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THE EFFECTS OF SOYBEAN LIPOXYGENASE-1 ON CHLOROPLASTS FROM WHEAT

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; isolated chloroplasts; lipoxygenase; lipids; fatty acids; photosynthetic electron transport.

Abstract—Chloroplasts were isolated from primary leaves of wheat 12 days after germination and incubated at 25° for 45 min in the dark with soybean lipoxygenase-1. The lipoxygenase action was evident from a weak oxygen uptake of *ca* 0.18 μmol/hr per mg chloroplast protein. The lipoxygenase treatment caused a marked decrease in the photochemical activity, as measured by the reduction rate of 2,6-dichlorophenolindophenol. However, both the content and composition of the lipids as well as those of total fatty acids remained largely unchanged except for a slight but significant decrease in the total linolenic acid content. It is proposed that soybean lipoxygenase-1 selectively attacks free linolenic acid present in chloroplasts, followed by a chlorophyll-catalysed reaction of hydroperoxylinolenic acid with components of the electron transfer system.

INTRODUCTION

Lipoxygenases (EC 1.13.11.12) catalyse the oxygenation of polyunsaturated fatty acids (for a review see, for example, ref. [1]) which are known to play an essential role in the structure and function of chloroplast membranes [2]. At least two types of lipoxygenases may be distinguished. The first type represented by soybean lipoxygenase-1 (Theorell enzyme), the best characterized lipoxygenase so far, attacks only unesterified polyenoic fatty acids; its action on chloroplast membranes would require, therefore, the preceding action of a lipid-hydrolysing enzyme. Another type of lipoxygenase is able to act directly on esterified polyenoic fatty acids in membrane phospholipids and to attack, therefore, biological membranes even in the absence of a lipid-hydrolysing enzyme. This type of lipoxygenase has been clearly demonstrated with an animal enzyme, the lipoxygenase of rabbit reticulocytes which initiates the degradation of mitochondria during the cell maturation process [3].

Many fruits and seeds have been shown to contain high lipoxygenase activity [4, 5]. Moreover, the lipoxygenase level exhibits in many cases biological dynamics in developmental and senescence processes and seems to be controlled by hormone actions [6, 7]. It is reasonable to speculate, therefore, that lipoxygenases may be involved in the breakdown of intracellular membranes during restructuring processes such as the germination of seeds, ripening of fruits, withering of leaves, etc. Fruit ripening is known to be connected with a transformation of green chloroplasts to chromoplasts or amyloplasts. To determine whether lipoxygenases are able to induce inactivation and breakdown of thylakoid membranes, we studied first the effect of soybean lipoxygenase-1 on chloroplasts. This lipoxygenase affords the advantage that it does not cause co-oxidation of chlorophyll, as is known, for example, for the lipoxygenase isoenzymes from peas [8], which would complicate the system under investigation.

RESULTS

Soybean lipoxygenase-1 produced an oxygen uptake when added to a chloroplast suspension in the dark (Fig. 1 and Table 1). The rate of oxygen uptake was relatively small as compared with the reaction with linoleic acid as substrate. It was not proportional to the amount of lipoxygenase added (Table 1), which may indicate that the reaction rate is limited by the accessibility of the chloroplast lipids to lipoxygenase. The kinetics show two phases: a fast initial rate followed by a slower but linear rate of *ca* 0.18 μmol/hr per mg protein. This behaviour is atypical for a reaction with polyenoic fatty acids as substrate and indicates the existence of two different substrate pools (e.g. free linolenic acid tightly bound in a different way to proteins). The slow linear phase may also be brought

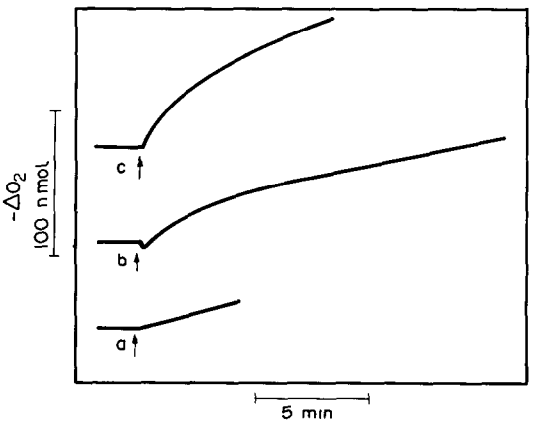

Fig 1. Oxygen uptake caused by the addition of lipoxygenase-1 to wheat chloroplasts. The arrows indicate the addition of 40 μg (a, c) or 400 μg (b) lipoxygenase per mg chloroplast protein in the absence (a, b) or presence of 0.2% sodium cholate (c).

Table 1. Rates of oxygen uptake caused by the action of soybean lipoxygenase-1 on wheat chloroplasts

Amount of lipoxygenase			Oxygen uptake	
nmol/ sample	μg/mg chloroplast protein	Presence of 0.2% cholate	Initial rate (nkat)	After 5–7 min (nkat)
1	40	–	0.076	
2	80	–	0.095	
10	400	–	0.147	0.069
20	800	–	0.208	0.069
1	40	+	0.474	0.093
0	0	+	0.051	
1	0.53 mM linoleic acid	–	10.8	

about by a continuous endogenous enzymatic liberation of free linolenic acid from the chloroplast lipids. This activity produces oxygenation roughly estimated as *ca* 11% of the total linolenic acid content per hr.* The detergent sodium cholate (final concentration 0.2%) markedly increased oxygen consumption, presumably by weakening protein–lipid interactions.

The treatment of wheat chloroplasts with soybean lipoxygenase-1 leads to a marked inhibition of the photochemical activity, as measured by the reduction rate of 2,6-dichlorophenolindophenol. The inhibition is linearly dependent on the concentration of lipoxygenase (Fig. 2) and increases continuously during the 45 min incubation period (Fig. 3). Using partial systems of electron transfer, it was established that the inhibitory site is located in photosystem II. The reduction of 2,6-dichlorophenolindophenol in the presence of the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone involving only photosystem II was strongly inhibited, whereas a stimulation was observed in electron transfer systems involving only photosystem I (Fig. 3). The effects of the lipoxygenase action on the photochemical electron transfer will be described in detail elsewhere [9].

In order to determine whether the effects on the photochemical electron transfer chain in chloroplasts might be due to changes of the membrane lipids, the lipid composition was analysed before and after lipoxygenase treatment (Fig. 4). The lipid composition of untreated wheat chloroplasts proved to be similar to that reported for spinach and pea chloroplasts [2]. A relatively high content of linolenic acid was found [2] which may be mainly responsible for the high degree of fluidity of thylakoid membranes [10]. After lipoxygenase treatment, no significant changes of the total lipid content of the main lipid classes in chloroplasts (mono- and digalactosyl diglycerides, phosphatidylcholine and phosphatidylethanolamine) were observed. In contrast, the total linolenic acid content was lowered significantly by $12.70 \pm 4.9\%$ ($P = 0.05$) whereas the other fatty acids remained largely unchanged. The extent of the selective loss of linolenic acid corresponds fairly closely to the oxygen uptake mentioned above.

*This estimate is based on the following assumptions: a lipid content of chloroplasts of 1 mg/mg protein, a mean M_r of the complex lipids of 800, two fatty acid residues per lipid molecule, 68% linolenic acid.

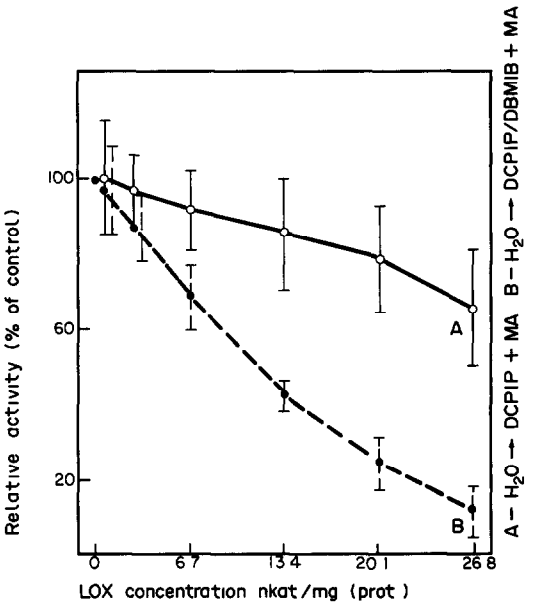

Fig. 2. Relative rates of dichlorophenolindophenol photoreduction by isolated wheat chloroplasts in the presence of different concentrations of soybean lipoxygenase-1. The dye photoreduction includes the action of both photosystem II and photosystem I (A) or only photosystem II (B). Rate of controls: (A) 171.7 ± 24.8, (B) 64.9 ± 6.4 μmol dichlorophenolindophenol–H_2/mg chlorophyll per hr; $n = 6$. Abbreviations: AS, ascorbate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP, dichlorophenolindophenol; DGDG, digalactosyl diglycerides; LA, linoleic acid; LNA, linolenic acid; LOX, lipoxygenase; LRA, lauric acid; MA (Fig. 4), myristic acid; MA, methylamine; MGDG, monogalactosyl diglycerides; MV, methylviologen; OA, oleic acid; PA, palmitic acid; PE, phosphatidyl ethanolamine; PC, phosphatidylcholine; SA, stearic acid.

DISCUSSION

In this work it is clearly shown that soybean lipoxygenase-1 affects isolated chloroplasts from wheat as is evident from (1) oxygen uptake in the dark, (2) inhibition of the photochemical activity at the level of photosystem II, and (3) a slight but significant decrease of the total linolenic acid content. Since this enzyme is scarcely able to attack esterified membrane lipids (experiments not shown) it may be concluded that the effects observed are due to a selective action on free linolenic acid, the percentage of which has been reported to be 3–5% of total linolenate in the chloroplasts of mature leaves [11]. Taking into account that the percentage may still increase during the preincubation period of 45 min at 25°, possibly via an endogenous lipase action, the amount of free linolenic acid appears to be sufficient to cover both the oxygen uptake and the loss of total linolenic acid observed. In as much as the content and composition of the

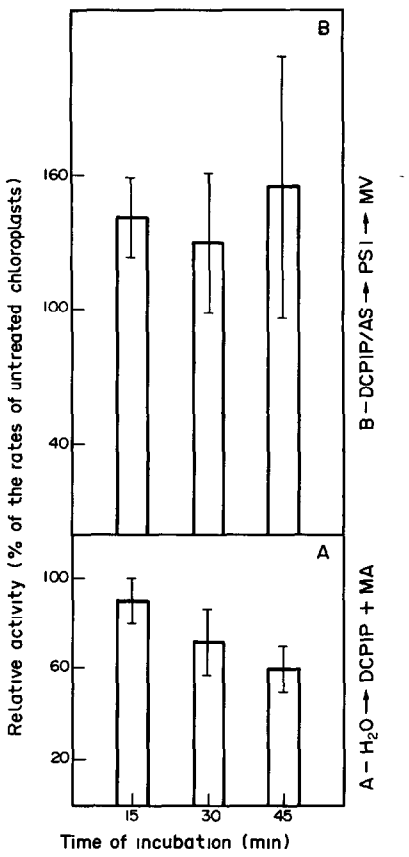

Fig. 3. Relative rates of dichlorophenolindophenol photoreduction via photosystems II and I and of oxygen uptake via photosystem I and methyl viologen after incubation of isolated wheat chloroplasts with soybean lipoxygenase-1 (13.4 nkat/mg protein, $n = 6$). For abbreviations see legend of Fig. 2.

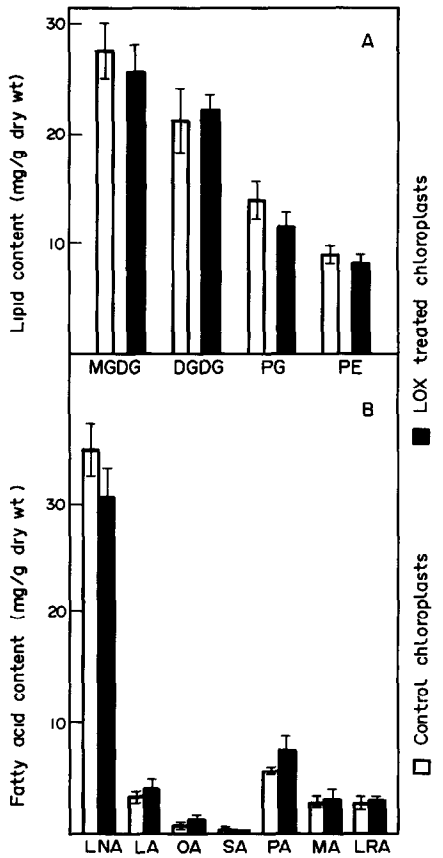

Fig. 4. Lipid- and fatty acid content of control and soybean lipoxygenase-1 (9 nkat/mg chloroplast protein) treated wheat chloroplasts ($n = 6$). For abbreviations see legend of Fig. 2.

membrane lipids remain unchanged, the inhibition of the electron transfer system has to be ascribed to the formation of hydroperoxylinolenic acid as the product of the reaction of the lipoxygenase with free linolenic acid, which may in turn affect components of the electron transfer system. A very similar effect was observed with the NADH oxidase system of beef heart submitochondrial particles; either soybean lipoxygenase-1 or hydroperoxylinolenic acid produced strong inhibition when haemoglobin was present as a catalyst of hydroperoxide-dependent reactions [12]. We failed to detect an additional effect of haemoglobin with chloroplasts (experiments not shown); however, chlorophyll was shown to exert lipohydroperoxidase activity like haemoglobin [13] and it may be involved in a similar manner in the lipoxygenase-mediated inhibition of the photochemical activity of chloroplasts.

Free fatty acids are known to inhibit photochemical electron transport [14]. This inhibition is probably caused by direct interaction with thylakoid membranes. The combined action of lipoxygenase and free linolenic acid may represent another type of damage to the chloroplast function. In the light of reports on the presence of lipoxygenase activity in isolated chloroplasts of peas [15] and wheat [16], this action may be involved in the senescence and breakdown of chloroplasts.

EXPERIMENTAL

Soybean lipoxygenase-1 was prepared according to ref. [17]. Lipoxygenase activity was measured with linoleic acid as substrate in 0.05 M Na borate buffer (pH 9), following the formation of conjugated dienes spectrophotometrically at 234 nm. Chloroplasts were isolated from 12-day-old primary leaves of *Triticum aestivum* L. in 50 mM Tricine (pH 7.5) containing 2.5% Ficoll, 0.25 mM sorbitol, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EDTA and 2 mM NaCl. The filtered homogenate was centrifuged at 9000 *g* for 2 min. The pellet was resuspended in the isolation medium and centrifuged at 9000 *g* for 10 min. These washed and resuspended chloroplasts were used immediately for the experiments. Treatment with soybean lipoxygenase-1 was carried out in the isolation medium at 25° for 45 min in the dark.

The action of the soybean lipoxygenase on wheat chloroplasts was followed by measurement of the O_2 uptake by means of a Clark oxygen electrode in a completely darkened reaction vessel (1.85 ml); the reaction medium contained 0.1 M Sörensen Pi buffer (pH 7.4) and 1.36 mg chloroplast protein. The photochemical activity of the chloroplasts was assayed according to ref. [18] by measuring the reduction rate of 2,6-dichlorophenolindophenol in the presence of 5.5 mM methylamine as uncoupler at 590 nm (Spekol photometer with booster amplifier, VEB Carl-Zeiss-Jena, G.D.R.) before and after a 2 min illumination period

(25°, 600 W/m²); the reference cuvet contained identical samples without illumination.

The chloroplast dry wt, lipid content and fatty acid composition were determined according to ref. [19]; the protein content according to ref. [20] using human serum albumin as the standard

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