Formation of Trierucoylglycerol (Trierucin) from 1,2-Dierucoylglycerol by a Homogenate of Microspore-Derived Embryos of *Brassica napus* L.[1]

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Homogenates of microspore-derived embryos of rape (*Brassica napus* L.) incubated with [1-^{14}C]erucoyl-CoA and 1,2-dierucoylglycerol are able to assemble trierucoylglycerol (trierucin). In addition, radioactive triacylglycerols are formed by transferring [1-^{14}C]-erucoyl moieties to endogenous lipid precursors. Stereospecific analysis of radioactive triacylglycerols revealed that labeled erucoyl moieties had been incorporated exclusively into the *sn*-1,3 positions with more than 95% of radioactivity in the *sn*-3 position. No incorporation of labeled erucic acid into the *sn*-2 position has been observed. All data agree with the involvement of 1,2-diacylglycerol acyltransferase (E.C. 2.3.1.20), which utilized 1,2-dierucoylglycerol as well as endogenous 1,2-diacylglycerols as acceptors of erucoyl moieties. This result is of particular interest for the genetic modification of rape and other Cruciferae for producing trierucin in their seed oils.

KEY WORDS: *Brassica napus*, 1,2-diacylglycerol acyltransferase, microspore-derived embryos, triacylglycerol biosynthesis, trierucoylglycerol (trierucin).

The modification of the acyl composition of triacylglycerols in oilseed crops is an important commercial objective. Economically valuable fats and oils, suitable for the oleochemical industry require a fairly uniform composition of specific acyl moieties. High levels of specific fatty acids in seed oils provide a great advantage for oleochemical processing by lowering production costs. In the northern hemisphere, there is considerable interest in developing genetically manipulated rapeseed (*Brassica napus*) as an improved oilseed crop for industrial use. Such biotechnological research on rapeseed is directed toward the production of trierucoylglycerol (trierucin) in the seed oil, hydrolysis of which yields erucic acid [(*Z*)-13-docosenoic acid], a valuable starting material for the production of plasticizers and lubricants (1,2).

Very long-chain fatty acids, such as erucic acid, are virtually excluded from the *sn*-2 position of triacylglycerols in various Cruciferae (3,4), suggesting a theoretical limit of 66% (*sn*-1 + 3) erucic acid in the seed oils of this plant family. Attempts have been made to introduce by conventional breeding erucoyl moieties into the *sn*-2 position of triacylglycerol species in high erucic acid rapeseed. These efforts were unsuccessful (5-8). Plant genetic engineering may make it possible to transfer isolated genes, *e.g.* from *Tropaeolum majus*, a seed known to contain substantial proportions of trierucin (3). This could lead to the oleochemically valuable trierucin.

Recently, we have shown that microspore-derived embryos of *B. napus* are sources of highly active enzymes of lipid biosynthesis (9) and are superior to zygotic embryos of this plant (10,11) in this respect. The similar acyl composition of storage lipids in both microspore-derived embryos and zygotic embryos of *B. napus* suggests that the former may be excellent model systems to facilitate studies on the formation of triacylglycerols and the regulation of lipid biosynthesis in oilseeds (12).

EXPERIMENTAL PROCEDURES

Reagents. [1-^{14}C]Erucic acid (1.92 GBq/mmol) was purchased from NEN Research Products (Mississauga, Ontario, Canada) and converted to [1-^{14}C]erucoyl-CoA by an enzymatic method described previously (13). Dierucoylglycerol, enriched in the *sn*-1,2 isomer (NuChek Prep, Elysian, MN), was purified and separated from small proportions of the *sn*-1,3 isomer by thin-layer chromatography (TLC) on Silica Gel 60 G (E. Merck, Darmstadt, Germany), impregnated with 10% (w/w) boric acid, by using hexane-diethyl ether (1:1, v/v) as the developing solvent (14). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade solvents were used throughout these experiments.

Culture conditions. Microspores of *Brassica napus* L. cv Reston (a high erucic acid variety) were isolated from anthers and cultured as described previously (9). Embryos at the mid-late cotyledonary stage, which had been cultured for about four weeks, were readily obtained by filtration of the cultures through sterilized nylon mesh with a pore size of 500 μm. Harvested embryos were rinsed with distilled water, and homogenized at 4°C by gentle grinding with a mortar and pestle in the presence of a small amount of acid-washed silica sand and 100 mM HEPES-KOH buffer, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA and 1 mM DTT. A ratio of 4 to 5 mL grinding medium/g embryo fresh weight was used. The homogenate was passed through two layers of Miracloth (Calbiochem, La Jolla, CA), and the filtered cell-free extract was utilized directly for *in vitro* studies of lipid biosynthesis.

Assay conditions. The incorporation of erucoyl moieties into acyl lipids was studied in a standard reaction mixture containing 80 mM HEPES-KOH buffer, pH 7.4, 1 mM ATP, 0.3 mM CoASH, 2 mM $MgCl_2$, 200 μM 1,2-dierucoylglycerol (emulsified in the HEPES-KOH buffer containing 0.2%, w/v, Tween-20), 40 μM [1-^{14}C]erucoyl-CoA (370 MBq/mmol) and 0.2 to 0.4 mg protein in a final volume of 0.5 mL. Reactions were stopped by the addition of 2 mL dichloromethane-methanol (1:2, v/v). The organic and aqueous phases were then separated by adding 2 mL dichloromethane followed by 2 mL 1 M KCl in 0.2 M H_3PO_4. The organic phase was removed, and the aqueous phase was washed twice with 2 mL dichloromethane; the organic phases were combined, and the solvents

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were removed under a stream of nitrogen to yield the total lipid fractions.

Lipid analysis. The radioactive lipids were separated by TLC on 0.5 mm layers of Silica Gel H. Plates were first developed 4 cm with diethyl ether, than fully with hexane-diethyl ether-acetic acid (70:30:1, v/v). The lipid standards, *i.e.*, monoacylglycerols (R_f 0.2), a mixture of diacylglycerols (R_f 0.4), fatty acids (R_f 0.6), and triacylglycerols (R_f 0.8), were resolved and located with light iodine staining. Radioactive lipids were located with a Berthold LB 285 TLC Analyzer (Berthold, Wildbad, Germany). Neutral lipids were scraped from the plates and eluted from the silica gel with water-saturated diethyl ether. The polar lipid fraction was recovered from the origin by elution with dichloromethane-methanol-water (1:2:0.8, v/v/v) (15) followed by phase separation as described above. Aliquots of the separated radioactive lipid fractions were quantitated by counting on an LKB 1219 Rack-Beta instrument (LKB-Wallac Oy, Turku, Finland) in either 2.5 mL of toluene containing 0.4% (w/v) PPO (NEN) for the neutral lipids, or in 2.5 mL Aquasol 2 (NEN) for the polar lipids.

Radioactive triacylglycerols were analyzed by high performance liquid chromatography (HPLC) in an SP 8700 liquid chromatograph (Spectra Physics, San José, CA) equipped with an ACS Model 750/14 Evaporative Analyzer (Applied Chromatography Systems, Macclesfield, United Kingdom) and a Flo-One/Beta radioactive flow detector with data processing program (Radiomatic Instruments and Chemical Co., Tampa, FL). The HPLC columns consisted of two Whatman PartiSphere C_{18} 5 μm reversed-phase cartridges (4.6 mm × 12.5 cm, Whatman, Clifton, NJ) in series. The triacylglycerols were resolved by means of the following gradient: 50% acetone (A)/50% acetonitrile (B) at time 0, to 60% A/40% B at 4 min, to 65% A/35% B at 20 min, to 100% A at 30 min (5 min isocratic period). The column eluate was split, with 10–20% going to the evaporative analyzer and the remainder going to the radioactive flow detector. Radioactive triacylglycerols were identified by co-chromatography with external standards detected with the evaporative analyzer and were confirmed by direct probe mass spectrometry of HPLC fractions.

Radioactive triacylglycerols, synthesized during *in vitro* incubations with a homogenate of microspore-derived embryos of *B. napus*, were analyzed by direct-probe mass spectrometry in a VG 70-250 SEQ hybrid mass spectrometer (VG Analytical, Wythenshawe, United Kingdom) in the electron impact (EI) mode at an electron energy of 70 eV (source temperature 250°C). Spectra were acquired by scanning from *m/z* 1300 to *m/z* 100 every 10 s with a 0.5-s settling time.

Positional analyses were performed on labeled triacylglycerols by treatment with pancreatic lipase and by a modified Brockerhoff stereospecific analysis (14).

RESULTS AND DISCUSSION

In seeds and fruits of oil plants, triacylglycerols are synthesized *de novo* via the Kennedy pathway (16,17). Thus, it is evident that the specific acyltransferases involved in triacylglycerol assembly play an important role in biotechnological studies aimed at modifying the acyl composition of seed oils.

Using particulate fractions of developing seeds of *B. napus*, Bernerth and Frentzen (18) recently showed that erucoyl-CoA was esterified to some extent into *sn*-1,3 positions of triacylglycerols. The erucoyl moieties were strongly excluded, however, from the *sn*-2 position, demonstrating the high specificity of lysophosphatidic acid acyltransferase for other than very long-chain acyl moieties. This enzyme seems to be mainly responsible for the biosynthesis of 1,3-dierucoyl-2-acylglycerols in seeds of *B. napus* and, most probably, other cruciferous plants.

In plants and animals, the final step of triacylglycerol assembly is catalyzed by 1,2-diacylglycerol:acyl-CoA acyltransferase (DGAT). In the Brassicaceae, it is not known whether or not this enzyme will accept 1,2-dierucoylglycerol as a substrate for the biosynthesis of trierucoylglycerol. This question is, however, of major importance for genetic engineering of cruciferous oilseed plants. For example, a genetically modified rapeseed variety forming 1,2-dierucoylglycerol as an intermediate substrate would not necessarily produce trierucoylglycerol if its DGAT could not accept such a substrate. Therefore, our initial studies (9,19) of the biosynthetic activity of DGAT in homogenates of microspore-derived embryos of *B. napus* were further extended in this investigation.

Incubations of homogenates were carried out with [1-^{14}C]erucoyl-CoA as substrate and with 1,2-dierucoylglycerol as an acyl acceptor or without an exogenous acyl acceptor. It is evident from the incorporation of radioactive erucoyl moieties that the basal rate (no acceptor) of biosynthesis of labeled triacylglycerols increased by more than five-fold when 1,2-dierucoylglycerol was included as an acceptor (Fig. 1). The rates of incorporation of erucoyl moieties into triacylglycerols were high, and they exceeded by far those observed in homogenates of various zygotic oilseeds, such as *B. napus* (10) and *Crambe abyssinica* (11).

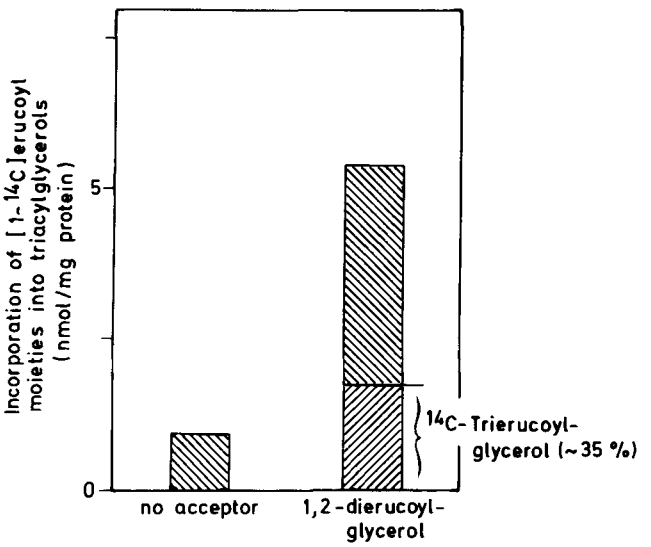

FIG. 1. Incorporation of radioactive erucoyl moieties into triacylglycerols by incubating a homogenate of cotyledonary stage microspore-derived embryos of *B. napus* L. cv Reston with [1-^{14}C]-erucoyl-CoA as substrate and 1,2-dierucoylglycerol as acyl acceptor or without an exogenous acyl acceptor. Values are the average of three determinations.

However, in the present study, labeled erucoyl moieties were not found to be incorporated in appreciable amounts into other intermediates of the Kennedy pathway, *e.g.*, phosphatidic acids, or into membrane lipids, *e.g.*, phosphatidylcholines, which is consistent with the findings of others (10,11).

HPLC analyses of the labeled triacylglycerols formed in the presence of 1,2-dierucoylglycerol as acceptor (Fig. 2) indicated that, in addition to various other labeled triacylglycerol species, the "non-indigenous" trierucoylglycerol was produced comprising about 25 to 35% of the labeled triacylglycerols (*cf.* Figs. 1 and 2). The specific activity of the biosynthesized ^{14}C-trierucoylglycerol was quite high, and thus a very low signal was detected on the mass trace from the evaporative analyzer (Fig. 2B). However, the radiolabeled trierucoylglycerol (Fig. 2C) exhibited a retention time identical to an authentic trierucoylglycerol external standard (27.2 min) as detected by the evaporative analyzer (Fig. 2A). From Figure 2 (and the results of the stereospecific analyses discussed below) it is evident that various endogenous 1,2-diacylglycerols were also used as acyl acceptors to form labeled triacylglycerols containing ^{14}C-erucoyl moieties.

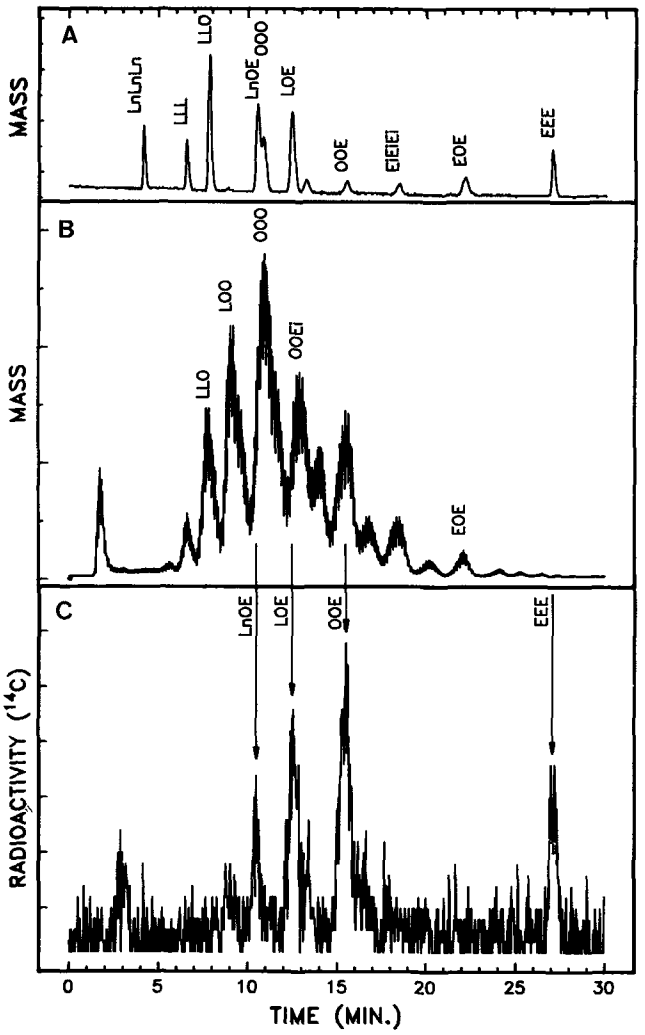

FIG. 2. Reversed-phase HPLC analysis of labeled triacylglycerols formed by a homogenate of cotyledonary stage microspore-derived embryos of *B. napus* L. cv Reston, upon incubation with [1-^{14}C]-erucoyl-CoA and 1,2-dierucoylglycerol. The radioactive triacylglycerol sample was split, with 10% going to an evaporative analyzer (panel B) and the remaining 90% going to the radioactive flow detector (panel C). Triacylglycerol species were identified by co-chromatography with external standards (panel A) and were confirmed by direct probe mass spectrometry of HPLC fractions. Abbreviations: EEE, LOL, etc. = triacylglycerol species composed of E = erucoyl, Ei = eicosenoyl, L = linoleoyl, Ln = linolenoyl, and O = oleoyl moieties.

The total radioactive triacylglycerol fraction was analyzed by direct-probe electron-impact mass spectrometry (EI-MS). Figure 3 shows the partial molecular ion region of a direct-probe EI mass spectrum of triacylglycerols formed upon incubation of homogenates of cotyledonary stage microspore-derived embryos of *B. napus* with [1-^{14}C]erucoyl-CoA and 1,2-dierucoylglycerol. In this part of the normalized spectrum, the most abundant ion is at *m/z* 1053, the molecular ion [M$^+$] for trierucoylglycerol. This mass peak was not detected, however, in EI-MS studies of endogenous triacylglycerols (data not shown). In addition, in Figure 3 there is a molecular ion observed at *m/z* 1025 for 1,3-dierucoyl-2-eicosenoylglycerol, a species found endogenously in these embryos (12). Both triacylglycerol species (Fig. 3) exhibit characteristic [M-18]$^+$ fragment ions.

Stereospecific analyses (14) of labeled triacylglycerols formed in the incubations with [1-^{14}C]erucoyl-CoA and 1,2-dierucoylglycerol demonstrated that more than 95% of the labelled erucoyl moieties had been incorporated into the *sn*-3 position. In addition, enzymatic hydrolysis of radioactive triacylglycerols with pancreatic lipase confirmed that [1-^{14}C]erucoyl moieties were esterified exclusively at the *sn*-1,3 positions. These results strongly suggest that erucoyl moieties were incorporated only *via* the 1,2-diacylglycerol acyltransferase reaction.

Currently, there is great interest in genetic modification of rapeseed to produce seed oils very high in erucic acid,

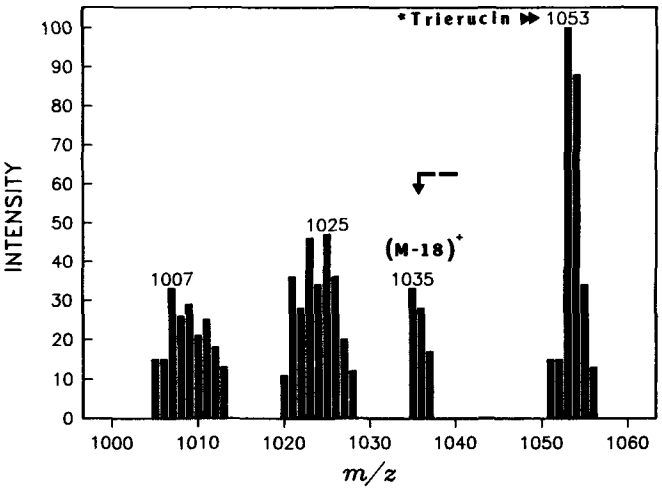

FIG. 3. Partial molecular ion region of a direct-probe electron-impact mass spectrum (EI-MS) of triacylglycerols formed by a homogenate of cotyledonary stage microspore-derived embryos of *B. napus* L. cv Reston, upon incubation with [1-^{14}C]erucoyl-CoA and 1,2-dierucoylglycerol.

by introducing a foreign lysophosphatidic acid acyltransferase gene, *e.g.*, from *Tropaeolum majus* or *Limnanthes* spp. (3,4,20), which would code for an enzyme capable of inserting erucoyl moieties into the *sn*-2 position of triacylglycerols of cruciferous oilseeds. However, the final success of such experiments would depend on whether, for example, 1,2-diacylglycerol acyltransferase and phosphatidic acid phosphatase of *B. napus* can accept 1,2-dierucoylglycerol and 1,2-dierucoylphosphatidic acid, respectively, as substrates. The present studies, where trierucoylglycerol is formed from erucoyl-CoA and 1,2-dierucoylglycerol in homogenates of microspore-derived embryos of *B. napus* L. cv Reston, suggest that 1,2-diacylglycerol acyltransferase of *B. napus* is quite capable of catalyzing such a conversion. Furthermore, the diacylglycerol acyltransferase from microspore-derived Reston embryos may have a stronger affinity for erucoyl over oleoyl moieties. Recent specificity studies have shown that in the presence of 1,2-dioleoylglycerol as an acyl acceptor, and either ^{14}C-oleoyl-CoA or ^{14}C-erucoyl-CoA as acyl donor, the radiolabeled fatty acids were incorporated into triacylglycerols at about equal rates, up to acyl-CoA concentrations of 5 μM *in vitro*. However, at concentrations above 5 μM, ^{14}C-erucoyl moieties were incorporated into triacylglycerols at significantly higher rates (21). Studies on the substrate specificity of the phosphatidic acid phosphatidase in *B. napus* are currently being conducted in this laboratory.

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