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Developmental Profile of Diacylglycerol Acyltransferase in Maturing Seeds of Oilseed Rape and Safflower and Microspore-Derived Cultures of Oilseed Rape[1]

Randall J. Weselake*, M. Keith Pomeroy, Tara L. Furukawa, Joanne L. Golden, Dawn B. Little, and Andre Laroche

Department of Chemistry, University of Lethbridge, Lethbridge, Alberta, Canada, T1K 3M4 (R.J.W., T.L.F., J.L.G., D.B.L.); Plant Research Centre, Agriculture Canada, Ottawa, Ontario, K1A 0C6 (M.K.P.); and Lethbridge Research Station, Agriculture Canada, Lethbridge, Alberta, Canada, T1J 4B1 (A.L.)

Diacylglycerol acyltransferase (EC 2.3.1.20) activity was assayed during the maturation of seeds of oilseed rape (*Brassica napus* L.) and safflower (*Carthamus tinctorius* L.). Developmental studies were also conducted with microspore-derived embryos of oilseed rape (*B. napus* L. cv Topas) and an embryogenic microspore-derived cell-suspension culture of winter oilseed rape (*B. napus* L. cv Jet Neuf). In the maturing seeds, diacylglycerol acyltransferase activity increased to a maximum during rapid accumulation of lipid and declined, thereafter, with seed maturity. In microspore-derived embryos of oilseed rape (cv Topas), high levels of diacylglycerol acyltransferase activity were found throughout the early torpedo to late cotyledonary developmental stages with maximum enzyme specific activity associated with the mid-cotyledonary developmental stage. The cell-suspension culture of winter oilseed rape (cv Jet Neuf) contained 3 to 4% triacylglycerol on a dry weight basis and represented about half of the total lipid. The fatty acid profile of total lipid and triacylglycerol in the cell-suspension culture was similar in samples taken during a 1-year period. The Jet Neuf culture contained diacylglycerol acyltransferase with specific activity similar to that of Topas microspore-derived embryos. Jet Neuf diacylglycerol acyltransferase also displayed an enhanced specificity for erucoyl-CoA over oleoyl-CoA when assayed with 14 μM acyl-coenzyme A in the reaction mixture. The specific activity of diacylglycerol acyltransferase in homogenates prepared from the Jet Neuf culture ranged from 5 to 15 pmol of triacylglycerol min^{-1} mg^{-1} of protein when assayed at intervals during a period of 1 year. Thus, the cell-suspension culture may represent an attractive tissue source for purification and characterization of triacylglycerol biosynthetic enzymes.

The determination of the quantity and composition of storage lipid during seed maturation in various oilseed crops has been reported in numerous studies such as those of Sims et al. (1961), Canvin (1963), Fowler and Downey (1970), Rubel et al. (1972), Norton and Harris (1975, 1983), and Luthra et al. (1991). The developmental profiles during seed maturation of enzymes participating in the pathway from glycerol-3-P to TG have been examined to a lesser extent. DGAT (EC 2.3.1.20) catalyzes the acylation of *sn*-1,2-diacylglycerol to form TG and is the only enzyme in the glycerol-3-P pathway that is exclusively committed to TG biosynthesis (Kennedy, 1961; Stymne and Stobart, 1987). Furthermore, DGAT may represent a rate-limiting step in TG formation (Ichihara et al., 1988). Studies of DGAT activity have been conducted mainly with the use of particulate fractions generated by differential centrifugation of seed homogenates. In each case the studies have utilized seeds harvested throughout a limited range of seed development (Ichihara and Noda, 1981b, 1982; Cao and Huang, 1986, 1987; Bernerth and Frentzen, 1990). Similar studies have also been conducted with germinating oilseeds (Kwanyuen and Wilson, 1986), which have been shown to synthesize TG (Ichihara and Noda, 1981a; Wilson and Kwanyuen, 1986).

Recently, MD embryos of oilseed rape have been shown to be useful in biochemical and molecular genetic studies of seed development (Taylor et al., 1990). MD embryos accumulate TG in a fashion similar to developing seeds (Pomeroy et al., 1991) and have been used to study TG bioassembly (Taylor et al., 1990, 1991; Wiberg et al., 1991; Taylor et al., 1992), fatty acid elongation (Holbrook et al., 1992), and the enzymology of glycolysis (Sangwan et al., 1992). A nondifferentiating embryogenic cell-suspension culture of winter oilseed rape (*Brassica napus* L. cv Jet Neuf), also derived from microspores, has been used in experiments involving ABA-inducible freezing tolerance (Orr et al., 1986; Johnson-Flanagan et al., 1991).

The properties of DGAT in homogenates and particulate fractions of MD embryos of oilseed rape (*B. napus* L. cvs Reston and Topas) have been examined (Taylor et al., 1991;

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* Corresponding author; fax 1-403-329-2057.

Abbreviations: ACP, acyl carrier protein; DGAT, diacylglycerol acyltransferase; FAMES, fatty acid methyl esters; HIP, hexane/isopropanol; MD, microspore-derived; TG, triacylglycerol; TL, total lipid; 18:1, oleic acid; 20:1, *cis*-9-eicosenoic acid; 22:1, erucic acid; 16:3, *cis*-7,10,13-hexadecatrienoic acid; 18:3, linolenic acid; 18:2, linoleic acid; 16:0, palmitic acid.

Weselake et al., 1991, 1992). In the current study, we have assayed DGAT activity in maturing seeds of oilseeds and MD cultures of oilseed rape at various stages of development to enhance our understanding of the regulation of TG formation.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Erucic acid (58 Ci mol^{-1}) was from NEN-Dupont (Mississauga, Ontario, Canada), and [1-^{14}C]oleic acid (54 Ci mol^{-1}) was from Amersham Canada Ltd. (Oakville, Ontario, Canada). [1-^{14}C]Acyl-CoAs were synthesized from radiolabeled fatty acids as described by Taylor et al. (1990). *sn*-1,2-Diolein and authentic methyl esters of straight-chain fatty acids were from Nu-Check-Prep, Inc. (Elysian, MN). Diolein was enriched in the *sn*-1,2 isomer by TLC with boric acid-impregnated silica gel G (Thomas et al., 1965). Dye reagent concentrate for protein assays was from Bio-Rad (Richmond, CA). HPLC-grade solvents were from BDH, Inc. (Toronto, Ontario, Canada). Silica gel 60 H was from E.M. Science (Gibbstown, NJ), and silicon carbide (400 mesh) was from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Methanolic HCl reagent was from Supelco, Inc. (Bellefonte, PA). Ecolite(+) biodegradable scintillant was from ICN Biomedicals, Inc. (Irvine, CA). Other biochemicals were from Sigma Chemical Co. and were of the highest purity available.

Developing Seeds

Oilseed rape (*Brassica napus* L. cv Westar) was field grown at the Agriculture Canada Research Station in Lethbridge, Alberta. Safflower (*Carthamus tinctorius* L. experimental line Lesaf 241L-138) was grown to maturity in a temperature-controlled growth chamber at 18°C day (14 h at 310 μE m^{-2} s^{-1} supplied by fluorescent cool-white light supplemented with 4% incandescent)/13°C night. Light-intensity measurements were taken 1 m above the pots. Oilseed rape flowers were tagged at anthesis, and the developing pods were harvested at about weekly intervals until maturation. With safflower, the flowering capitula were tagged, and thereafter capitula were harvested at weekly intervals. Fresh weight of randomly selected seeds was determined, and dry weight of seeds was recorded after the seeds were dried for 24 h at 70°C. Seeds were also frozen with liquid N_2 and stored at −20°C for future analysis of TL, DGAT activity, and protein content.

MD Embryos and Embryogenic MD Cell-Suspension Culture

Microspores of oilseed rape (*B. napus* L. cv Topas) were isolated and induced to form MD embryos (Pomeroy et al., 1991). MD embryos at specific stages of development were carefully picked from culture and blotted dry, and fresh weight was determined. The blotted embryos were frozen in liquid N_2 and stored at −20°C for analysis of DGAT activity.

An embryogenic MD cell-suspension culture of winter oilseed rape (*B. napus* L. cv Jet Neuf) was provided by Dr. J. Singh of the Plant Research Centre, Agriculture Canada, Ottawa. The cell suspension was maintained according to the method of Orr et al. (1986). Before maceration or freezing, the cells were washed with water over a nylon sieve (60 μm) and blotted dry, and fresh weight was determined.

Lipid Analysis

TLs were extracted from all tissues using the HIP procedure of Hara and Radin (1978), and TG was separated from HIP extracts by TLC. Aliquots of TL were spotted as narrow bands on HPTLC-Fertigplatten Kieselgel 60 plates (Mandel Scientific Co., Toronto, Ontario, Canada), and the plates were developed in hexane:diethylether:acetic acid (82:18:1, v/v/v). The plates were dried under N_2 gas, the sample areas for fatty acid analysis covered by a clean glass plate, and the lipid classes identified by placing plates briefly in iodine vapor. The lipids were scraped from the plates and transferred to screw-cap tubes with Teflon-lined caps. FAMES of TL extracts and TG/silica fractions were prepared according to the method of Browse et al. (1986), and GLC analysis was carried out using a Varian 3400 GC with a 30-m DB-Wax Megabore Column (Chromatographic Specialties Inc., Brockville, Ontario, Canada) according to the method of Pomeroy et al. (1991).

Preparation of Homogenate and Assay of DGAT

All procedures were conducted at 0 to 4°C unless indicated otherwise. Frozen tissue was thawed on ice. Seeds were ground using a mortar and pestle and acid-washed silica, with 8 volumes of grinding medium per g of tissue. MD embryos and Jet Neuf cells were macerated with 4 volumes of grinding medium per g of tissue. To effectively grind MD embryos at the heart-shaped stage, silicon carbide replaced acid-washed silica as an abrasive. The grinding medium consisted of 0.2 M Hepes-NaOH buffer (pH 7.4) containing 0.5 M Suc and 1 mM DTT. Homogenates derived from tissue more advanced than the heart stage were filtered through a nylon sieve (60 μm). The volume of heart-stage homogenate was relatively low. Thus, further manipulation of this homogenate was avoided to minimize sample loss.

DGAT assays were performed as described by Weselake et al. (1991), with 0.2 M Hepes-NaOH (pH 7.4) as the reaction mixture buffer. The protein content of plant tissue extracts was determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure, with BSA as the standard.

RESULTS AND DISCUSSION

Developmental Studies with Maturing Seeds

DGAT activity was determined at various stages of seed maturation in oilseed rape (Fig. 1) and safflower (Fig. 2). The general pattern of storage lipid accumulation was consistent with other studies of lipid deposition in developing seeds of oilseed rape (Fowler and Downey, 1970; Norton and Harris, 1975, 1983). Norton and Harris (1983) have shown that the neutral lipid fraction accounts for the largest proportion of newly synthesized lipid during seed maturation in oilseed rape, with TG representing a large fraction of the neutral lipid.

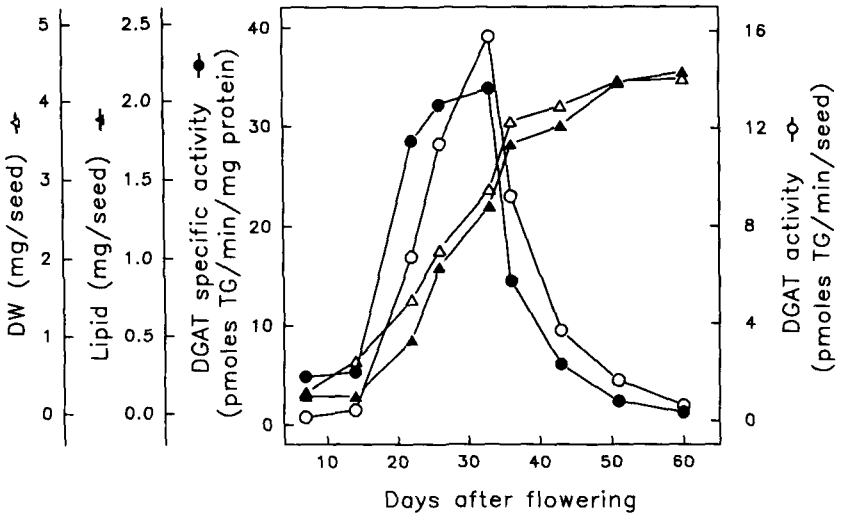

Figure 1. DGAT activity, lipid content, and dry weight (DW) of maturing seeds of oilseed rape (*B. napus* L. cv Westar). Groups of 25 seeds, obtained from plants grown in the field at the Lethbridge Research Station, were used for each type of analysis. Enzyme assays were conducted with 14 μM [1-^{14}C]oleoyl-CoA as the donor substrate and 10 μL of seed homogenate.

The appearance of DGAT activity was expressed in terms of specific activity and activity per seed. In oilseed rape (Fig. 1), the two DGAT activity profiles were similar. Enzyme activity increased from 14 DAF to a maximum at 33 DAF, and thereafter the activity decreased rapidly until about 40 DAF. The appearance of DGAT activity was coincident with the onset of lipid accumulation. Maximum DGAT activity occurred during the rapid phase of lipid accumulation, and the activity rapidly decreased as the lipid content of the seeds reached a plateau. A similar relationship between DGAT activity and lipid accumulation was found in studies with maturing seeds of safflower (Fig. 2). Both DGAT-specific activity and the activity per seed reached a maximum at 14 DAF, during the rapid phase of lipid accumulation. The enzyme activity decreased markedly between 14 and 21 DAF, and soon thereafter the seed lipid level began to reach a plateau. A preliminary investigation with maturing soybean (*Glycine max* L. Merr cv Maple Amber) seeds indicated a similar developmental profile for DGAT with maximum activity per seed and specific activity occurring in the range of 20 to 30 DAF. The soybean plants were grown in a growth chamber (14-h day at 21/17°C night) with lighting conditions similar to those used during growth of safflower plants.

The in vitro DGAT rate for lipid accumulation was estimated to be 33 and 15% of the in vivo lipid accumulation rate for oilseed rape (33 DAF) and safflower (13 DAF), respectively, at the time when DGAT activity was maximal in the developing seeds. Thus, in vitro DGAT activity did not entirely account for the in vivo rate of lipid accumulation. This was not surprising, because the in vitro assay conditions were different from the true physiological environment of the enzyme in the maturing seed.

The developmental profile of DGAT activity in safflower was similar to the profile for incorporation of radioactivity from [U^{14}C]acetate into the TG fraction of developing safflower seeds reported by Ichihara and Noda (1980). The investigators found that the maximum incorporation rate of label into the TG fraction took place during the active phase of TG accumulation. In a more recent study with safflower, Ichihara et al. (1990) found that membrane-bound phosphatidate phosphatase activity reached a maximum at 13 DAF, with rapid accumulation of oil occurring 13 to 15 DAF. Sharp

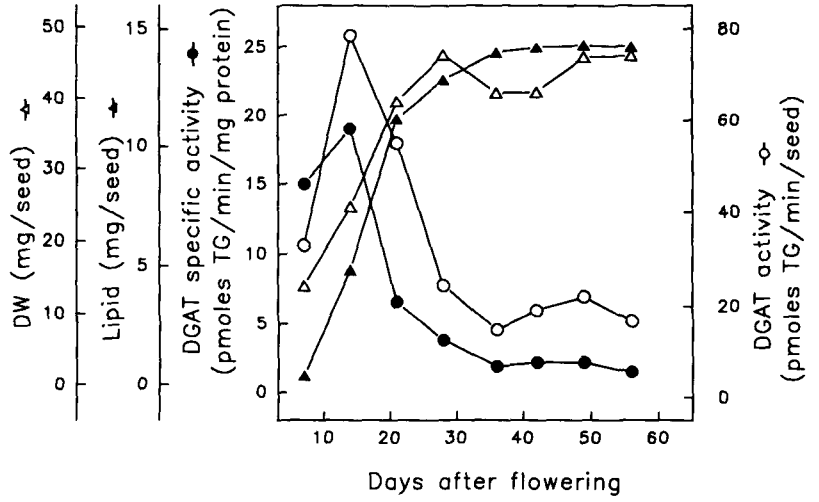

Figure 2. DGAT activity, lipid content, and dry weight (DW) of maturing seeds of safflower (*C. tinctorius* L. experimental line Lesaf 241L-138). Plants were grown in a growth chamber under the conditions indicated in "Materials and Methods." Groups of 25 seeds were used for each type of analysis. Enzyme assays were conducted with 14 μM [1-^{14}C]oleoyl-CoA as the donor substrate and 10 μL of seed homogenate.

peaks of specific activity for phosphatidate phosphatase and DGAT, with enzyme activity maximums occurring 30 DAF, have also been reported for microsomes isolated from developing groundnut seeds (Sukumar and Sastry, 1987).

Acetyl-CoA carboxylase activity has also been shown to increase and decrease during maturation of castor seeds (Simcox et al., 1979) and seeds of oilseed rape (Turnham and Northcote, 1983). In each case maximum enzyme activity occurred during the rapid phase of oil accumulation. In contrast, components of the fatty acid synthase complex in developing seeds of oilseed rape have been shown to be induced largely before oil accumulation, and their activity levels (per seed) remained high even after the seed lipid level reached a plateau (Slabas et al., 1986, 1987; MacKintosh et al., 1989). However, a study with maturing soybean seeds has indicated that ACP levels decreased to about half the maximal level following the active phase of oil accumulation (Ohlrogge and Kuo, 1984). Developmental profiles obtained using enzymic and immunochemical assays of ACP were similar, suggesting that soybean ACP underwent turnover and degradation during seed maturation.

Overall, the developmental profiles reported for components of the fatty acid synthase complex in maturing seeds have indicated that relatively high activity levels of the associated proteins were still persistent after seed lipid levels reached a plateau. There was a marked decrease following the lipid plateau, as previously reported for acetyl-CoA carboxylase in castor seed (Simcox et al., 1979) and oilseed rape (Turnham and Northcote, 1983), for phosphatidate phosphatase in safflower (Ichihara et al., 1990) and groundnut (Sukumar and Sastry, 1987), for DGAT in groundnut (Sukumar and Sastry, 1987), and for the developmental profile of DGAT activity in oilseed rape and safflower reported in the current study. Thus, the sharp activity peaks during seed maturation reported for acetyl-CoA carboxylase, phosphatidate phosphatase, and DGAT suggest that these enzymes may be subject to regulation in the biosynthesis of TG.

Developmental Studies with MD Embryos of Oilseed Rape

Development of MD embryos is not synchronous in that at any one time during culture there are embryos present at various stages of development (Pomeroy et al., 1991). In studies with a high-erucic acid cultivar of oilseed rape (cv Reston), Taylor et al. (1991) found a strong similarity in the relative proportions of 18:1, 20:1, and 22:1 when comparing zygotic and MD embryos at the same developmental stages. In the current study, developmental stages of MD embryos of distinct morphology were isolated from cultures to examine the relationship between DGAT activity and stage of embryo development. High levels of DGAT activity were found throughout the early torpedo to late cotyledonary developmental stages.

The specific activity of DGAT as a function of developmental phase of MD embryos of cv Topas is shown in Figure 3. DGAT-specific activity was greatest in embryos at the mid-cotyledonary phase. The specific activity of the enzyme at this stage represented about a 3-fold increase over the specific activity of the enzyme in the heart to early torpedo stage. The higher specific activity at the mid-cotyledonary phase

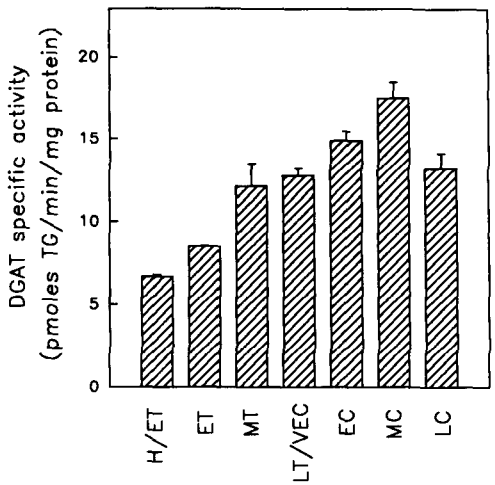

Figure 3. DGAT activity in MD embryos of oilseed rape (*B. napus* L. cv Topas) at various stages of development. Enzyme assays were conducted with 14 μM [1-^{14}C]oleoyl-CoA as the donor substrate and 10 μL of homogenate. Error bars represent the SE for triplicate assays. Developmental stages: H, Heart; ET, early torpedo; MT, mid-torpedo; LT, late torpedo; VEC, very early cotyledonary; EC, early cotyledonary; MC, mid-cotyledonary; and LC, late cotyledonary.

suggested that relatively more protein synthesis may have been committed to DGAT formation at later stages of development. The specific activity of DGAT in the mid-cotyledonary phase was about 2-fold lower than the maximum specific activity of the enzyme in the developing seed (Fig. 1). Intercultivar genetic differences together with environmental conditions may have had an impact on the relative levels of DGAT activity in the two systems (zygotic versus MD embryos).

Lipid Composition and DGAT Activity in an Embryogenic MD Cell-Suspension Culture of Oilseed Rape

Cell-suspension cultures of various oilseeds have been shown to contain TG (Radwan and Mangold, 1976), and culture conditions have been manipulated to alter lipid content and composition (Radwan and Mangold, 1976; Dutta and Appelqvist, 1989). In the current study, an embryogenic MD cell-suspension culture of oilseed rape (cv Jet Neuf), originally described by Orr et al. (1986), was examined for content and composition of acyl lipid and DGAT activity.

The dry weight/fresh weight, TL, and TG content of the Jet Neuf cell-suspension culture were compared to those of mature seeds and leaves of the same cultivar (Table I). Acyl lipid levels were reported as FAMES. TL-FAMES constituted about 7% of the dry weight, and TG represented about 45 to 50% of TL, which translates to a TG content of about 3 to 4% of the dry weight of the cells. In comparison, TG constituted about 88 and 4% of TL, respectively, in seeds and leaves of Jet Neuf.

The acyl compositions of TL and TG in the cell-suspension culture, seeds, and leaves are shown in Table II. In the culture, 18:1 represented about 55% of the fatty acids in TG. The

Table I. *TL and TG content in embryogenic MD cell-suspension culture, seeds, and leaves of B. napus L. cv Jet Neuf*

Isolation of the TG fraction and subsequent methylation of TL and TG were performed as described in "Materials and Methods." FAMES were determined by GLC and FAME/dry weight (DW) values were based on the total integrated area of a chromatogram representing a sample. Data are provided for three samples taken during a period of 1 year. Values representing the cell suspension are the means of duplicate analyses from two separate experiments. Values representing seeds and leaves are the means of triplicate analyses. FW, Fresh weight.

Tissue	DW/FW	TL-FAMES/DW	TG-FAMES/TL
		%	
October 15, 1990 culture	16.3	6.8	44.7
July 26, 1991 culture	15.5	7.4	49.8
October 18, 1991 culture	14.7	7.2	45.8
Seed	95.0	37.7	87.6
Leaves	12.5	3.1	3.8

level of 18:1 was somewhat greater in seeds. In the culture, 16:0 and 18:3 were the next predominant species, respectively, representing 12.6 to 15.5 and 7.0 to 12.1% of the total fatty acids in TG. In contrast, 18:2 was the next predominant species in seeds, representing 19.2% of the total. The level of 18:1 in the TG of leaves was 50.2%, with 16:0 and 16:3 representing the next two major species, at 16.6 and 16.1% of the total, respectively.

The low but significant level of TG in the cell-suspension culture prompted an analysis of DGAT activity. Jet Neuf DGAT activity was about 2-fold greater when [1-^{14}C]erucoyl-CoA was substituted for [1-^{14}C]oleoyl-CoA in the reaction mixture. The enhanced specificity for erucoyl-CoA displayed by Jet Neuf DGAT was consistent with the reported specificity of DGAT in microsomal fractions prepared from MD embryos of oilseed rape (Taylor et al., 1991; Weselake et al., 1991). MD embryos from both high- and low-erucic acid cultivars (Reston and Topas, respectively) displayed a greater specificity for erucoyl-CoA under these assay conditions (Weselake et al., 1991, 1992).

If the Jet Neuf cells were cultured without changing medium for up to 6 weeks, there was little effect on the fatty acid profiles of TL and TG (data not shown). By the 6th week, however, cultures without media replacement were brown, and viability was markedly decreased. DGAT activity was also determined in cell-suspension cultures that were allowed to propagate without medium replacement (Fig. 4). DGAT-specific activity was relatively constant during a 2-week culture period. Both enzyme-specific activity and activity per g fresh weight decreased significantly by the 3rd week of culture. Thus, in terms of preserving DGAT specific activity, the regular 2-week subculturing period was essential. Under these optimal subculturing conditions, DGAT activity varied from 5 to 15 pmol of TG min^{-1} mg^{-1} of protein over a period of 1 year.

The Jet Neuf culture appeared to pass through phases of rapid and slower growth, and at present we are uncertain why this occurred. This variability in culture growth may have had an impact on the level of DGAT activity in the cells. The concentration of DGAT activity per g fresh weight in the Jet Neuf cells was 10- to 15-fold lower than the tissue concentration of enzyme activity in MD embryos. The specific activity of DGAT in the Jet Neuf cells, however, was similar

Table II. *Fatty acid composition of TL and TG in embryogenic MD cell-suspension culture, seeds, and leaves of B. napus L. cv Jet Neuf*

Isolation of the TG fraction, methylation of TL and TG, and separation of FAMES by GLC were performed as described in "Materials and Methods." Data are provided for three samples taken during a period of 1 year. Values representing the cell suspension are the means of duplicate analyses from two separate experiments. Values representing seeds and leaves are the means of triplicate analyses.

Tissue	Lipid Class	Fatty Acid Composition, Wt								
		16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0
						%				
October 15, 1990 culture	TL	9.8	2.7	2.9	49.0	6.8	11.8	5.3	tr[b]	11.7
	TG	12.6	3.8	3.6	55.8	6.8	11.7	2.6	tr	2.9
July 26, 1991 culture	TL	14.8	3.2	4.5	52.1	6.4	7.1	4.9	tr	7.0
	TG	15.5	3.5	4.6	57.4	6.4	7.0	2.8	tr	2.5
October 18, 1991 culture	TL	12.4	2.7	3.6	46.8	7.4	12.6	5.6	tr	8.9
	TG	12.4	3.6	4.2	55.0	6.8	12.1	2.8	tr	2.8
Seed	TL	5.2	tr	1.2	60.9	21.8	9.2	tr	0.8	tr
	TG	5.5	tr	1.2	64.3	19.2	7.8	tr	0.9	tr
Leaves	TL[a]	9.9	0.7	1.7	3.2	12.7	50.2	tr	tr	tr
	TG	16.6	3.5	5.2	50.2	9.5	12.6	1.6	tr	0.8

[a] Leaf TL also contained 3.1% 14:0, 2.4% 16:2, and 16.1% 16:3. [b] tr, Trace, <0.5%.

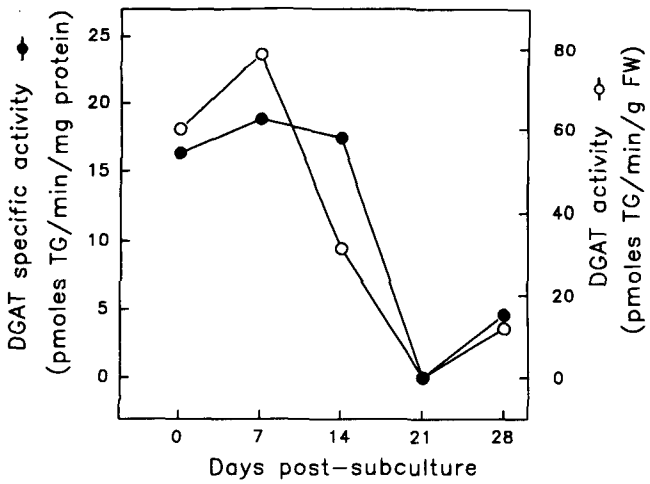

Figure 4. DGAT activity in MD embryogenic cell suspension of oilseed rape (*B. napus* L. cv Jet Neuf) as a function of days in culture without subsequent subculturing. Samples of cell suspension were removed at intervals, filtered, and frozen with liquid N_2 and stored at −20°C. Before DGAT activity was assayed the frozen cells were thawed on ice and homogenized. Enzyme assays were conducted with 14 μM [1-^{14}C]oleoyl-CoA as the donor substrate and 15 μL of homogenate. FW, Fresh weight.

to the range of specific activities observed with enzyme in homogenates of MD embryos (Fig. 3). The relatively high specific activity of DGAT in the cell-suspension culture, ease of culture maintenance, and possibility for scale-up are factors that make this system an attractive source of DGAT for purification purposes.

CONCLUSIONS

The current study has provided fundamental knowledge concerning the induction of DGAT activity during development of seeds and MD embryos of oilseed crops. The information will contribute to our understanding of the possible regulation of DGAT activity and TG biosynthesis. The rapid increase in DGAT activity (specific activity and activity per seed) during active lipid accumulation in developing oilseed rape and safflower seed, and the marked decrease in activity when oil levels reach a plateau, suggests that this enzyme activity is somehow coordinated in TG biosynthesis. Also, DGAT may prove to be a useful enzymic marker for oil formation, providing valuable information for plant breeders and agronomists. The significant level of DGAT activity in the embryogenic MD cell-suspension culture suggests that this nondifferentiating system may be useful in the purification of DGAT and other TG bioassembly enzymes.

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