Solubilization and characterization of diacylglycerol acyltransferase from microspore-derived cultures of oilseed rape

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Particulate fractions prepared from microspore-derived (MD) embryos of oilseed rape (*Brassica napus* L. cv. Reston) and an embryogenic MD cell suspension culture of oilseed rape (*B. napus* L. cv. Jet Neuf) were used as a source of diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) for enzyme characterization and development of a solubilization procedure. DGAT activity in the 1500–100000 *g* fraction from MD embryos was stimulated 4–5-fold by 3 to 4 mg of BSA/ml of reaction mixture. DGAT activity from MD embryos was stimulated 2–3-fold by fluoride salts and 1.4-fold by NaCl, whereas iodide salts caused substantial inhibition of enzyme activity. The effect of the various 1:1 electrolytes on enzyme activity appeared to be related more to their differential effects on solution structure rather than ionic strength. DGAT was solubilized from membranes of MD embryos and the cell suspension culture by about 80 and 50% respectively, using 2 M NaCl in 1% (w/v) octanoyl-*N*-methylglucamide (MEGA-8) (pH 8.0 buffer) at a detergent to protein ratio of 2:1. The specific activity of solubilized DGAT was about 2-fold greater than that of the particulate enzyme. The mechanism of solubilization appeared to be related to the lowering of the critical micellar concentration of MEGA-8 in the presence of NaCl. DGAT, solubilized from MD embryos, eluted with an M_r of about 2×10^6 during gel-filtration chromatography on a Superose 6 column equilibrated in buffer containing 0.1% (w/v) MEGA-8. The solubilized enzyme exhibited optimal activity at pH 7. At concentrations above 2 μM acyl-CoA, the specificity of solubilized DGAT for oleoyl-CoA and palmitoyl-CoA was considerably greater than for stearoyl-CoA.

INTRODUCTION

In developing oilseeds, triacylglycerol (TG) biosynthesis occurs according to the glycerol 3-phosphate or Kennedy pathway (Kennedy, 1961; Stymne and Stobart, 1987). Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyses the final and committed step in this pathway utilizing fatty acyl-CoA as a donor substrate in the acylation of diacylglycerol to form TG (Stymne and Stobart, 1987). The DGAT-catalysed reaction may represent a rate-limiting step in seed oil biosynthesis (Ichihara et al., 1988; Perry and Harwood, 1993). DGAT activity has been shown to reach a maximum during the active phase of oil accumulation in maturing seeds of oilseed rape (Tzen et al., 1993; Weselake et al., 1993a), suggesting that the enzyme is coordinated with TG accumulation. If DGAT activity is both rate-limiting and tightly regulated then genetic alteration of the expression of DGAT may have a profound effect on the quality and/or quantity of seed oil produced during seed maturation. The TG formed by extracts of microspore-derived (MD) embryos of oilseed rape is not subject to acyl-exchange (Taylor et al., 1991). This finding, together with the committed nature of the DGAT-catalysed reaction, suggests that stable modifications of acyl composition can be made at the *sn*-3 position of TG. DGAT and other TG bioassembly enzymes, however, are membrane bound and have proven recalcitrant to purification. This has been a major obstacle in making advances in the genetic modification of oilseeds to produce seed oils with desired characteristics.

DGAT from germinating soybean was reported to have been purified to homogeneity by Kwanyuen and Wilson (1986) but a recent report has suggested that the preparation also contained lipid body proteins (Wilson et al., 1993). DGAT was solubilized from microsomes of germinating soybean by treatment with 3-cholamidopropyl-dimethylammonio-1-propane sulphonate (CHAPS) but the specific activity of the solubilized enzyme was reduced by about 60-fold when compared with the particulate enzyme fraction used for solubilization (Kwanyuen and Wilson, 1986). DGAT has also been solubilized by treatment of a membrane fraction of *Mycobacterium smegmatis* with acetone and subsequent extraction of the acetone powder with 0.15 M KCl (Akao and Kusaka, 1976). The M_r of the solubilized bacterial DGAT was estimated to be about 50000 based on gel-filtration chromatography. DGAT has also been solubilized and partially purified from rat liver microsomes (Hosaka et al., 1977; Polokoff and Bell, 1980). Recently, rat liver DGAT was purified to near homogeneity using gel-filtration, ion-exchange and immunoaffinity chromatography (Andersson et al., 1994). The enzyme preparation was enriched in a polypeptide with M_r of 60000 based on analysis by SDS/PAGE. A TG synthetase complex, containing DGAT activity, was solubilized from microsomes of the villus cells of rat intestinal mucosa with sodium taurocholate and further purified by chromatography on phenyl-Sepharose (Manganaro and Kuksis, 1985).

DGAT activity in MD cultures of oilseed rape has been partially characterized (Taylor et al., 1991, 1992; Weselake et al., 1991, 1993a). Furthermore, particulate fractions containing DGAT activity have been partially dispersed with octanoyl-*N*-methylglucamide (MEGA-8) but the dispersion could be recovered in a pellet following centrifugation at 105000 *g* for 1 h (Weselake et al., 1993b). The current study examines the effects of BSA and various salts on the activity of DGAT in particulate fractions from MD cultures of oilseed rape (*Brassica napus* L.). A combination of high salt concentration and detergent was effective in the solubilization of DGAT with enhanced specific activity. Some characteristics of the solubilized enzyme are described.

Abbreviations used: CHAPS, 3-cholamidopropyl-dimethylammonio-1-propanesulphonate; CMC, critical micellar concentration; DGAT, diacylglycerol acyltransferase; DTT, dithiothreitol; MEGA-8, octanoyl-*N*-methylglucamide; MD, microspore-derived; TG, triacylglycerol.

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EXPERIMENTAL

Materials

[1-^{14}C]Oleic acid (54–56 Ci/mol) and [1-^{14}C]stearic acid (51 Ci/mol) were obtained from Amersham Canada Ltd., Oakville, ON, Canada. [1-^{14}C]Palmitic acid (8.4 Ci/mol) was from NEN Research Products, Mississauga, Ontario, Canada. Acyl-CoAs were synthesized from radiolabelled fatty acids using the method described by Taylor et al. (1990). Silica Gel H was from E. Merck, Darmstadt, Germany. Glycerol, inorganic salts and h.p.l.c.-grade solvents were from the Fisher Scientific Co., Canada. Ultrapure urea was from Schwarz/Mann Biotech, ICN Biomedicals, Cleveland, OH, U.S.A. Sepharose CL-4B and a Superose 6 (HR 10/30) column were from Pharmacia LKB Biotechnology International AB, Uppsala, Sweden. Ecolite (+) biodegradable scintillant was from ICN Biomedicals, Irvine, CA, U.S.A. Chemicals for electrophoresis and gelatin were obtained from Bio-Rad, Richmond, CA, U.S.A. Horse heart cytochrome *c*, bovine blood haemoglobin (type I), bovine milk α-lactalbumin, chicken egg ovalbumin, *sn*-1,2-diolein and other biochemicals were from the Sigma Chemical Co., St. Louis, MO, U.S.A.

MD cultures and developing seeds of oilseed rape

Microspores of oilseed rape (*Brassica napus* L. cv. Reston) were isolated and induced to form MD embryos according to Pomeroy et al. (1991). The embryos in the early to mid-cotyledonary stages of development were blotted dry and the fresh wt. was determined. The embryos were frozen in liquid N_2 and stored at −20 °C until homogenization. An embryogenic MD cell suspension culture of winter oilseed rape (*B. napus* L. cv. Jet Neuf) was maintained according to Orr et al. (1986). Harvested cells were washed on a 60 μm nylon mesh, blotted dry and the fresh wt. was determined. Oilseed rape (*B. napus* L. cv. Westar) plants were field grown at the Agriculture and Agri-Food Canada Research Centre in Lethbridge. Pods were harvested 3 to 4 weeks after flowering and the maturing seeds were frozen in liquid N_2 and stored at −20 °C.

Preparation of particulate fractions

All procedures were conducted at 0–4 °C. Frozen tissue was thawed on ice. Tissues were ground using a mortar and pestle and acid-washed silica with 4 vol. of grinding medium per g of tissue for the MD embryos and cell suspension whereas 8 vol. of grinding medium per g of tissue were used for the developing seeds. The grinding medium consisted of 0.2 M Hepes/NaOH buffer, pH 7.4, containing 0.5 M sucrose and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 1500 ***g*** for 15 min and the resulting pellet discarded. The supernatant was filtered through glass wool to remove the lipid layer and centrifuged at 100000 ***g*** for 2 h. The resulting pellet was resuspended in 10 mM Hepes/NaOH buffer, pH 7.4, containing 0.2 M KCl and 1 mM DTT. One millilitre of resuspension buffer was used for every 10 g of original tissue wt. The resuspended particulate fractions were divided into 100 μl aliquots which were frozen with liquid N_2 and stored at −20 °C.

Enzyme solubilization

Particulate fractions used in solubilization experiments were prepared as described previously up to the 1500 ***g*** centrifugation step. The 1500 ***g*** supernatant was further centrifuged at 105000 ***g*** for 1 h. The pellet was resuspended in 10 mM Tris/HCl buffer, pH 8.0, containing 20% (w/v) glycerol and 1 mM DTT using 10 ml of buffer per g of original tissue wt. Following protein determination, aliquots of the resuspension, containing 500–1000 μg of protein, were distributed into microcentrifuge tubes and were centrifuged at 105000 ***g*** for 1 h. The resulting pellets were resuspended at a detergent to protein ratio of 2:1 using solubilization buffer which consisted of 10 mM Tris/HCl buffer, pH 8.0, containing 20% (w/v) glycerol, 1 mM DTT, 1% (w/v) MEGA-8 and various concentrations of NaCl ranging from 0 to 4 M. The microcentrifuge tubes, containing the solubilization mixtures, were secured on a shaker (IKA-VIBRX VXR, Janke and Kunkel Gmbh Co., KG, Germany) set up at 4 °C. The samples were vibrated at 1800 rev./min for 30 min and then centrifuged at 105000 ***g*** for 1 h. Resulting supernatants were designated as solubilized fractions. Pellets were resuspended with their corresponding solubilization buffers to the same volume used during solubilization. Solubilized fractions and resuspended pellets were assayed for DGAT activity and protein content.

Determination of critical micellar concentration (CMC)

The CMC of MEGA-8 in the absence or presence of salt solution was determined using a modification of the procedure of Vulliez-Le Normand and Eiselé (1993). The ink (blue) from a permanent fine tip Staedtler Lumocolor 313 pen was extracted with 5 ml of ethanol and filtered. Portions (30 μl) of the ink solution were transferred to 1.5 ml microcentrifuge tubes and the ethanol was allowed to evaporate overnight. Buffer solutions (1 ml), containing various concentrations of MEGA-8 (±2 M NaCl) were added to the dried ink. The tubes were sealed and shaken horizontally at 1800 rev./min on a shaker rack for 16 h at room temperature. The samples were then centrifuged at 16000 ***g*** for 5 min in a microcentrifuge. The absorbances of the supernatants were determined at 613 nm.

DGAT assays and protein determination

Enzyme assays were performed with some changes to the method described by Weselake et al. (1991). Assays were conducted in a total volume of 60 μl in 3 ml glass tubes at 30 °C in a shaking waterbath using enzyme extract (5–10 μl) to initiate the reaction. The standard reaction mixture consisted of 0.2 M Hepes/NaOH buffer, pH 7.4, containing 3 mM $MgCl_2$, 1 mM ATP, 330 μM coenzyme A, 330 μM *sn*-1,2-diolein, 0.02% Tween-20, 0.5% (w/v) BSA and 15 μM [1-^{14}C]oleoyl-CoA. Unless otherwise indicated, enzyme reactions were allowed to proceed for 10 min and were terminated by adding 10 μl of 5% (w/v) SDS. Aliquots of 50 μl were applied directly to plates coated with 0.5 mm of Silica Gel H. Moisture was removed from the origin with a gentle stream of warm air and the plate developed with hexane/diethyl ether (4:1, v/v). The TG spot was scraped from the plate, combined with 5 ml of scintillant and analysed for radioactivity. Enzyme assays were performed in duplicate or triplicate. The protein content of plant tissue extracts and column fractions was determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure, with BSA as the standard.

Electrophoresis

Particulate and solubilized fractions, prepared from MD embryos, were also analysed by SDS/PAGE using the Laemmli (1970) procedure with the exception that 4.2 M urea was incorporated into the separating gel. The NaCl content of the solubilized fraction was reduced by washing with 125 mM Tris/HCl (pH 6.8) in a Centricon 10 microconcentrator (Amicon, W. R. Grace and Co., Danvers, MA, U.S.A.). The following components were incorporated into the washed protein: 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol

and 0.005% (w/v) Bromophenol Blue. The entire concentrator unit was boiled for 5 min to disrupt proteins in the solution above the ultrafiltration membrane and proteins which may have adsorbed to the membrane during centrifuge-mediated concentration. Electrophoresis was conducted using the Bio-Rad Mini-Protean II apparatus and 1-mm-thick polyacrylamide gel (12% total monomer concentration; 3% cross-linking monomer concentration). The total monomer concentration of the stacking gel was 4%. Following electrophoresis, the separated polypeptides in the gel were stained using the Bio-Rad silver staining method based on the procedure of Merril et al. (1981).

Gel-filtration chromatography

Gel-filtration chromatography was conducted using a Superose 6 column (HR 10/30) equilibrated with either 0.1% or 1% (w/v) MEGA-8 in 10 mM Tris/HCl buffer, pH 8.0, containing 2 M NaCl, 20% (w/v) glycerol and 1 mM DTT. Samples (200 μl) of solubilized enzyme were applied to the gel-filtration column which was operated at a flow rate of 12 ml/h with an f.p.l.c. system (Pharmacia LKB Biotechnology International AB, Uppsala, Sweden). Column fractions of 1 ml were collected and assayed for DGAT activity and protein content.

RESULTS AND DISCUSSION

Assay of DGAT activity and the effect of BSA

In the course of developing an effective solubilization procedure for DGAT from MD cultures of oilseed rape, a number of agents were found to stimulate DGAT activity. BSA has previously been shown to stimulate Kennedy pathway enzymes *in vitro* and is often added during the assays of these enzymes (Hershenson and Ernst-Fonberg, 1983; Stobart and Stymne, 1990; Oo and Chew, 1992). In the current study, BSA stimulated DGAT activity in particulate fractions of maturing seeds, MD embryos and an embryogenic MD cell suspension culture of oilseed rape. Thus BSA was routinely incorporated into the reaction mixture to increase the sensitivity of enzyme detection. In the presence of BSA (5 mg/ml), DGAT activity in the 1500–100000 *g* particulate fraction of MD embryos of oilseed rape responded linearly up to about 100 μg of particulate protein (Figure 1). Therefore the quantity of microsomal protein in the assay mixture was routinely kept below 100 μg. In studies with MD embryos, the stimulatory effect of BSA on DGAT activity was concentration-dependent with maximum stimulation of enzyme activity at a concentration of 3–4 mg of BSA/ml of reaction mixture (Figure 2a). The degree

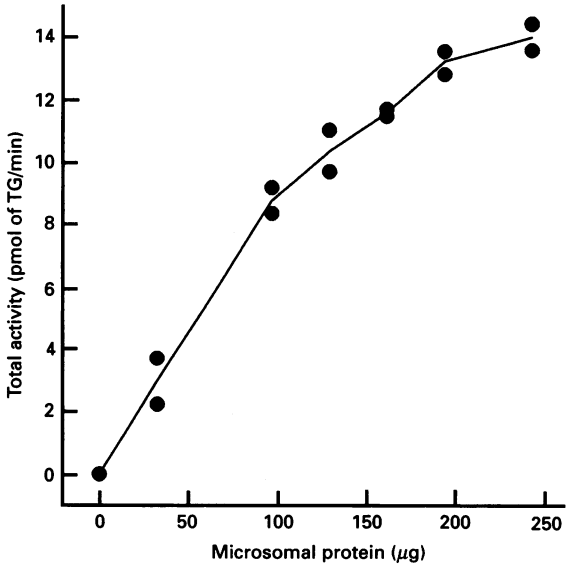

Figure 1 Dependence of DGAT activity on the quantity of particulate enzyme from MD embryos of oilseed rape

Assays were conducted with the 1500–100000 *g* particulate fraction of MD embryos of *B. napus* L. cv. Reston for 10 min in the presence of 5 mg of BSA/ml. Results of duplicate assays are shown with the plot line connecting the means of the duplicate values.

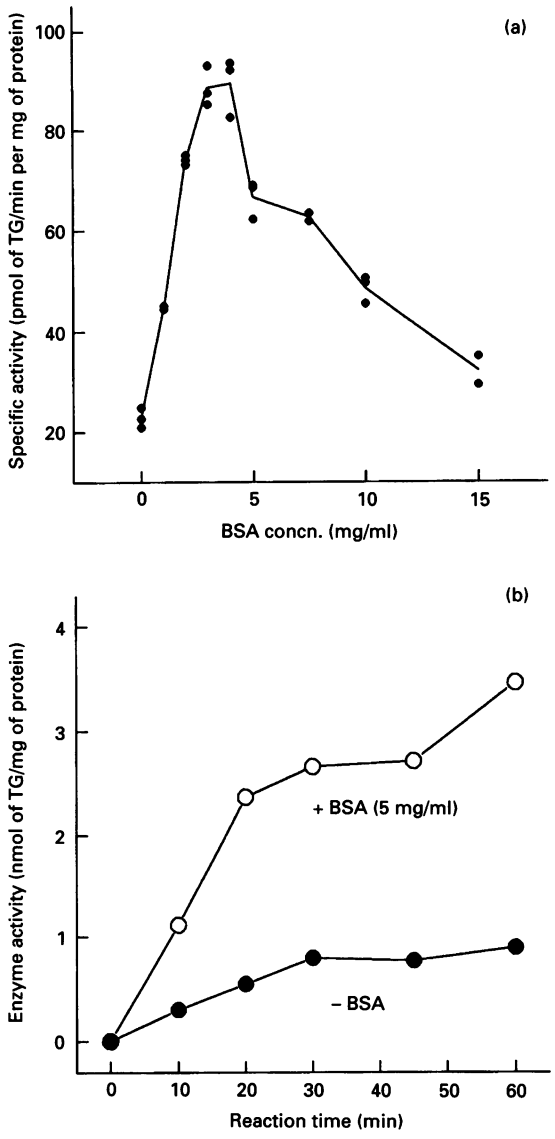

Figure 2 Effect of BSA on DGAT activity in the particulate fraction from MD embryos of oilseed rape

The 1500–105000 *g* particulate fraction of MD embryos of *B. napus* L. cv. Reston was used as a source of enzyme. (**a**) Effect of BSA concentration on DGAT activity. Reactions were allowed to proceed for 10 min using 80 μg of microsomal protein. Results of duplicate or triplicate assays (at each BSA concentration) are shown with the plot connecting the means of the replicate points. (**b**) Time course for the formation of TG catalysed by DGAT in the absence or presence of BSA (5 mg/ml). Assays were conducted with 30 μg of microsomal protein. Each data point represents the mean of duplicate assays.

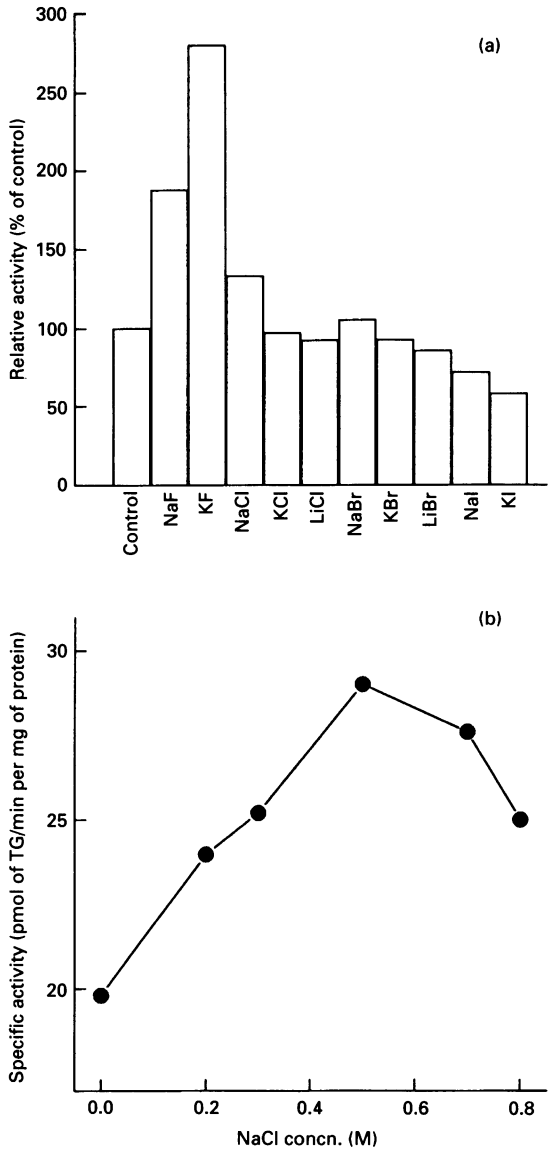

Figure 3 Effect of 1:1 electrolytes on particulate DGAT activity from MD embryos of oilseed rape

The 1500–100000 ***g*** particulate fraction of MD embryos of *B. napus* L. cv. Reston was used as a source of enzyme. (**a**) Effect of fluoride, chloride, bromide and iodide salts on DGAT activity. Enzyme reaction mixtures contained 5 mg of BSA/ml, 0.5 M salt and 80 μg of microsomal protein. Assays were allowed to proceed for 10 min. Bar heights represent the means of triplicate assays. (**b**) Effect of NaCl concentration on DGAT activity. Enzyme reaction mixtures contained 5 mg of BSA/ml and 80 μg of microsomal protein. Reactions were allowed to proceed for 10 min. Each data point represents the mean of triplicate assays.

of stimulation ranged from 4- to 5-fold at this concentration. The time courses of TG formation, catalysed by microsomes of MD embryos, are shown in the absence or presence of BSA (5 mg/ml) (Figure 2b). Both of these time courses for TG formation were linear for at least 20 min. Thus, end-point assays used in other experiments were based on a 10 min reaction in order to maintain linear kinetics. The degree of experimental uncertainty depicted in Figures 1 and 2(a) was characteristic of other experiments.

Studies with BSA and other proteins at the same concentration (5 mg/ml) indicated that BSA was the most effective in stimulation of DGAT activity in microsomes of MD embryos. In these experiments BSA stimulated DGAT activity by about 3-fold. Haemoglobin and α-lactalbumin stimulated DGAT activity by about 1.4- and 1.5-fold respectively. Cytochrome *c*, ovalbumin and gelatin had almost no effect on DGAT activity whereas soybean trypsin inhibitor inhibited DGAT activity by about 35%.

Oo and Chew (1992) have reported that BSA (2 mg/ml) stimulated microsomal and lipid body DGATs from oil palm mesocarp by about 3-fold. BSA has also been shown to have a concentration-dependent influence on the acyl selectivity of lysophosphatidate acyltransferase (Bafor et al., 1990). In studies with developing safflower seeds, 0.25 mg of BSA/ml of reaction mixture stimulated acylation of lysophosphatidate by 2-fold with equal incorporation of oleoyl and linoleoyl moieties, whereas 0.5 mg of BSA/ml resulted in preferential incorporation of linoleoyl moieties. It has been suggested that the mechanism of acyltransferase stimulation by BSA involves the binding of acyl-CoA to BSA thus preventing the formation of acyl-CoA micelles (in the micromolar range) which are believed to inhibit acyltransferase activity (Stobart and Stymne, 1990). Bound acyl-CoAs may also be better substrates for acyltransferases (Hershenson and Ernst-Fonberg, 1983). It has also been suggested that BSA may protect against the action of endogenous thioesterase which could erode the acyl-CoA pool (Stymne et al., 1983). Binding studies have confirmed the interaction of BSA with acyl-CoA (Richards et al., 1990). In the current study, the decrease in DGAT activity observed beyond 4 mg of BSA/ml, as shown in Figure 2(a), suggested that a sufficiently high concentration of BSA could limit the amount of acyl-CoA available to the enzyme.

Effect of 1:1 electrolytes on the assay of DGAT

The effect of 0.5 M concentrations of various salts on DGAT activity in the 1500–100000 ***g*** particulate fraction from MD embryos of oilseed rape is shown in Figure 3(a). Fluoride salts and NaCl stimulated DGAT activity by 2–3-fold and 1.4-fold respectively. In general, KCl and bromide salts had little effect on DGAT activity whereas iodide salts appeared to substantially inhibit enzyme activity. DGAT activity in the embryogenic MD cell suspension culture was also stimulated by NaCl.

The variable effects of the different salts, at constant concentration, ruled out ionic strength as the sole factor in altering DGAT activity. The effectiveness of activation by anions followed the Hofmeister series (Hatefi and Hanstein, 1969, 1974), to some extent, with $F^- > Cl^-$ and Br^-, and $Br^- > I^-$. The ability of these salts to differentially alter solution structure may have caused changes in the conformation of DGAT or DGAT–membrane complex, resulting in altered enzyme activity.

Concentrations of NaCl beyond 0.5 M resulted in a progressive decrease in enzyme activity (Figure 3b). At this point the activating effect of salt may have been counteracted to some extent by the formation of inhibitory micelles of oleoyl-CoA. Ray and Némethy (1971) have reported that salts lower the CMCs of detergents. Thus it is possible that high concentrations of salt may have lowered the CMC of oleoyl-CoA which has the properties of a detergent.

Solubilization of DGAT

Recently, we have reported that MEGA-8 was effective in the dispersion of membranes from MD embryos of oilseed rape containing DGAT activity, but the dispersed enzyme could be sedimented following centrifugation at 105000 ***g*** for 1 h (Weselake et al., 1993b). Mono Q ion-exchange chromtography

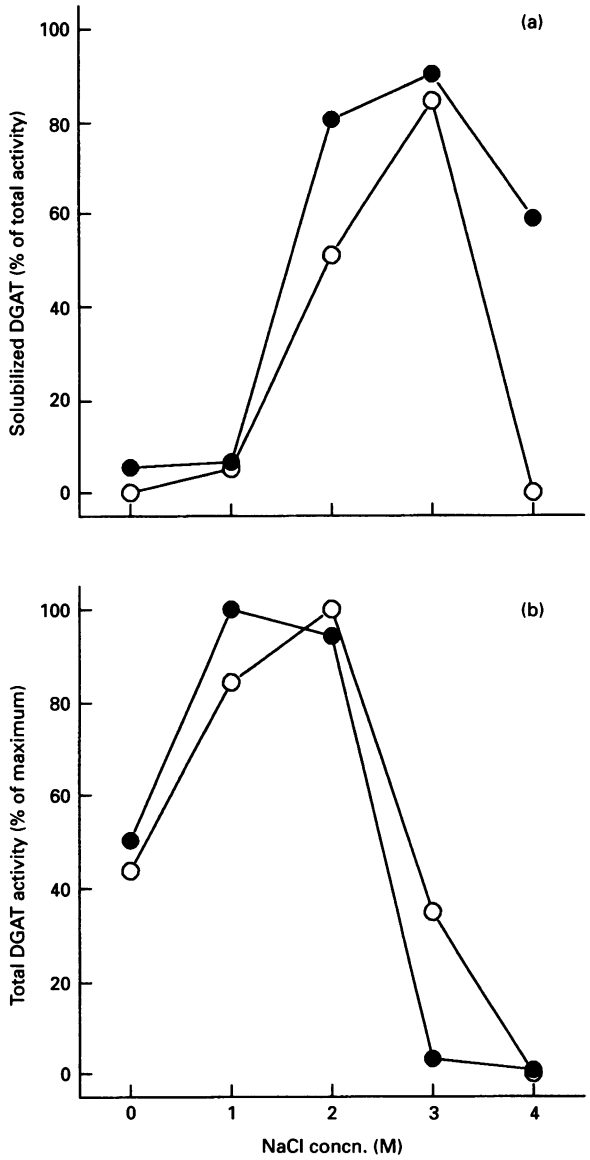

Figure 4 Solubilization of DGAT from particulate fractions of MD cultures of oilseed rape

●, MD embryos of *B. napus* L. cv. Reston; ○, embryogenic MD cell suspension culture of *B. napus* L. cv. Jet Neuf. (**a**) DGAT solubilized as a function of NaCl concentration. (**b**) Total recovery of DGAT as a function of NaCl concentration. Particulate fractions (1500–105000 ***g***) were treated with various concentrations of NaCl in 10 mM Tris/HCl buffer, pH 8.0, containing 20% (w/v) glycerol, 1% (w/v) MEGA-8 and 1 mM DTT. Following centrifugation at 105000 ***g*** for 1 h, both the supernatant and pellet were assayed for DGAT activity. The protein contents and volumes of the solubilization mixtures were 570 μg and 114 μl, and 885 μg and 177 μl, respectively, for MD embryos and the embryogenic MD cell suspension culture.

of the dispersion at pH 8.0, in the presence of MEGA-8, resulted in the elution of some unbound DGAT activity and at least two peaks of enzyme activity which were eluted with a NaCl gradient. Since then, we have hypothesized that release of DGAT activity from the ion-exchange column may have involved salt-mediated solubilization of enzyme from the column matrix. Subsequently, the possible effect of NaCl on DGAT solubilization was further investigated.

In the current study, MD embryos, the embryogenic MD cell supsension culture and developing seeds of oilseed rape were

Table 1 DGAT specific activity of particulate and detergent-treated fractions of MD cultures of oilseed rape

Particulate fractions (1500–100000 ***g***) from MD embryos of *B. napus* L. cv. Reston and the embryogenic MD cell suspension culture of *B. napus* L. cv. Jet Neuf were used as a source of enzyme. DGAT specific activity was determined in the supernatant and 105000 ***g*** pellet following treatment of particulate fractions with 10 mM Tris/HCl buffer (pH 8.0) containing 2 M NaCl, 1% (w/v) MEGA-8, 20% (w/v) glycerol and 1 mM DTT at a detergent to protein ratio of 2:1. Results are the means of triplicate assays.

	Specific activity of DGAT (pmol of TG/min per mg of protein)	
Fraction	MD embryos	Cell suspension
Particulate	40.2	21.5
Solubilized	95.4	35.3
Insoluble	55.7	47.3

examined as sources of membranes for solubilization of DGAT. Particulate fractions (1500–105000 ***g***) were treated with 1% (w/v) MEGA-8, at a detergent to protein ratio of 2:1, at various concentrations of NaCl followed by centrifugation at 105000 ***g*** for 1 h to obtain the soluble enzyme. This detergent to protein ratio was previously determined to be the most effective condition for the dispersion of DGAT from membranes of MD embryos of oilseed rape (Weselake et al., 1993b). All MEGA-8/NaCl mixtures used in these experiments were stable and optically clear. Thus high concentrations of NaCl did not appear to induce separation of the solution into a detergent-rich and aqueous phase as has been recently described for Triton X-100 and Nonidet P-40 (Fricke, 1993). Solubilization results for particulate fractions from MD embryos and the embryogenic MD cell suspension system are shown in Figure 4. The percentage of enzyme solubilized as a function of NaCl concentration is shown in Figure 4(a) and the total recovery of enzyme activity, in the supernatant and pellet combined, as a function of salt concentration is shown in Figure 4(b). Using both tissues, DGAT was solubilized with maximum recovery of enzyme activity at a concentration of 2 M NaCl (Figure 4b). The enzyme was solubilized approx. 80 and 50% respectively, from membranes of MD embryos and the embryogenic MD cell suspension culture (Figure 4a). More vigorous centrifugation (200000 ***g*** for 1 h) of the NaCl/detergent-treated membranes of the cell suspension culture did not result in further sedimentation of the enzyme. The overall recovery of enzyme activity was increased by about 2-fold at 1 and 2 M NaCl respectively, for extracts of the MD embryos and embryogenic MD cell suspension culture (Figure 4b). A concentration of 3 M NaCl resulted in increased solubilization of DGAT but the overall recovery of enzyme activity was diminished. Furthermore, the original activity of the deactivated enzyme could not be restored by the removal of NaCl.

The specific activities of DGAT in non-treated membranes and the solubilized and insoluble fractions obtained following treatment of membranes with solubilization buffer containing 2 M NaCl are shown in Table 1. The specific activity of solubilized DGAT was 2.4- and 1.6-fold greater than that of the particulate enzyme from the MD embryos and the cell suspension culture respectively. The increased specific activity of the solubilized and insoluble fractions could be partially attributable to stimulation

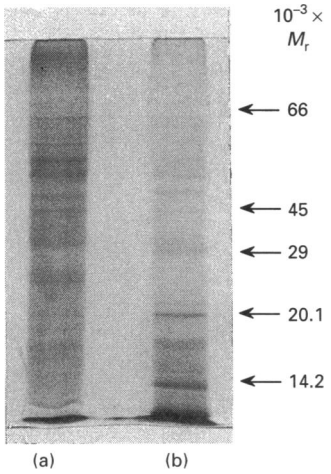

Figure 5 SDS/PAGE of particulate and solubilized DGAT from MD cultures of oilseed rape (*B. napus* L. cv. Reston)

(**a**) Particulate fraction (1500–100000 *g*) about 2.5 μg of protein applied. (**b**) Solubilized fraction. M_r makers from top to bottom were BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin (positions indicated by arrows).

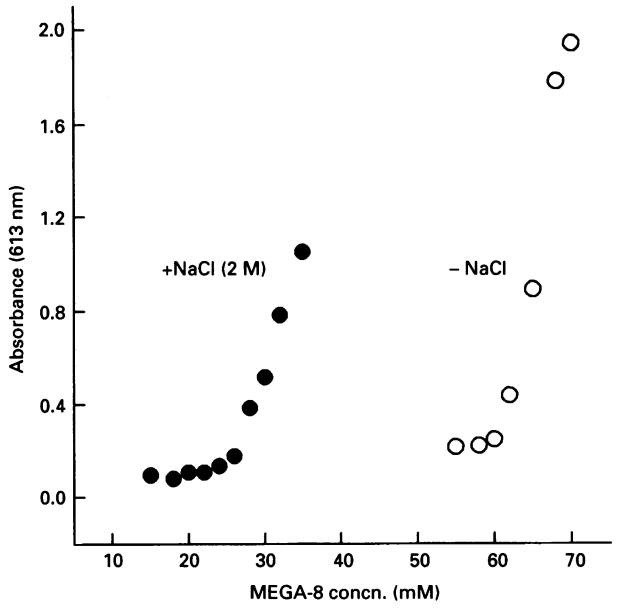

Figure 6 Absorbances of MEGA-8–ink solutions at various concentrations of detergent in the absence or presence of 2 M NaCl

The detergent buffer consisted of 10 mM Tris/HCl (pH 8.0), 20% (w/v) glycerol and 1 mM DTT. Prior to absorbance determination, detergent solutions were allowed to interact with the dye from a blue permanent marker pen as described in the Experimental section. Absorbance values represent the means of triplicate determinations. CMCs were estimated from the breaks in the two plots according to Vulliez-Le Normand and Eiselé (1993).

of DGAT activity by residual MEGA-8 and NaCl carried over into the reaction mixture. Weselake et al. (1993b) have reported that the DGAT activity of particulate fractions of MD embryos of oilseed rape was stimulated by about 25% at a concentration of 0.05% (w/v) MEGA-8 in the reaction mixture. Analysis of the particulate and solubilized fractions from MD embryos by SDS/PAGE, however, indicated that proteins were removed following enzyme solubilization, suggesting that DGAT was partially purified as a result of solubilization (Figure 5). Urea was incorporated into the separating gel because it has been shown to increase the resolution of membrane proteins during electrophoresis (Kadenbach et al., 1983).

DGAT was also solubilized from membranes prepared from developing seeds of oilseed rape. The combination of detergent and salt, however, resulted in large decreases in DGAT activity at all concentrations of salt tested (results not shown). The poor solubilization recovery of DGAT activity from developing seeds may have been related to the presence of the ovule. In this regard, the MD embyros and the embryogenic MD cell suspension system have a distinct advantage over the developing seed as a tissue source of DGAT. Furthermore, with the cell suspension system it is not necessary to isolate microspores on a continual basis and the system is also amenable to scale-up for obtaining suitable quantities of tissue for enzyme isolation (Weselake et al., 1993a).

Other plant acyltransferases have also been solubilized using detergent/salt mixtures. Frentzen (1990) reported that glycerol-3-phosphate acyltransferase was optimally solubilized from microsomes of spinach leaves by treatment with 3% (w/v) Tween 40 and 0.8 M NaCl. Hares and Frentzen (1991) also reported that a combination of 0.4% CHAPS (at a detergent to protein ratio of 1.5:1) and 0.5 M NaCl was optimal for the solubilization of acyl-CoA:1-acyl-*sn*-glycerol-3-phosphate acyltransferase from microsomes of etiolated pea shoots. In both of the above cases, however, the specific activity of the solubilized enzyme was slightly lower than that of the particulate enzyme.

In general, membrane proteins are most effectively solubilized at concentrations above the CMC of the detergent used for solubilization (Thomas and McNamee, 1990). The mechanism of solubilization of DGAT from MD cultures of oilseed rape may be linked to a lowering of the CMC of MEGA-8 caused by the presence of NaCl. The CMC of non-ionic detergents is lowered in the presence of salts (Ray and Némethy, 1971). The 1% (w/v) solution of MEGA-8 used in the current study was equivalent to a concentration of about 30 mM which was two times lower than the CMC of 60 mM (available from Boehringer–Mannheim, Laval, Quebec, Canada). As shown in Figure 6, 2 M NaCl was effective in lowering the CMC of MEGA-8 from about 60 to 25 mM. Thus 2 M NaCl may lower the CMC of MEGA-8 to a concentration where it is effective in solubilizing DGAT. It is possible that other factors are operative in promoting solubilization such as the combined requirement of high salt concentration for disruption of ionic interactions and of detergent for mobilization of hydrophobic regions. Recently, Banerjee et al. (1993) have proposed that the structural features of a detergent were more important in solubilizing membrane lipids than the CMC value of the detergent.

Properties of solubilized DGAT

The native M_r of DGAT, solubilized from MD embryos of oilseeed rape, was examined using Superose 6 gel-filtration chromatography. Chromatography in the presence of 0.1% (w/v) MEGA-8 resulted in a major peak of DGAT activity eluting at the same position as Blue Dextran, indicating an M_r of about 2×10^6 (Figure 7). Gel-filtration chromatography of proteins at detergent concentrations 10- to 100-fold lower than the detergent concentration used for solubilization has been known to result in non-specific aggregation of solubilized protein (Thomas and McNamee, 1990). Thus DGAT may have aggregated during gel-filtration chromatography in the presence of 0.1% (w/v) MEGA-8. Chromatography of solubilized DGAT

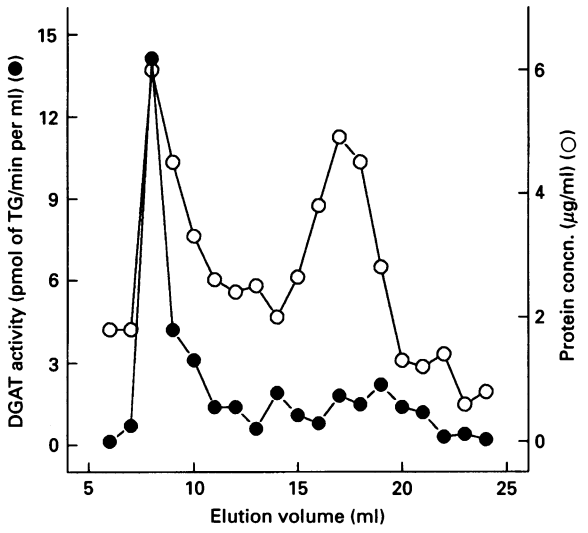

Figure 7 Superose 6 gel-filtration chromatography of solubilized DGAT from MD embryos of oilseed rape

The column was equilibrated with 10 mM Tris/HCl buffer (pH 8.0) containing 2 M NaCl, 0.1% (w/v) MEGA-8, 20% (w/v) glycerol and 1 mM DTT. Solubilized DGAT (200 μl; 380 μg of protein), prepared from *B. napus* L. cv. Reston, were injected on to the column which was operated at a flow rate of 12 ml/h using an f.p.l.c. system. Fractions of 1 ml were collected. Enzyme activity values are based on the means of duplicate assays.

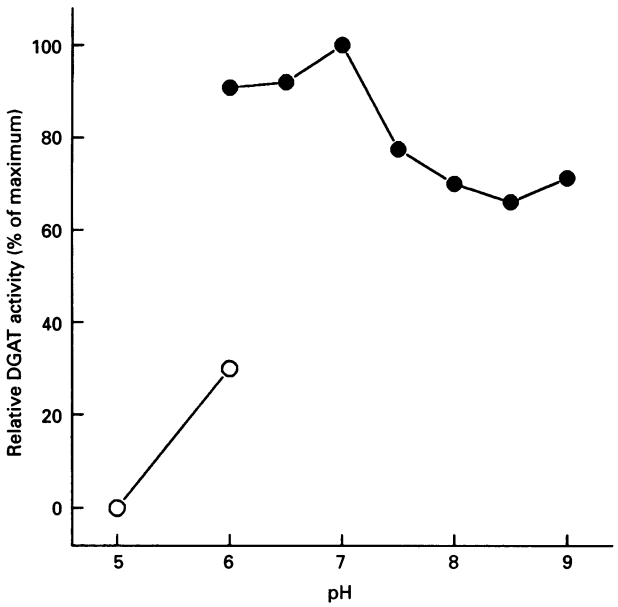

Figure 8 pH dependence of DGAT activity in the solubilized fraction from MD embryos of oilseed rape

The following 200 mM buffers were used: ●, 1,3-Bis(tris[hydroxymethyl]-methylamino)-propane/HCl; ○, succinic acid/NaOH. Reactions were allowed to proceed for 10 min using 10 μg of protein solubilized from *B. napus* L. cv. Reston. Each data point represents the mean of duplicate assays.

in the presence of 1% (w/v) MEGA-8, however, led to inactivation of the enzyme. In some runs minor activity peaks were apparent within the sieving range of the gel but the results were not reproducible. Similar results were found when using Sepharose CL-4B as a sieving matrix. Preincubation of the eluted fractions with egg yolk phosphatidylcholine prior to assaying the enzyme failed to restore any DGAT activity. It is possible that DGAT was irreversibly denatured by extensive contact with MEGA-8 during passage of the enzyme through the gel-filtration matrix equilibrated in 1% (w/v) MEGA-8. The elution of DGAT activity in association with a high-M_r complex after solubilization from microsomes, however, is consistent with the gel-filtration behaviour of solubilized DGATs from germinating soybean (Kwanyuen and Wilson, 1986, 1990) and rat liver (Polokoff and Bell, 1980; Andersson et al., 1994). The pH dependency of DGAT activity in the solubilized fraction from MD embryos is shown in Figure 8. DGAT activity was optimal at pH 7. The pH dependency of DGAT activity in its solubilized form was similar to the pH dependency for activity previously described for the particulate form of the enzyme (Weselake et al., 1991).

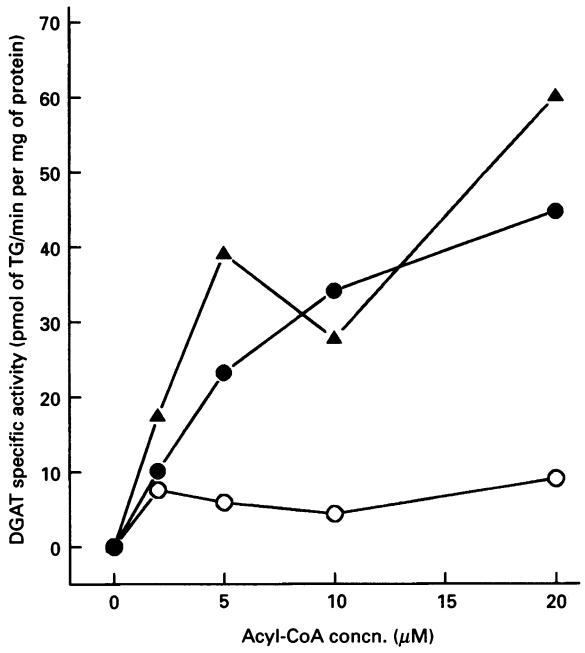

Figure 9 Acyl-CoA specificity of solubilized DGAT from MD embryos of oilseed rape

●, Oleoyl-CoA; ○, stearoyl-CoA; ▲, palmitoyl-CoA. Reaction mixtures contained 330 μM exogenous *sn*-1,2-diolein but did not contain BSA. Reactions were allowed to proceed for 10 min using 20 μg of protein solubilized from *B. napus* L. cv. Reston. Each data point represents the mean of duplicate assays.

The acyl-CoA specificity of solubilized DGAT was examined over a range of substrate concentrations using three different acyl donors (Figure 9). Bafor et al. (1990) have shown that BSA can alter the results of specificity studies with lysophosphatidic acid acyltransferase. Thus the current specificity study was conducted in the absence of BSA. At concentrations above 2 μM acyl-CoA, solubilized DGAT exhibited a considerably greater specificity for both oleoyl-CoA and palmitoyl-CoA than for stearoyl-CoA. Bernerth and Frentzen (1990) reported an enhanced specificity for palmitoyl-CoA over oleoyl-CoA in specificity studies of particulate DGAT in 5000–20000 *g* fractions prepared from developing seeds of two different cultivars of oilseed rape.

Addition of exogenous *sn*-1,2-diolein (330 μM) had essentially no effect on the rate of TG formation when implementing the

solubilized fraction from MD embryos of oilseed rape in DGAT assays. Previous studies with MD embryos of oilseed rape have shown that substantial TG formation occurred when particulate fractions were incubated with acyl-CoAs in the absence of exogenous diacylglycerol (Weselake et al., 1991; Taylor et al., 1992). For example, when homogenates of MD embryos were incubated for 60 min in the presence of [1-^{14}C]oleoyl-CoA and exogenous *sn*-1,2-diolein, 30% of the radiolabel in TG was found in trioleoylglycerol with the remaining label associated with dioleoylmonolinoleoylglycerol and dioleoylmonolinolenoylglycerol (Weselake et al., 1991). Presumably, the solubilization procedure implemented in the current study resulted in endogenous diacylglycerols being made even more accessible to DGAT than in the particulate fraction. Thus meaningful specificity studies with exogenous diacylglycerols are not possible until endogenous diacylglycerols are removed from the solubilized enzyme.

Conclusion

MD cultures of oilseed rape continue to be useful in studies of TG biosynthesis. In the current study, BSA, fluoride salts and NaCl markedly stimulated DGAT activity in particulate fractions from MD cultures of oilseed rape. NaCl was of further benefit in the solubilization of DGAT from these particulate fractions when combined with MEGA-8 at high salt concentration. Both MD embryos and an embryogenic MD cell suspension culture of oilseed rape were suitable tissue sources for solubilization of DGAT. The solubilization step enhanced the specific activity of DGAT and provided an enzyme solution for characterization and further purification.

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