $CaCl_2$ and 100 μmol of NaCl. At the end of the incubation period, the chloroplast suspension was subjected to centrifugation. The obtained chloroplast pellet was washed twice with McIlvaine's buffer (pH 6.3), was resuspended in McIlvaine's buffer, pH 6.3, and served as an enzyme solution for assay of C_6 aldehyde formation.

Crude lipids from tea chloroplast pellet were prepared according to the method of Folch et al. (1957). Dried crude lipids were dissolved in chloroform, after being weighed, and then applied on a silica gel column and eluted with solvent systems of chloroform–acetone and chloroform–methanol (Ohmori and Yamada, 1974).

Table I. Lipid Composition of Tea Chloroplasts Treated with or without Potato LAHase[a]

component	amounts −LAHase (A)	amounts +LAHase (B)	ratio B/A
crude total lipids[b]	29.0	18.4	0.63
MGDG[c]	3.8	0.6	0.16
DGDG[c]	1.7	0.4	0.24
phospholipids[c]	8.6	2.8	0.33
chlorophyll[b]	4.5	4.9	1.09
protein[b]	39.3	27.2	0.69

[a] Crude total lipids prepared by the method of Folch et al. (1957) were subjected to silica gel column chromatography to separate and to determine galactolipids and phospholipids. Chlorophyll and protein were determined by the methods described under Experimental Section using the same lot of treated chloroplasts as that used for lipid analysis. [b] Milligrams per gram of chloroplast pellet. [c] Micromole(s) per gram of chloroplast pellet.

Chlorophyll was determined by the method of Mackinney (1941), and protein was determined by the method of Lowry et al. (1951).

Sucrose density gradient centrifugation was carried out in a 60-mL centrifugation tube. On 5 mL of McIlvaine's buffer (pH 6.3) containing 70% sucrose, 15 mL of the same buffer containing 60, 40, and 20% (w/v; at 20 °C) sucrose, respectively, was gently layered, and 5 mL of sample was layered on the top. The solution in a tube was centrifuged at 20000 rpm (Hitachi RPS 25-2A rotor) for 60 min. After centrifugation, the solution was fractionated, and chlorophyll, protein, oxygen uptake, and the 1-hexanal-forming activity were determined.

RESULTS AND DISCUSSION

Hatanaka et al. (1979) demonstrated that the enzyme system responsible for C_6 aldehyde formation from LA and LNA was bound to chloroplast lamellae and the activity of C_6 aldehyde formation was reduced by the treatment with potato LAHase which catalyzes acyl hydrolysis of glactolipids and phospholipids (Galliard, 1971; Hirayama et al., 1975). The maximal rate of reduction of 1-hexanal formation by potato LAHase was 50–70%, even though the concentration of LAHase was increased (Figure 1). Formation of hexenals (*cis*-3-hexenal and *trans*-2-hexenal) from LNA was also reduced similarly by potato LAHase. Treatment of chloroplasts with snake venom phospholipase A_2 caused no loss of the enzyme activity for 1-hexanal formation. Lipid composition of chloroplasts treated with or without potato LAHase was examined. Galactolipids, phospholipids, and protein were lost from chloroplasts by the treatment with potato LAHase but not chlorophyll (Table I). Monogalactosyldiacylglyceride (MGDG), digalactosyldiacylglyceride (DGDG), and phospholipids were

0021-8561/82/1430-0183$01.25/0

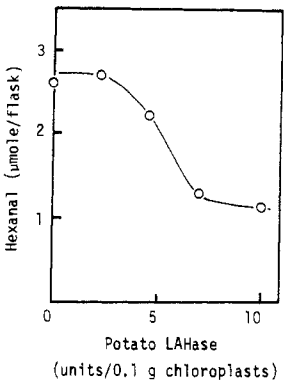

Figure 1. Inactivation of 1-hexanal-forming activity by potato LAHase. 1-Hexanal formation from LA (60 μmol) by chloroplasts treated with potato LAHase was determined by headspace vapor analysis.

removed from chloroplasts by 84, 76, and 67%, respectively, whereas protein and crude total lipids decreased to a lesser extent (ca. 40 and 30%, respectively). Ratios of galactolipids and phospholipids to protein also decreased by the treatment with LAHase. These findings indicate that degradation of galactolipids, rather than phospholipids, causes reduction of 1-hexanal-forming activity. In contrast, activities for photoelectron transport system of chloroplasts were reduced by the treatment with potato LAHase and phospholipase A_2 (Hirayama and Matsui, 1976). The decrease in protein suggests the possibility of removal of protein other than the expected enzymes or degradation of surface protein by proteolytic enzymes contaminated in LAHase preparation. However, the possibility that C_6 aldehyde forming enzymes are inactivated by proteolytic enzyme may be excluded, because of trypsin resistance of C_6 aldehyde forming activity in chloroplasts (Hatanaka et al., 1979). The 1-hexanal-forming activity of chloroplasts treated with potato LAHase appeared in the same fractions as those without LAHase treatment, when chloroplasts were subjected to sucrose density gradient centrifugation (Figure 2). The 1-hexanal-forming activity after the treatment with LAHase was still associated with oxygen uptake and chlorophyll. The C_6 aldehyde forming enzymes are considered to be located in the inner part of lamellae membrane, as the enzymes were resistant to trypsin digestion. Therefore, the residual activity of 1-hexanal formation in chloroplasts after the treatment with potato LAHase seems to be associated with undegraded chloroplast fragments although it was 30–50% of the original activity.

The decreased enzyme activity of chloroplasts caused by potato LAHase was not restored by adding MGDG and DGDG (30–120 μg as glucose per flask) in the presence or absence of 0.005% Triton X-100. Crude total lipids extracted from tea chloroplasts and crude total phospholipids, which were separated from nonpolar lipids and galactolipids by silica gel column chromatography (Ohmori and Yamada, 1974), also had no effect on the decreased enzyme activity for 1-hexanal formation. The sonication (30 s; 4 times) in addition to MGDG and/or DGDG had no effect on restoration. Addition of purified MGDG (60 μg as glucose) to untreated chloroplast (0.1 g) caused a remarkable inhibition (60%) of 1-hexanal-forming activity but DGDG did not. The reason why MGDG inhibits the enzyme activity for 1-hexanal formation has not been known so far.

Recently the enzyme system responsible for C_6 aldehyde formation in isolated tea chloroplasts was reported to be composed of two enzymes, lipoxygenase and fatty acid

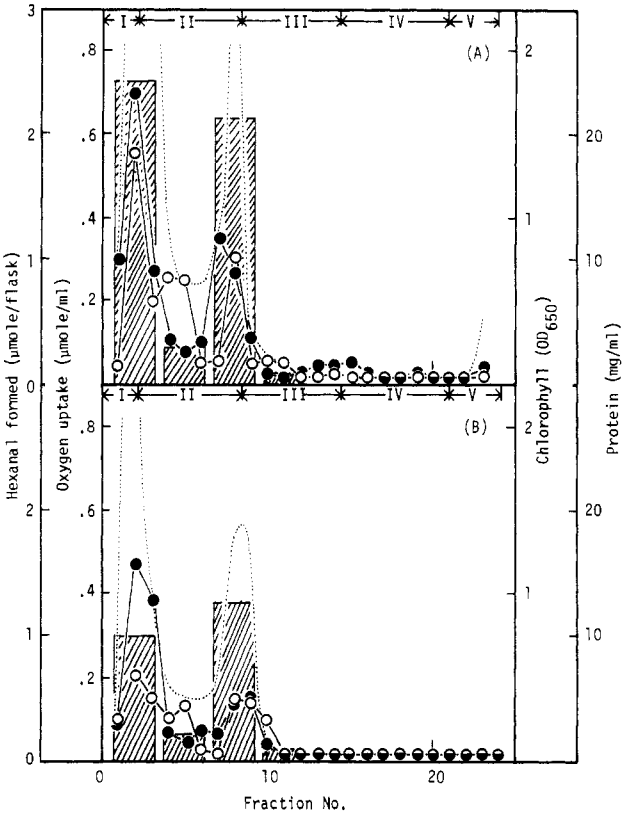

Figure 2. Sucrose density gradient centrifugation of chloroplasts treated without (A) and with (B) potato LAHase. I, II, III, IV, and V indicate 70, 60, 40, and 20% sucrose and sample layer, respectively. (○) indicates oxygen uptake; (●) protein; (···) chlorophyll determined from OD_{650}. Shaded bars indicate 1-hexanal-forming activity for three fraction tubes.

Table II. Effect of Potato LAHase on 1-Hexanal-Forming Activities[a]

treatment	fraction	1-hexanal formed, μmol/flask LA	LA-13-HPO
−LAHase	pellet	2.60	3.07
	supernatant	0.04	0.24
+LAHase	pellet	1.38	3.28
	supernatant	0.01	0.26

[a] Chloroplast pellet and supernatant after treatment with or without potato LAHase, followed by centrifugation at 25000*g* for 20 min, were incubated with LA (6 μmol) or LA-13-HPO (6 μmol) to determine 1-hexanal-forming activity by the headspace method.

hydroperoxide cleavage enzyme (hydroperoxide lyase) (Hatanaka et al., 1981). 1-Hexanal formation from LA decreased by 50% after potato LAHase treatment, whereas hydroperoxide lyase activity was maintained even after the treatment with potato LAHase (Table II). Therefore, reduction of 1-hexanal-forming activity by potato LAHase could be attributed to reduction of activity for the first step of 1-hexanal formation, oxygenation of LA, rather than hydroperoxide lyase. However, oxygen uptakes of chloroplasts treated with various preparations of potato LAHase ranged from 60 to 120% of that of chloroplasts treated without potato LAHase. This was probably due to contamination of potato lipoxygenase in the potato LAHase preparation.

2,6-Dichlorophenolindophenol, SKF 525-A [2-(dimethylamino)ethyl 2,2-diphenylvalerate], and cytochrome *c*, which are potent inhibitors for the enzyme system in chloroplasts (Hatanaka et al., 1979), also inhibited the

residual 1-hexanal-forming activity of chloroplasts treated with potato LAHase at the same inhibition rate as the enzyme activity of untreated chloroplasts.

During harvesting, storage, and processing of vegetables including green leaves, LAHase may attack membrane lipids, as LAHase involved in green leaves hydrolyzes both galactolipids and phospholipids (Matsuda et al., 1979; Matsuda and Hirayama, 1979). Free C_{18} fatty acids thus released by hydrolysis of lipids may be better substrates than esters of fatty acids (e.g., glycerolipids) for C_6 aldehyde formation in tea leaves (Sekiya et al., 1976) and cucumber fruits (Galliard and Phillips, 1976). However, degradation of membrane lipids during harvesting, storage, and processing of vegetables may cause inactivation of the enzyme system reponsible for C_6 aldehyde formation from C_{18} fatty acids, even though degradation of membrane lipids provides free fatty acids. Therefore, it is important to know rates of acyl hydrolysis of lipids and inactivation of C_6 aldehyde forming activity by leaf LAHase to evaluate flavor (C_6 aldehyde) formation.

ACKNOWLEDGMENT

We express our thanks to Smith Kline & French Laboratories, Philadelphia, PA, for their kind gift of SKF 525-A.

LITERATURE CITED

Folch, J.; Lees, M.; Stanley, G. H. S. *J. Biol. Chem.* **1957**, *226*, 497–509.
Galliard, T. *Biochem. J.* **1971**, *121*, 379–390.
Galliard, T. "Biochemistry of Wounded Plant Tissues"; Kahl, G., Ed.; Walter de Gruyter: Berlin, West Germany, 1978; p 155.
Galliard, T.; Phillips, D. R. *Biochim. Biophys. Acta* **1976**, *431*, 278–287.
Hatanaka, A.; Harada, T. *Phytochemistry* **1973**, *12*, 2341–2346.
Hatanaka, A.; Kajiwara, T. *Z. Naturforsch., B: Anorg. Chem., Org. Chem.* **1981**, *36*, 755–756.
Hatanaka, A.; Kajiwara, T.; Sekiya, J.; Imoto, M.; Inouye, S. *Plant Cell Physiol.* **1981**, in press.
Hatanaka, A.; Sekiya, J.; Kajiwara, T.; Miura, T. *Agric. Biol. Chem.* **1979**, *43*, 735–740.
Hirayama, O.; Matsuda, H.; Takeda, H.; Maenaka, K.; Takatsuka, H. *Biochim. Biophys. Acta* **1975**, *384*, 127–137.
Hirayama, O.; Matsui, T. *Biochim. Biophys. Acta* **1976**, *423*, 540–547.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
Mackinney, G. *J. Biol. Chem.* **1941**, *140*, 315–322.
Matsuda, H.; Hirayama, O. *Biochim. Biophys. Acta* **1979**, *573*, 155–165.
Matsuda, H.; Tanaka, G.; Morita, K.; Hirayama, O. *Agric. Biol. Chem.* **1979**, *43*, 563–570.
Ohmori, M.; Yamada, M. *Plant Cell Physiol.* **1974**, *15*, 1129–1132.
Sekiya, J.; Numa, S.; Kajiwara, T.; Hatanaka, A. *Agric. Biol. Chem.* **1976**, *40*, 185–190.

Jiro Sekiya*
Tadahiko Kajiwara
Masaya Imoto
Satoshi Inouye
Akikazu Hatanaka

Department of Agricultural Chemistry
Faculty of Agriculture
Yamaguchi University
Yamaguchi 753, Japan

Received for review April 7, 1981. Revised manuscript received August 17, 1981. Accepted October 21, 1981.

Convenient Preparation of Chlordecone Alcohol (Kepone Alcohol) and Its Deuterated, Tritiated, and Dechlorinated Derivatives

Successful synthesis of chlordecone alcohol and its deuterated, tritiated, and dechlorinated analogues is described. Borohydride reduction of chlordecone or of one of its dechlorinated derivatives resulted in the formation of the corresponding alcohol in greater than 60% yield. Characterization of reduction products was established by chromatographic (GLC; TLC) and spectral (IR; NMR; MS) analyses.

Chlordecone (CD; 1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one), an organochlorine pesticide reported to be a liver carcinogen in rats and mice (Reuber, 1978), was manufactured in Hopewell, VA, from 1966 to 1975. Due to inappropriate industrial disposal procedures, primarily by a small, independent company, this pesticide now contaminates the rivers and marine life in the Tidewater region of Virginia. It is anticipated that area residents will be exposed to small amounts of this chemical for the foreseeable future since CD undergoes minimal degradation in the environment.

The only alteration products of CD detected in environmental samples (water, soil, and fish) are low levels of dechlorinated derivatives presumably formed by photolysis (Harless et al., 1978; Carver and Griffith, 1979). In contrast, a major pathway for CD (1) metabolism in humans has recently been discovered. The first step in the biotransformation of CD is its reduction to chlordecone alcohol (2) (CDOH; 1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-ol). In chemical workers poisoned with large quantities of CD, reduction appears to be the major pathway for CD inasmuch as 75% of the total pesticide excreted in bile is CDOH (Fariss et al., 1980). Because it was previously believed that CD is not subject to metabolism, little information is available regarding the measurement or the toxicology of CDOH. In order to acquire needed information regarding the pharmacokinetics and the toxicity of this reduced form of CD, synthetic procedures were developed to provide pure standards of CDOH and related derivatives.

EXPERIMENTAL SECTION

Materials. CD (1) (99% pure), monohydrochlordecone (5) (monohydro-CD; 1a,3,3a,4,5,5,5a,5b,6-nonachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one) (95% pure), and dihydrochlordecone (6) (dihydro-CD; 1a,3,4,5,5,5a,5b,6-octachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one) (98% pure) were provided by the Environmental Protection Agency (Research Triangle Park, NC). Sodium borodeuteride (99% deuterated) was purchased from Stohler Chemicals (Waltham, MA) and tritiated sodium borohydride (260.2 mCi/mM) from New England Nuclear (Boston, MA).

0021-8561/82/1430-0185$01.25/0