Inhibition of *de novo* Fatty Acid Biosynthesis in Isolated Etioplasts by Herbicides

Klaus Kobek and Hartmut K. Lichtenthaler

Botanisches Institut der Universität (Pflanzenphysiologie), Kaiserstraße 12, D-7500 Karlsruhe 1, Bundesrepublik Deutschland

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It is demonstrated that – similar to chloroplasts – etioplasts isolated from leaves of pea (*Pisum sativum* L.) and oat seedlings (*Avena sativa* L.) also possess a high capacity for *de novo* fatty acid biosynthesis starting from [1-^{14}C]acetate. The etioplast system proved to be a suitable test system for inhibitors of *de novo* fatty acid biosynthesis such as cycloxydim, sethoxydim and diclofop. In contrast to the chloroplast test system, the etioplast system is independent of light and also permits screening of photosynthetic herbicides as potential inhibitors of fatty acid biosynthesis.

Introduction

The *de novo* fatty acid biosynthesis of higher plants is bound to the chloroplast (plastid) compartment [1]. Isolated chloroplasts also possess *de novo* fatty acid biosynthesis capacity [2–5]. Isolated intact chloroplasts of monocotyledonous and dicotyledonous plants exhibit a linear incorporation of [^{14}C]acetate into the total fatty acid fraction for about 30 min [2–4]. This *de novo* fatty acid formation is mainly dependent upon light and photosynthesis, which provides the cofactors ATP and NADPH needed for fatty acid synthesis. In the dark the *de novo* fatty acid synthesis of isolated chloroplasts amounts to only *ca.* 10% of that of illuminated chloroplasts [3]. In order to establish a test system for inhibitors of fatty acid biosynthesis which is based on isolated plastids and is highly active and independent of light, we studied the capacity of etioplasts isolated from oat and pea seedlings to synthesize fatty acids from [^{14}C]acetate. The results with these etioplast systems are described here and contrasted with those of chloroplasts isolated from the same plants.

Abbreviations: Bentazone, 3-isopropyl-1H-2,1,3-benzodiathiazin-4(3H)-one 2,2-dioxide; BSA, bovine serum albumin; cycloxydim, 2-(1-ethoxyimino)butyl-5-(thian-3-yl)-2-cyclohexane-1,3-dione; diclofop, 2-(4-(2,4-dichlorophenoxy-)phenoxy-)propionic acid; DTE, Dithioerythritol; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; sethoxydim, 2-(1-ethoxyimino) butyl-5-(2-ethylthiopropyl)-3-hydroxy-2-cyclohexen-1-one.

Reprint requests to Prof. Dr. H. K. Lichtenthaler.

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Materials and Methods

Oat (*Avena sativa* L. var. Flämingnova) and pea (*Pisum sativum* var. Lisa) seedlings were cultivated on peat (TKS II) in the light (light intensity: 1500 μmol · m^{-2} · s^{-1}; light source: Osram HQI-E 400 W; 5 cm water filter) or in the dark. Chloroplasts were isolated from primary leaves of 7 d old oat and 14 d old pea seedlings and etioplasts from primary leaves of yellowish 6 d old oat and 8 d old pea seedlings. The isolation medium contained 330 mM sorbitol, 50 mM phosphate buffer (pH 8.0), 2 mM $MgCl_2$ and 0.2% BSA. Homogenization of the leaf material (*ca.* 40 g in 150 ml buffer, 0 °C) was carried out in a self-constructed mixer with replaceable razor blades [6] within 10 s. After filtering through 10 layers of cheesecloth and centrifugation at 2000 × *g* (chloroplasts) or 4000 × *g* (etioplasts) for 90 s at 4 °C, the supernatant was decanted, the pellet resuspended in chilled isolation medium without BSA and filtered through 2 layers of miracloth. These plastid suspensions were used for the incorporation studies.

The incubation with [1-^{14}C]acetate was carried out at 20 °C in 500 μl plastid suspensions in the dark (in the case of etioplasts) or in light (1400 μmol · m^{-2} · s^{-1}; in the case of chloroplasts). The reaction mixture contained 300 mM sorbitol, 50 mM N-tris (hydroxymethyl)methylglycine (Tricine), 50 mM potassium phosphate (pH 7.9 for chloroplasts, pH 8.1 for etioplasts) and cofactors such as DTE, ATP, CoA and $MgCl_2$. Applied were 37 kBq [1-^{14}C]acetate (35 μM) of which 2 to 3% were incorporated into fatty acids in the case of oat and pea etioplasts and in the case of chloroplasts 3 to 4% (oat) or 6 to 7% (pea). The plastid concentration in the 0.5 ml sus-

pension amounted to *ca.* 0.5 and 1.5 μg carotinoids (pea and oat etioplasts) and *ca.* 15 to 20 μg carotinoids (oat and pea chloroplasts). The reaction was started by addition of the freshly isolated plastids to the incubation medium already containing the labelled acetate and the herbicides. The latter were added in methanolic solution, the final methanol concentration in all suspensions including controls was 0.2% (w/v). After an incubation time of 20 min, the reaction was stopped by adding 500 μl 30% (w/v) KOH and the acyllipids were hydrolyzed at 70–80 °C for 90 min. After acidification the fatty acids and remaining non-acyl lipids were extracted twice with 5 ml of light petrol (b.p. 50–70 °C) [4, 7]. After evaporation of the light petrol, the radioactivity was determined in a liquid-scintillation counter (Packard Tri-carb 2000 CA, quench correction by external standard). Separation of the saponified lipid extracts by TLC revealed that more than 97% of the [1-^{14}C]acetate taken up into the lipid fraction was incorporated into the total fatty acids [3, 4]. The chlorophyll and carotinoid content of the isolated plastids was determined using the new extinction coefficients and equations of Lichtenthaler [8].

Results

Etioplasts, isolated from dark-grown oat and pea seedlings, incorporated [1-^{14}C]acetate into the total fatty acid fraction at very high rates. On a carotinoid basis the incorporation rates of etioplasts for a 20 min dark-incubation period were higher than those of isolated oat and pea chloroplasts where the incorporation of acetate is a light-dependent process (Table I). The acetate incorporation was 750 nmol per mg carotinoids and hour in oat and 540 nmol per mg carotinoids and hour in pea etioplasts as compared to 140 and 120 nmol per mg carotinoids and hour in oat and pea chloroplasts.

The cyclohexane-1,3-dione-type herbicide cycloxydim inhibited the *de novo* fatty acid synthesis from [1-^{14}C]acetate in chloroplasts and etioplasts of the herbicide-sensitive oat seedlings in a dose-dependent manner (Table I). In contrast, in chloroplasts and etioplasts of the herbicide-tolerant pea seedlings no inhibition by cycloxydim of *de novo* fatty acid biosynthesis was detected (Table I). Similar inhibition results were obtained in oat etioplasts with sethoxydim, another cyclohexane-1,3-dione derivative [2], as well as with diclofop (Table II), a phenoxyphenoxy-propionic acid type herbicide [4]. Pea etioplasts were tolerant towards sethoxydim. A slight inhibition of fatty acid biosynthesis by diclofop was detected only at a very high concentration of 10^{-4} M.

Table I. Influence of cycloxydim on the incorporation of [^{14}C]acetate into the fatty acid fraction of chloroplasts and etioplasts isolated from primary leaves of sensitive oat plants and tolerant pea seedlings. The incorporation rate is expressed in the case of chloroplasts as kBq per mg chlorophyll $a+b$ and also per mg carotenoids $(x+c)$ and in the case of etioplasts per mg carotenoids $(x+c)$. Incorporation time 20 min. Mean of 4 determinations with standard deviation (SD). The chloroplast pigment ratio (chlorophylls to carotenoids) was found to be 4.9 (± 0.3) for chloroplasts of both plants.

		$\frac{\text{kBq}}{x+c}$	SD	$\frac{\text{kBq}}{a+b}$	% inhibition
oat chloroplasts					
control		96	4	20	–
+ cycloxydim	10^{-7} M	58	11	12	40
+ cycloxydim	10^{-6} M	17	7	3	82
+ cycloxydim	10^{-5} M	3	1	0	97
oat etioplasts					
control		516	12	–	–
+ cycloxydim	10^{-7} M	351	19	–	32
+ cycloxydim	10^{-6} M	92	3	–	82
+ cycloxydim	10^{-5} M	16	1	–	97
pea chloroplasts					
control		85	8	17	–
+ cycloxydim	10^{-6} M	83	7	17	2
+ cycloxydim	10^{-5} M	86	4	18	0
+ cycloxydim	10^{-4} M	88	8	18	0
pea etioplasts					
control		378	37	–	–
+ cycloxydim	10^{-6} M	375	36	–	1
+ cycloxydim	10^{-5} M	365	23	–	3
+ cycloxydim	10^{-4} M	391	39	–	0

The herbicides bentazone and diuron block the photosynthetic electron transport [9–12]. In the isolated oat chloroplast test system, which is light-dependent, the *de novo* fatty acid biosynthesis from [^{14}C]acetate was affected by both herbicides. Bentazone inhibited fatty acid formation to about 70% in a concentration of 1 mM, and 100 μM diuron blocked fatty acid synthesis to about 90% (Fig. 1). This inhibition is due to the fact that the chloroplast test system is dependent on the photosynthetic regeneration of ATP and NADPH needed in fatty acid biosynthesis. The remaining incorporation rate of *ca.* 10% in presence of diuron and light correspond to that of oat chloroplasts which were incubated with [^{14}C]acetate

Table II. Influence of the herbicides diclofop and sethoxydim on the incorporation of [^{14}C]acetate into the fatty acid fraction of etioplasts isolated from sensitive oat leaves (primary leaves) and tolerant pea seedlings (primary leaves). The incorporation rate is expressed as kBq per mg carotenoids $(x+c)$. Incubation time 20 min. Mean of 6 (diclofop) and 4 (sethoxydim) determinations from 2 etioplast isolations.

		$\frac{\text{kBq}}{x+c}$	SD	% inhibition
oat etioplasts				
control		516	12	–
+ diclofop	10^{-7} M	446	20	14
+ diclofop	10^{-6} M	143	3	73
+ diclofop	10^{-5} M	17	1	97
+ sethoxydim	10^{-7} M	380	23	26
+ sethoxydim	10^{-5} M	27	2	95
pea etioplasts				
control		372	38	–
+ diclofop	10^{-6} M	365	14	2
+ diclofop	10^{-5} M	358	40	3
+ diclofop	10^{-4} M	280	12	25
+ sethoxydim	10^{-4} M	361	15	3

in darkness. In contrast to the chloroplast test system, bentazone and diuron had no effect on fatty acid biosynthesis in the oat etioplast test system. A slight decrease by diuron of *de novo* fatty acid biosynthesis in the oat etioplast test system was only detected at the rather high concentration of 1 mM. The I_{50}-values for 50% inhibition of *de novo* fatty acid biosynthesis obtained in the oat etioplast test system are about the same or only slightly lower than those found in the chloroplast test system described before [3, 4]. They are about 0.2 μM for cycloxydim and diclofop and *ca.* 0.5 μM for sethoxydim.

Discussion

Isolated intact chloroplasts had been established before as a suitable test system for inhibitors of *de novo* fatty acid biosynthesis starting from [^{14}C]acetate [2–5]. By measuring the incorporation of [^{14}C]acetate into the fatty acid fraction of isolated chloroplasts, a good estimate of the effectiveness of inhibitors *e.g.* those of the acetyl-CoA carboxylase such as cycloxydim, sethoxydim, clethodim and the diphenoxypropionic acid type compounds diclofop, fenoxaprop or fluazifop is possible [2–5, 13]. However, there is one disadvantage when using isolated oat chloroplasts as a test system. Since the cofactors ATP and NADPH necessary for *de novo* fatty acid biosynthesis are regenerated by the photosynthetic light reactions, the chloroplast test system is highly light-dependent. Inhibitors of photosynthetic electron transport such as diuron and bentazone therefore decrease *de novo* fatty acid biosynthesis in an indirect manner by blocking the regeneration of ATP and NADPH from ADP and NADP (Fig. 1).

In contrast, the *de novo* fatty acid synthesis of the isolated etioplasts is independent of light and the photosynthetic electron transport. In this etioplast test system one can therefore also screen inhibitors of photosynthetic electron transport to see whether or not they also affect *de novo* fatty acid biosynthesis. This is not the case with the herbicides bentazone and diuron, as is shown here (Fig. 1). By us-

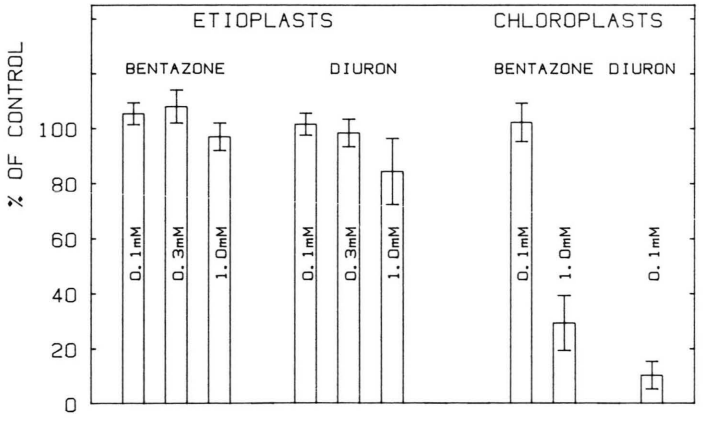

Fig. 1. Effect of different concentrations of the photosynthesis herbicides bentazone and diuron on the *de novo* fatty acid synthesis of etioplasts and chloroplasts, isolated from 6 d old oat seedlings during a 20 min incubation. The latter was carried out in the dark in the case of etioplasts and in the light in the case of chloroplasts. Mean of 4 determinations from 2 plastid isolations.

ing isolated etioplasts as test system for herbicides, an indirect inhibition of *de novo* fatty acid biosynthesis *via* inhibition of photosynthetic electron transport can be excluded.

The effectiveness of the acetyl-CoA carboxylase inhibitors cycloxydim, sethoxydim and diclofop in the oat etioplast test system was comparable to that in the chloroplast test system [2–4]. The I_{50}-values for fatty acid biosynthesis inhibition were nearly the same as in the chloroplast test system. In addition, the tolerance of dicot plants and chloroplasts against cyclohexane-1,3-diones and phenoxyphenoxy-propionic acids [2–5] was also found on the etioplast level. The fatty acid biosynthesis of pea etioplasts was affected neither by cycloxydim or sethoxydim nor by diclofop. Only 100 μM diclofop caused a slight inhibition of fatty acid formation in pea etioplasts. The fatty acid biosynthesis in etioplasts from the sensitive oat plants, however, is 100 times more sensitive than that of etioplasts from the rather tolerant pea plants. Our results show that isolated etioplasts are a very suitable test system for inhibitors of fatty acid biosynthesis which, as compared to isolated chloroplasts, has the great advantage that it is independent of light and the photosynthetic electron transport. In this test system it can be studied whether photosynthetic herbicides also affect fatty acid biosynthesis.

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[1] J. B. Ohlrogge, D. N. Kuhn, and P. K. Stumpf, Proc. Natl. Acad. Sci. U.S.A. **76,** 1194–1198 (1979).
[2] H. K. Lichtenthaler, K. Kobek, and K. Ishii, Z. Naturforsch. **42c,** 1275–1279 (1987).
[3] K. Kobek, M. Focke, H. K. Lichtenthaler, G. Retzlaff, and B. Würzer, Physiol. Plant. **72,** 492–498 (1988).
[4] K. Kobek, M. Focke, and H. K. Lichtenthaler, Z. Naturforsch. **43c,** 47–54 (1988).
[5] H. H. Hoppe and H. Zacher, Pestic. Biochem. Physiol. **24,** 298–305 (1985).
[6] C. G. Kannangara, S. P. Gough, B. Hansen, J. N. Rasmussen, and D. J. Simpson, Carlsberg. Res. Commun. **42,** 431–439 (1977).
[7] R. G. Roughan, R. Holland, and C. R. Slack, Biochem. J. **184,** 193–202 (1979).
[8] H. K. Lichtenthaler, in: Methods in Enzymologie (R. Douce and L. Packer, eds.), **Vol. 148,** pp. 350–382, Academic Press Inc., New York 1987.
[9] K. Pfister, C. Buschmann, and H. K. Lichtenthaler, Proceed. 3rd Internat. Congr. Photosynth. (M. Avron, ed.), **Vol. I,** 675–681, Elsevier, Amsterdam 1974.
[10] G. Retzlaff, J. L. Hilton, and G. B. St. John, Z. Naturforsch. **34c,** 994–997 (1979).
[11] J. S. C. Wessels and R. van der Veen, Biochim. Biophys. Acta **19,** 548–549 (1956).
[12] K. Pfister and C. J. Arntzen, Z. Naturforsch. **34c,** 996–1009 (1979).
[13] M. Focke and H. K. Lichtenthaler, Z. Naturforsch. **42c,** 1361–1363 (1987).