CHARACTERIZATION OF TRIACYLGLYCEROL BIOSYNTHETIC ENZYMES FROM MICROSPORE-DERIVED CULTURES OF OILSEED RAPE

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ABSTRACT

Particulate and solubilized preparations of phosphatidate (PA) phosphatase (EC 3.1.3.4) and diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) from microspore-derived (MD) cultures of *Brassica napus* L. cv Topas were characterized. The activity of solubilized PA phosphatase decreased by about 50% following storage for 24 h at 4°C, whereas the activity of DGAT decreased by 30%. Bovine serum albumin increased the stability of both enzymes. Both preparations were enriched in the target enzyme and thus, may be useful in studies of regulation with limited influence by the other Kennedy pathway enzymes. Solubilized PA phosphatase was shown to dephosphorylate a number of phosphate-containing compounds and showed a preference for dioleoyl-PA and dipalmitoyl-PA over other forms of PA tested. Microsomal PA phosphatase from MD embryos was partially dependent on Mg^{2+} and partially inhibited by the thioreactive agent, N-ethylmaleimide (NEM). The partial sensitivity to NEM suggested that MD embryos of *B. napus* may contain forms of PA phosphatase involved in glycerolipid synthesis and signal transduction. NEM-sensitive and NEM-insensitive PA phosphatase activity was found in microsomes of a cell suspension culture of *B. napus* L. cv Jet Neuf. PA phosphatase, solubilized from MD embryos, was partially purified using ammonium sulfate fractionation followed by anion exchange chromatography. PA phosphatase was resolved into two distinct peaks following anion-exchange chromatography. The peaks contained both NEM-sensitive and NEM-insensitive PA phosphatase activity. Following gel filtration, solubilized PA phosphatase displayed a minimum apparent M_r of about 40 000. Antibodies raised against partially purified preparations of PA phosphatase and DGAT from MD embryos of *B. napus* L. cv Topas were used in the development of immunochemical probes for these enzymes. Inhibitory anti-PA phosphatase antibodies were developed. Attempts were also made to identify a sub-class of antibodies which could interact with both denatured and native DGAT.

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Ab	antibody
ACCase	acetyl-coenzyme A carboxylase
ACP	acyl carrier protein
ASD-CoA	12-[(4-azidosalicyl)amino]dodecanoyl-coenzyme A
B. napus	*Brassica napus*
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CDP-DG	cytidine diphosphate-diacylglycerol
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CoA	coenzyme A
CO_2	carbon dioxide
CPT	CDP-choline: 1,2-diacylglycerol cholinephosphotransferase
DE	denatured enzyme
DG	*sn*-1,2-diacylglycerol
DGAT	diacylglycerol acyltransferase
DGDG	digalactosyldiacylglycerol
DHAP	dihydroxyacetone phosphate
DTT	dithiothreitol
DW	dry weight
EC	early cotyledonary
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
FPLC	fast performance liquid chromatography
FW	fresh weight
G3P	*sn*-glycerol-3-phosphate
GPAT	glycerol-3-phosphate acyltransferase

HEPES	N-[2-hydroxyethyl]peperazine-N'-[2-ethanesulfonic acid]
IgG	immunoglobulin G
LC	late cotyledonary
LPA	lysophosphatidate
LPAAT	lysophosphatidate acyltransferase
LPCAT	acyl-CoA: lysophosphatidylcholine acyltransferase
MC	mid-cotyledonary
MD	microspore-derived
MEGA-8	octanoyl-N-methylglucamide
MG	monoacylglycerol
MGDG	monogalactosyldiacylglycerol
MOPS	(3-N-morpholino)propane sulfonic acid
M_r	apparent molecular weight
N_2	nitrogen gas
NC	nitrocellulose
NE	native enzyme
NEM	N-ethylmaleimide
*p*NPP	*p*-nitrophenyl phosphate
PA	phosphatidate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethylene glycol 8000
PG	phosphatidylglycerol
P_i	inorganic orthophosphate
PI	phosphatidylinositol
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sn	stereochemical numbering
SQDG	sulfoquinovosyldiacylglycerol
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TG	triacylglycerol
TLC	thin layer chromatography
TRIS	tris(hydroxymethyl)aminomethane
UDP-galactose	uridine-diphosphate-galactose

INTRODUCTION

Canada produces about 18% of the world's oilseed rape requirements (Anonymous, 1996). In Alberta, canola (*Brassica napus* L.) is an economically valuable oilseed crop. The production of canola in Alberta represents over 36% of Canada's overall production (Anonymous, 1996). Oil and meal are both important products obtained from canola. The oil has many uses ranging from cooking oils and salad dressings to industrial lubricants. Canola is credited for its superior quality of oil for human nutrition. The seeds have an oil content of about 40 - 50% and the oil is comprised of low levels of erucic acid and saturated fatty acids (Battey *et al.*, 1989). As well, the highly nutritional meal obtained from canola seeds, following oil extraction, contains low levels of glucosinolates (Sonntag, 1991). Oil from other *Brassica* species contain higher levels of erucic acid and are more suitable in the production of lubricants (Perry and Harwood, 1993a). The rising economic demands for seed oils required for both edible and industrial uses has resulted in extensive research in the area of plant lipid biochemistry.

Oil, or triacylglycerol (TG), is the major form of storage lipid in oilseeds. The TG molecule is comprised of three fatty acyl chains which are esterified to a glycerol backbone (Stymne and Stobart, 1987). The type of fatty acyl moieties in TG determines the potential usefulness of the oil. For example, edible oils are composed of varying amounts of saturated fatty acids such as palmitic acid (16:0) and stearic acid (18:0) and unsaturated fatty acids, such as oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) (Roughan and Slack, 1982). Seed oils containing longer fatty acids, such as erucic acid (22:1), are more suited to industrial applications such as high temperature lubricants, nylons and plasticizers (Sonntag, 1991). Increasing the overall oil content of seeds and manipulating fatty acid composition represent important goals

in oilseed breeding. Molecular approaches for the development of seeds with elevated oil content and desired fatty acid composition would benefit from a detailed knowledge of the pathways and regulatory mechanisms involved in fatty acid synthesis and TG bioassembly. The purification and characterization of the enzymes involved in these pathways is a prerequisite for designing experiments to study the regulation of TG biosynthesis.

In developing oilseeds, TG biosynthesis occurs via the enzyme-catalyzed reactions of the Kennedy pathway (Kennedy, 1961; Stobart and Stymne, 1990). This pathway involves the stepwise transfer of fatty acyl moieties onto a glycerol backbone to produce TG (Stobart and Stymne, 1990). Phosphatidate (PA) phosphatase (EC 3.1.3.4) and diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyze the penultimate and final steps of TG biosynthesis, respectively. PA phosphatase catalyzes the dephosphorylation of PA to produce *sn*-1,2-diacylglycerol (DG) which, in turn, is the substrate for the acylation by DGAT to produce TG (Kennedy, 1961; Stymne and Stobart, 1987). DG is also an important substrate in the formation of membrane phospholipids (Brindley, 1984; Ichihara *et al.*, 1989). Studies of both mammalian (Tijburg *et al.*, 1989) and plant systems (Kocsis and Weselake, 1996) have suggested that these enzymes may represent important regulatory points in TG biosynthesis. Identification of the genes which encode the enzymes of TG bioassembly has been hampered by the difficulties in isolating the enzymes from the membranes of the endoplasmic reticulum (ER) (Weselake *et al.*, 1993a; Little *et al.*, 1994; Kocsis *et al.*, 1996). The purification and characterization of PA phosphatase and DGAT would increase our understanding of seed oil formation and provide important tools for the genetic modification of oilseed metabolism.

The intent of this thesis was to further purify PA phosphatase and DGAT from microspore-derived (MD) cultures of *B. napus*. In order to assist this process, this thesis has investigated properties of these enzymes from membrane-bound and

solubilized preparations of MD cultures of *B. napus*. Optimal conditions for stability of enzyme activity and identification of N-ethylmaleimide (NEM)-sensitive and NEM-insensitive forms of PA phosphatase were investigated. These characterization studies may help researchers to design alternate purification strategies that may lead to the eventual purification of these important oil formation enzymes. These studies may also provide useful background information for studying the regulation mechanisms of these enzymes and thus enhance our understanding of TG biosynthesis in *B. napus* and in other oilseeds.

LITERATURE REVIEW

TG Biosynthesis in Developing Oilseeds

There are hundreds of varieties of plants which have oil-bearing seeds. Some of the major commercial varieties include: soybean, cotton, sunflower, groundnut, palm, coconut and rapeseed. Different varieties contain oil and products specific for different economic purposes. Some plants contain edible oils for human consumption or animal feeds, whereas others supply the industrial market with commodities such as paints, lubricants and cosmetics. TG is the major form of lipid stored within the seeds of developing oilseeds (Ohlrogge and Browse, 1995). The storage lipid serves as a source of energy during seedling germination. In avocado, olive, and palm, however, oil is stored within the mesocarp of the fruit surrounding the seed (Gurr, 1980).

The basic structure of TG consists of a glycerol backbone to which three fatty acids or acyl groups are esterified at the *sn*-1, *sn*-2, and *sn*-3 carbon positions of the glycerol molecule (Stymne and Stobart, 1987). The glycerol molecule does not exhibit rotational symmetry and thus the carbon atoms are classified by stereochemical numbering (*sn*). The composition of fatty acids in TG generally determines the quality of oil and subsequently its economic use. Hence, a variety of fatty acids in varying combinations have been found between different plant species. The most abundant fatty acids found to occur in TG are palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) (Ohlrogge and Browse, 1995) and, at least in plants, a higher proportion of unsaturated fatty acids are more common (Gurr, 1980).

The accumulation of TG follows a temporal pattern as seeds mature (Harwood, 1989). In zygotic embryos of oilseed rape, the onset of TG formation has been shown to occur between 14 and 33 days after flowering, coinciding with the appearance of TG biosynthetic enzyme activity (Weselake *et al.*, 1993b). In mature seeds, TG is found to occur in densely packed oil bodies or oleosomes (Murphy, 1993). These spherical

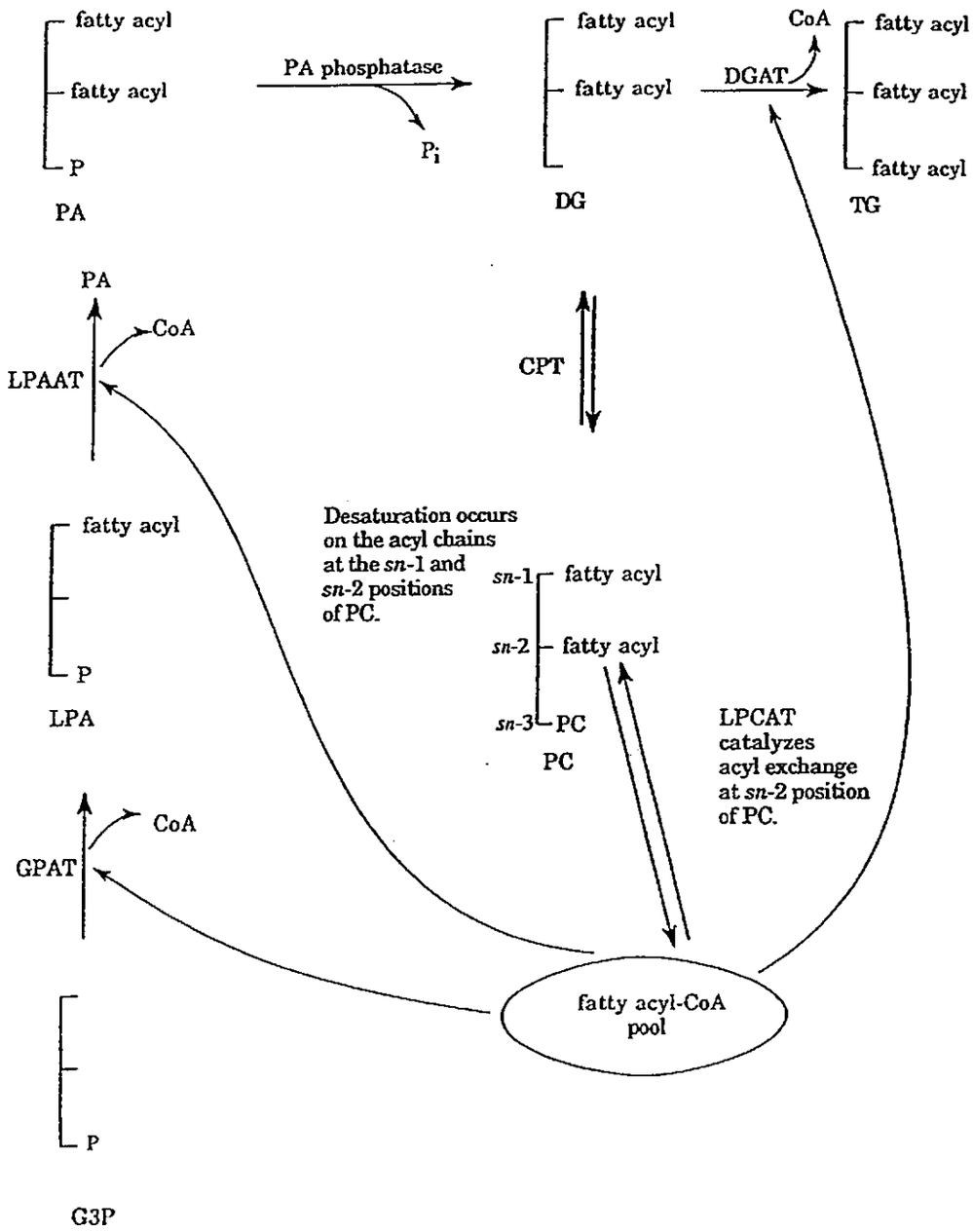
bodies are about 1 μm in diameter and increase in number with the accumulation of TG (Huang, 1992; Murphy, 1993). The TGs are surrounded by a monolayer of membrane which contains both phospholipids and proteins known as oleosins (Huang, 1992; Murphy, 1993).

Fatty acids used in TG formation are synthesized within the plastid of the plant cell. Fatty acid biosynthesis *de novo* has been reviewed extensively (Gurr, 1980; Gurr and Harwood, 1991; Sparace and Kleppinger-Sparace, 1993; Ohlrogge and Browse, 1995). In the first step of fatty acid biosynthesis, acetyl-coenzyme A (CoA) carboxylase (ACCase) catalyzes the formation of malonyl-CoA from acetyl-CoA and a carbon dioxide (CO_2) molecule, which is derived from the atmosphere. In plants, there are two different structures of ACCase which have been shown to differ in subcellular localization and the type of plant in which it occurs. One form of ACCase (also referred to as prokaryotic) found within the plastid of dicots is a multisubunit complex containing three separate and functional subunits: biotin carboxylase, biotin carboxyl carrier protein and carboxytransferase (Ohlrogge and Browse, 1995). A second form of ACCase (also referred to as eukaryotic) is similar to the ACCase found in the cytosol of both yeast and mammalian systems. This form of ACCase is a single, multifunctional complex which contains the three functional regions within a single polypeptide. This complex is located in the cytosol of dicots and occurs in both the cytosol and plastids of graminaceous plants. The ACCase found within the plastids is involved in supplying malonyl-CoA for *de novo* fatty acid biosynthesis whereas the ACCase found within the cytosol supplies malonyl-CoA for other pathways in the cytosol, including fatty acid elongation (Ohlrogge and Browse, 1995). Malonyl-CoA is the main carbon donor for fatty acid synthesis where carbon atom chain lengths for palmitic, stearic and oleic fatty acids are synthesized. The malonyl group is transferred to an acyl carrier protein (ACP) and converted to malonyl-ACP. Repeated reactions catalyzed by condensing enzymes (3-ketoacyl-ACP synthases) followed sequentially by

a reduction, dehydration and a second reduction reaction catalyzed by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and enoyl-ACP reductase, respectively, are required to produce an 18-carbon fatty acid. The 18:0-ACP may then be converted to 18:1-ACP by a stearoyl-ACP desaturase. The fatty acids are available for plastidial glycero- and galacto- lipid biosynthesis. Free fatty acids are released by hydrolysis catalyzed by an acyl-ACP thioesterase. The free fatty acids are able to pass through the membrane of the plastid into the cytoplasm. An acyl-CoA synthetase located on the outer membrane of the chloroplast envelope catalyzes the re-esterification of fatty acid with CoA which then becomes available for acyltransferase reactions to form glycerolipids in the ER (Ohlrogge and Browse, 1995).

The pathway of TG biosynthesis in relation to phosphatidylcholine (PC) synthesis is shown in Figure 1. The mainstream of TG biosynthesis follows the stepwise acylation of *sn*-glycerol-3-phosphate (G3P) to produce TG (Kennedy, 1957; Kennedy, 1961). The glycerol backbone of the TG molecule is derived from G3P which is considered to be generated in the cytosol via the reduction of dihydroxyacetone phosphate (DHAP) or from the direct phosphorylation of glycerol catalyzed by the enzyme glycerol kinase (Gurr, 1980). The four main enzymes which catalyze the reactions in the mainstream of TG biosynthesis (the Kennedy pathway) are primarily located within the membrane of the ER (Kennedy, 1961; Stymne and Stobart, 1987). The first enzyme, glycerophosphate acyltransferase (GPAT, EC 2.3.1.15), catalyzes the acylation of G3P at the *sn*-1 position forming lysophosphatidate (LPA). Recently, GPAT was solubilized and partially purified from avocado mesocarp (Eccleston and Harwood, 1995). The second enzyme, lysophosphatidate acyltransferase (LPAAT, EC 2.3.1.51), catalyzes the acylation of LPA at the *sn*-2 position to produce phosphatidate (PA). Davies *et al.* (1995) reported on a successful solubilization of LPAAT from immature coconut endosperm which further led to the partial purification and cloning of a cDNA that encoded LPAAT (Knutzon *et al.*,

Figure 1. Schematic representation of TG biosynthesis, in relation to phosphatidylcholine (PC) synthesis and modification, in developing oilseeds. Adapted from Stymne and Stobart (1987) and Stobart and Stymne (1990).


fatty acyl
fatty acyl
P
PA
PA phosphatase
P_i
fatty acyl
fatty acyl
DG
CoA
DGAT
fatty acyl
fatty acyl
fatty acyl
TG
PA
CoA
LPAAT
CPT
fatty acyl
P
LPA
Desaturation occurs on the acyl chains at the sn-1 and sn-2 positions of PC.
sn-1
fatty acyl
sn-2
fatty acyl
sn-3
PC
PC
LPCAT catalyzes acyl exchange at sn-2 position of PC.
CoA
GPAT
fatty acyl-CoA pool
P
G3P

1995). Later, Lassner *et al.* (1995) isolated a cDNA that encoded LPAAT from meadowfoam and was further able to express this enzyme in maturing rapeseed embryos. PA phosphatase catalyzes the dephosphorylation of PA to produce inorganic orthophosphate (P_i) and DG. DGAT catalyzes the final and committed step of TG biosynthesis by acylation of DG at the *sn*-3 position to yield TG. DGAT, however, may not be the only enzyme solely committed to TG bioassembly in plants. Recently a diacylglycerol transacylase was shown to catalyze a transesterification reaction using two molecules of diacylglycerol to form TG and monoacylglycerol (Lehner *et al.*, 1993).

TG bioassembly has been studied extensively in microspore-derived (MD) cultures from *Brassica napus* (Taylor *et al.*, 1990; Taylor *et al.*, 1991; Taylor and Weber, 1994). The MD embryo culture has been shown to accumulate TG in a similar manner as zygotic embryos (Taylor *et al.*, 1990, Pomeroy *et al.*, 1991). The TG produced has a fatty acid composition similar to that of the developing seed lipid (Taylor *et al.*, 1991). MD embryos can be cultured to yield large amounts of tissue at specific stages of development and thus have served as a useful system for studying TG biosynthesis (Pomeroy *et al.*, 1991; Taylor *et al.*, 1991). MD cell suspension cultures of *B. napus* L. cv Jet Neuf have also been shown to synthesize TG (Weselake *et al.*, 1993b). The cell suspension culture was accidentally developed in the early 1980s during an attempt to produce MD embryos (Simmonds *et al.*, 1991). Both the MD embryos and the MD cell suspension cultures have been used in the characterization, solubilization and partial purification of the TG biosynthetic enzymes (Weselake *et al.*, 1993b; Taylor *et al.*, 1993; Little *et al.*, 1994; Kocsis *et al.*, 1996).

Although some progress has been made in the solubilization and partial purification of the Kennedy pathway enzymes, further studies with purified enzymes and isolated genes are required to elucidate regulatory mechanisms underlying TG

biosynthesis. A detailed understanding of TG biosynthesis will set the foundation in a systematic approach to improving the fatty acid composition of seed oil.

Glycerolipids not only function as a storage source for fuel in the form of TG, but they also serve as the major constituents of biological membranes (Harwood, 1989). It is difficult to discuss TG biosynthesis without discussing membrane lipids. There are two major forms of glycerolipids distinguished by the polar head group, those containing a phosphorous molecule (phosphoglycerols) and those without phosphorous but containing a sugar molecule (glycosylglycerols) (Harwood, 1989). Glycerolipids are also divided into two groups according to the pattern of the acyl groups esterified to the *sn*-1 and *sn*-2 position of the glycerol backbone (Frentzen, 1993). One group contains C_{16} fatty acids at the *sn*-2 position, whereas the *sn*-1 position is more commonly comprised of C_{18} fatty acids (Frentzen, 1993). This pattern which is characteristic for cyanobacteria is termed "prokaryotic" and is found exclusively in the plastid of higher plant cells. The "eukaryotic" pattern of acyl distribution occurs both inside and outside of the plastid and displays C_{18} fatty acids at the *sn*-2 position and either C_{16} or C_{18} fatty acids at the *sn*-1 position (Harwood, 1989; Frentzen, 1993). The biosynthesis of glycerolipids occurs as in the first two steps of the Kennedy pathway where PA is produced via the stepwise acylation of G3P at the *sn*-1 and *sn*-2 position of the glycerol backbone (Frentzen, 1993). PA, via cytidine diphosphate (CDP)-diacylglycerol, is a key intermediate in the biosynthesis of phosphatidylglycerol (PG) and phosphatidylinositol (PI) (Frentzen, 1993). It is also the intermediate for the phospholipase A catalyzed hydrolysis reaction which yields G3P, and the PA phosphatase catalyzed dephosphorylation reaction to produce DG (Harwood, 1989). DG is required for the formation of important membrane phospholipids such as PC and phosphatidylethanolamine (PE), and for TG storage lipid biosynthesis (Harwood, 1989).

Within the chloroplasts, the biosynthesis of the glycosylglycerols yields three major lipid forms. Monogalactosyldiacylglycerol (MGDG) is formed by the transfer of galactose from uridine diphosphate (UDP)-galactose to diacylglycerol. Digalactosyldiacylglycerol (DGDG) can be synthesized either by the transfer of a second galactose from UDP-galactose to MGDG or inter-lipid transfer between two MGDG molecules (Harwood, 1989). There has been much debate on the mechanism for the formation of the third major glycosylglyceride, sulphoquinovosyldiacylglycerol (SQDG) although the enzymes necessary for sulpholipid synthesis are thought to be present in chloroplasts (Gurr and Harwood, 1991). In eukaryotes, MGDG and DGDG typically contain polyunsaturated fatty acids at both *sn*-1 and *sn*-2 positions of the glycerol backbone (Harwood, 1980). Some plants containing, predominantly, the fatty acid linolenic (18:3) are called "18:3" plants whereas other plants containing appreciable amounts of hexadecatrienoic acid (16:3) are called "16:3" plants (Joyard and Douce, 1987). In MGDG, 16:3 is only present at the *sn*-2 position of the glycerol backbone. MGDG has been shown to contain both eukaryotic and prokaryotic patterns of fatty acyl structure (Harwood, 1989).

The acyl CoA pool within the cytoplasm of the developing seed contributes to the vast complexity of lipids. These variations of common 16- and 18- carbon fatty acids found in membrane lipids occur via an oleate-containing DG molecule which is incorporated into PC by the action of CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (CPT) (Stobart and Stymne, 1990). The action of this enzyme in relation to TG biosynthesis is shown in Figure 1. The oleate-containing PC molecule is the substrate for the desaturation of oleate to linoleate and further desaturation to linolenate. Other fatty acyl moieties derived from acyl CoAs can be transferred to the *sn*-2 position of PC by the action of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) (Stobart and Stymne, 1990). This reaction is also reversible and similarly allows for the incorporation of

polyunsaturated fatty acids back into the acyl CoA pool for acylation of other positions of the glycerol backbone (Stobart and Stymne, 1990). Desaturation of acyl chains in PC and the action of LPCAT are depicted in Figure 1.

Unusual fatty acid structures including medium chain fatty acids (C_8-C_{12}), very long chain fatty acids (C_{20}-C_{24}) or substitutions such as hydroxyl, epoxy, and cyclopropane groups do not accumulate within the membrane lipid fractions but are targeted to TG (Battey and Ohlrogge, 1989; Vogel and Browse, 1996). This may be a result of a spatial separation of two different DG pools (Vogel and Browse, 1996). Stearate is the longest fatty acyl chain produced by the reactions catalyzed by the fatty acid synthase complex. Very long chain fatty acids (eg 20:1, 22:1) are formed via a malonyl-CoA elongation of preformed acyl chains on the ER (Harwood, 1989). In very long chained unsaturated fatty acids, the double bond occurs during the desaturation of stearate to oleate and is subsequently retained during elongation (Harwood, 1989).

PA Phosphatases of Plants

The study of PA phosphatase of mammals and yeast is considerably more advanced than what we know about this enzyme in higher plants. In plants, PA phosphatase catalyzes the formation of DG, which is a key intermediate in the biosynthesis of both membrane lipids and TG lipids (Stymne and Stobart, 1987). DG is the substrate for the synthesis of plastidial galactosylglycerolipids including MGDG, DGDG and SQDG, and important phospholipids including PC, PE, PI, PG and phosphatidylserine (PS) (Stobart and Stymne, 1990). PA phosphatase was first observed in subcellular fractions of spinach leaves by Kates (1955) and has since been further characterized in a number of plant systems.

In the leaves of broad bean (*Vicia faba*), PA phosphatase was found to be associated with the membrane of the ER but was also observed in a soluble form

(Königs and Heinz, 1974). Very little activity was associated with the chloroplast. The ER-enzyme, however, was solubilized by sonication in the presence of 0.2% (w/v) Triton X-100. Following centrifugation of the solubilized fraction at 100 000 x *g*, the activity of the membrane-bound form of PA phosphatase was located in the supernatant fraction. The PA phosphatase was inhibited by sucrose and Ca^{2+} ions.

Studies on the leaves of groundnut (*Arachis hypogaea*) revealed a plastidial and a microsomal PA phosphatase (Rajaskeharan and Sastry, 1989; 1990). Both phosphatases were insensitive to phenoxy, thiocarbamate, urea and uracil herbicides.

The majority of studies on PA phosphatases from leaves have focused on a plastidial PA phosphatase. An alkaline PA phosphatase was found in the envelope membrane of both spinach chloroplasts (Joyard and Douce, 1977; 1979) and pea chloroplasts (Andrews *et al.*, 1985). The enzyme was further localized in the inner envelope membrane of chloroplasts and was found to be strongly inhibited by Mg^{2+} (Block *et al.*, 1983; Andrews *et al.*, 1985; Malherbe *et al.*, 1992). The formation of DG from the dephosphorylation of PA catalyzed by PA phosphatase is an important step in the synthesis of MGDG, DGDG and SQDG in chloroplasts. Chloroplast PA phosphatase may play a regulatory role in the formation of these galactolipids and may be linked to the occurrence of "16:3" and "18:3" plants. The activity of chloroplast PA phosphatase was found to be higher in "16:3" plants as compared to "18:3" plants (Gardiner and Roughan, 1983; Heinz and Roughan, 1983; Gardiner *et al.* 1984; Malherbe *et al.*, 1995). In "16:3" plants, DG is more readily formed from PA containing 18:1 and 16:0 fatty acids at the *sn*-1 and *sn*-2 positions, respectively, of the glycerol backbone. Subsequently, synthesis of glycerolipids from these fatty acids precursors results in lipids which display this "prokaryotic" DG structure. Plants comprising the "18:3" category exhibit a lower rate of DG synthesis and cannot synthesize MGDG with 16:3 fatty acids at the *sn*-2 position of the glycerol backbone.

The properties of extraplastidial PA phosphatases from plant tissues are similar to those of mammalian and yeast PA phosphatases. The properties of chloroplast PA phosphatase, however, are quite different. The chloroplast enzyme is tightly bound to the inner envelope membrane (Joyard and Douce, 1977; Block *et al.*, 1983) whereas in mammalian cells (Bell and Coleman, 1983) and yeast (Morlock *et al.*, 1988), PA phosphatase activity is found in both the cytosol and microsome. The pH optimum of plastidial PA phosphatase was 9.0 and was inhibited by Mg^{2+} (Block *et al.*, 1983). Enzymes involved in fatty acid biosynthesis also have been shown to exhibit an alkaline pH optimum (Sauer and Heiss, 1983). In mammals and yeast, the pH optimum for PA phosphatase activity was around neutrality and the enzymes were Mg^{2+}-dependent (Bell and Coleman, 1983).

In other experiments with chloroplast inner envelope membrane, Malherbe *et al.* (1992) demonstrated that DG was a competitive inhibitor of PA phosphatase. This feedback inhibition may indicate a regulatory mechanism for galactolipid synthesis. Malherbe *et al.* (1995) solubilized PA phosphatase from spinach chloroplast envelope membranes in the presence of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). DG was also shown to increase solubilization of PA phosphatase and may be an activator of the enzyme. Solubilized PA phosphatase was inhibited by Mg^{2+} as well as by other divalent cations including Mn^{2+}, Zn^{2+}, and Ca^{2+}. DG and P_i inhibited PA phosphatase activity suggesting a regulatory role for these compounds. The addition of nucleotides (di- or tri- phosphates) had no effect on PA phosphatase activity. The solubilized enzyme was stable following storage for 24 h at 4°C in CHAPS but further storage resulted in a dramatic decrease in enzyme activity. The addition of ß-mercaptoethanol and glycerol helped to stabilize the enzyme activity. The enzyme was partially purified by hydroxyapatite chromatography. Three peaks of protein were resolved following hydroxyapatite chromatography. The third peak displayed a specific activity which was 4- to 5- fold greater than that of the

solubilized enzyme. The peak contained a major polypeptide with an apparent M_r of 37 000, as well as other minor polypeptides which ranged in M_rs from about 20 000 to 100 000.

A number of studies with germinating seeds have identified extraplastidial forms of PA phosphatases. Two separate PA phosphatase activities were present in imbibed mung bean (*Vigna radiata*) cotyledons (Herman and Chrispeels, 1980). One form of PA phosphatase was bound to the ER and displayed a pH optimum of 7.5. The second PA phosphatase was associated with protein bodies and had a pH optimum of 5.0. Following separation from non-specific acid phosphatase activity by gel filtration chromatography, the apparent M_r of the acidic PA phosphatase was about 37 000. The PA phosphatase activity was shown to increase during seedling growth. It was speculated that the PA phosphatase identified may play a role in phospholipid breakdown during seedling germination rather than TG formation.

Studies with endosperm from germinating castor bean (*Ricinus communis*) seeds revealed a PA phosphatase associated with the ER when assays were conducted at pH 7.4 (Moore *et al.*, 1973). Later, Moore and Sexton (1978) identified two separate PA phosphatases from the same tissue. A soluble PA phosphatase was observed when fractions were assayed at pH 4.25 and a microsomal PA phosphatase was identified when assays were conducted at pH 6.0. The total activity of the soluble PA phosphatase was 12-fold greater than that of the microsomal PA phosphatase. Both phosphatases were inhibited by Mg^{2+}.

In senescing tissues and germinating seeds, PA phosphatase appears to be involved in membrane metabolism. PA phosphatase activity from senescing carnation (*Dianthus caryophyllus*) flowers was strongly stimulated by Ca^{2+} and mediated through calmodulin (Paliyath *et al.*, 1987). Similarly, microsomal membrane from senescing bean (*Phaseolus vulgaris*) cotyledons also showed PA phosphatase activity (Paliyath and Thompson, 1987). The enzyme was tightly bound to the membrane

since attempts to solubilize the enzyme with detergent resulted in a pellet enriched in enzyme activity as compared to microsomal activity. The activity was stimulated by Ca^{2+} and was calmodulin-dependent. Ca^{2+} may serve as a secondary messenger and thus PA phosphatase may be a regulator of phospholipid degradation.

In order to study PA phosphatase involved in TG formation, it is more desirable to use developing oilseeds as a tissue source because these tissues exhibit a high level of TG synthesis (Stobart and Stymne, 1990). Oilseeds synthesize TG in a discontinuous manner (Harwood, 1989; Stobart and Stymne, 1990). Shortly after flowering, there is no accumulation of oil but, eventually, a rapid phase of storage lipid accumulation occurs. Finally, as the mature seed undergoes dehydration, there is very little further synthesis of lipids. In studies with developing oilseeds, activities of TG biosynthetic enzymes have been shown to increase during the rapid phase of oil formation (Sukumar and Sastry, 1987; Ichihara *et al.*, 1990; Tzen *et al.*, 1993; Weselake *et al.*, 1993b). In developing groundnut (*Arachis hypogaea*) seeds, Sukumar and Sastry (1987) identified PA phosphatase activities in both mitochondria and microsomal fractions. There was very little activity associated with the oil bodies in the "fat fraction" and there was no activity observed in the cytosol. Both phosphatases, however, displayed a sharp increase in specific activity at 30 days after fertilization which coincided with the active period of lipid accumulation (20 - 35 days after fertilization). In maturing safflower (*Carthamus tinctorius*) seeds, Ichihara *et al.* (1990) observed a cytosolic and microsomal form of PA phosphatase. It was shown that the combined and relative activities of these two forms of PA phosphatases varied with seed maturation. At the onset and during the final stages of seed maturation, PA phosphatase was found in the cytosol and during rapid TG accumulation, most of the PA phosphatase activity was associated with the microsomal fraction. This translocation of PA phosphatase from a reservoir in the cytosol to the membrane of the ER has been reported in mammalian systems (Brindley, 1984). In developing safflower seeds, the fatty acids palmitate,

stearate, and oleate, which were formed in the plastids, initiated the translocation of PA phosphatase from the cytosol to the microsome (Ichihara *et al.*, 1990). This translocation was reversible upon the removal of the fatty acid. Oleoyl-CoA, was ineffective in facilitating PA phosphatase translocation in developing safflower. In contrast, this acyl-CoA was effective in promoting translocation in mammals (Martin-Sanz *et al.*, 1984). Laurate and linoleate, which were not products of fatty acid biosynthesis in plastids, were also ineffective in promoting translocation of PA phosphatase. Ichihara *et al.* (1990) also demonstrated that the cytosolic PA phosphatase from safflower seeds could be induced to translocate to the microsome of sunflower but not to the microsomes of oilseed rape or soybean.

Most of the research on PA phosphatase in developing safflower seeds has focused on characterization of the enzyme in the microsomal fraction. The activity of PA phosphatase in the fraction was optimal between pH 6 - 7 (Ichihara *et al.*, 1989). Activity was inhibited in the presence of ethylenediamine tetraacetate (EDTA) but this inhibition was overcome with the addition of Mg^{2+} (Griffiths *et al.*, 1985; Ichihara *et al.*, 1989). The effect of divalent cations was the opposite to that observed by plastidial PA phosphatase. Mn^{2+} slightly stimulated PA phosphatase and Ca^{2+} showed no stimulatory effect (Ichihara *et al.*, 1989). The enzyme from chloroplast envelope membranes was inhibited in the presence of Mg^{2+} and other divalent cations such as Mn^{2+}, Ca^{2+} and Zn^{2+} (Block *et al.*, 1983; Malherbe *et al.*, 1995). PA phosphatase from safflower was also stimulated by PC and bovine serum albumin (BSA) (Ichihara *et al.*, 1989). Both of these compounds were routinely incorporated into the assay mixture (Ichihara *et al.*, 1989). PC and BSA appeared to provide a membrane-like environment in which PA may have been more accessible to PA phosphatase. Ichihara *et al.* (1989) noted that the enzyme was more thermolabile than other acyltransferases of the Kennedy pathway but when stored at -20°C, it was stable for three weeks.

Regulation studies of PA phosphatase indicated that P_i did not inhibit PA phosphatase activity but the activity was inhibited by spermidine and spermine (Ichihara *et al.*, 1989). Polyamines, such as spermidine and spermine, may play a role in regulating TG biosynthesis as they are thought to be able to bind to negatively charged phospholipid head groups or other anionic sites on membranes, thus altering the stability and fluidity of the membranes. Polyamines may facilitate the interaction of PA phosphatase with the membrane or lipid components of the membrane which may result in increased translocation of PA phosphatase from the cytosol to the membrane where TG biosynthesis occurs. Specificity and selectivity studies with maturing safflower suggested that PA phosphatase did not affect fatty acid composition of TG (Ichihara, 1991). Microsomal PA phosphatase was more specific and selective for PA containing unsaturated fatty acyl groups but could also accept PA containing saturated fatty acids to some extent.

Recently, PA phosphatase from developing seeds and MD cultures of oilseed rape (*B. napus*) was partially characterized (Kocsis *et al.*, 1996). Soluble and microsomal PA phosphatase activities were identified in developing seed (*B. napus* L. cv Westar) and were distinguished by their activities at various pHs. The microsomal PA phosphatase had a pH optimum of 6 - 7 whereas the soluble PA phosphatase had a pH optimum of 5.0 and may have been a non-specific acid phosphatase. Microsomal PA phosphatase was also identified in MD embryo and cell suspension cultures. PA phosphatase was further solubilized from microsomes of MD embryos using 0.4% (w/v) Tween 20 at a detergent to protein ratio of 1:1 (w/w). The solubilized enzyme remained in the supernatant following centrifugation at 105 000 *g* for 1 h. Solubilization was confirmed by the fact that enzyme activity eluted within the sieving range of a Superose 6 gel filtration column. The specific activity increased two- to three- fold over that in the microsomes following solubilization with 0.4% (w/v) Tween 20. Kocsis *et al.* (1996) reported that the solubilized PA phosphatase was less

stable than the microsomal PA phosphatase. About 50% of the enzyme activity was lost upon freezing and thawing, and also when the enzyme was stored for 24 h at 4°C.

DGATs of Plants

DGAT catalyzes the final and committed step in TG biosynthesis (Stymne and Stobart, 1987). The enzyme catalyzes the acylation of the *sn*-3 position of DG to yield TG using acyl-CoA as an acyl donor. A number of studies have indicated that this step of the Kennedy pathway may be the rate-limiting step in TG biosynthesis. For example, Ichihara *et al.* (1988) reported that the specific activity of the acylation reaction by DGAT in maturing safflower seeds was about 3 nmol/min per mg protein, which was much lower than those reported for the other enzymes of the Kennedy pathway which are involved in TG synthesis. This accumulation of DG was also observed in work with maturing safflower seeds when radioactivity from [^{14}C]acetate was detected in DG before its accumulation in TG (Ichihara and Noda, 1980). Perry and Harwood (1993a, 1993b) also observed a build up of DG during the rapid phase of oil accumulation in maturing *B. napus*.

Ichihara and Noda (1982) have identified DGAT activity in the microsomal fraction of maturing safflower seeds. The activity could be stimulated by *sn*-1,2-DGs immersed in a gelatin solution but, the activity was inhibited by metal ions such as Mg^{2+} and Ca^{2+}. DGAT displayed a broad specificity and was inhibited in the presence of BSA. Martin and Wilson (1983, 1984, 1985) identified DGAT activity in chloroplast envelopes and oil body organelles of spinach leaves. In this system, there were insignificant levels of DGAT activity observed in the ER. DGAT activity was optimum at pH 8.0 and was stimulated about two-fold by Mg^{2+} and Mn^{2+}. DGAT in leaves might play a role in the synthesis of intraplastidial oil bodies which have been observed in micrographs of chloroplasts from many tissues (Martin and Wilson, 1983). It is possible that the mechanism for TG synthesis in leaf tissue may be quite different

from plant organs, such as seed, which accumulates great quantities of TG (Martin and Wilson, 1985). Recently, TG has been shown to accumulate within the root plastids of peas (Xue *et al.*, 1996). Martin and Wilson (1985) also identified DGAT activity associated with the plastid in developing soybean seed. DGAT activity was shown to be localized in the ER-containing fractions of germinating soybean cotyledons (Settlage *et al.*, 1995).

Other studies of DGAT from developing seeds have reported activity associated with both the microsomal and oil body fractions (Murphy and Mukherjee, 1987; Murphy, 1988). DGAT has also been investigated in the microsomal fraction of maturing maize scutellum, soybean cotyledon, peanut cotyledon and castor bean endosperm (Cao and Huang, 1986). In maize, DGAT had a pH optimum of 6 - 7 and was associated with the rough ER. Acyl-CoA preference of DGAT appeared to be species-dependent (Cao and Huang, 1987). Ichihara *et al.* (1988) observed in maturing safflower, that there was no strict selectivity for acyl-CoA by DGAT. The fatty acids at the *sn*-3 position depended upon the acyl-CoA pool within the cell. It was also observed that the acyl-CoA specificity of DGAT differed with plant species. Spinach, castor bean and maize were specific for 16:0-CoA and 18:2-CoA whereas groundnut and safflower showed very broad specificities. DGAT from oil body and microsome fractions from oil palm mesocarp displayed higher specificity for 18:1-CoA over 16:0-CoA and 18:0-CoA (Chew and Oo, 1989; Oo and Chew, 1992). The oil body fraction was selective for 18:1-CoA whereas microsomal DGAT did not show any significant acyl-CoA selectivity. DGAT from oil body fractions also showed a requirement for Mg^{2+}, dithiothreitol (DTT), gelatin and fat free BSA for optimum activity.

Sukumar and Sastry (1987) identified DGAT activity in maturing groundnut seed. The activity was mainly microsomal with a pH optimum of 8.0. Similar to PA phosphatase, DGAT activity may be coordinated with the accumulation of lipid during seed maturation. DGAT was demonstrated to have a sharp peak of activity at 30 days

after fertilization which falls within the period where groundnut is actively accumulating lipid as TG. This has also been observed in other developing oilseeds, such as maize, oilseed rape and safflower (Tzen *et al.*, 1993; Weselake *et al.*, 1993b).

The only extensive purification of DGAT from a plant source was reported by Kwanyuen and Wilson (1986). In studying developing oilseeds, it is often difficult to obtain tissues that are simultaneously at the same stage of development. Wilson and Kwanyuen (1986) found that germinating soybean (*Glycine max* L.) cotyledons were capable of some degree of TG biosynthesis. The investigators concluded that germinating soybean was a uniform source of the enzyme which, was suitable for purification work (Kwanyuen and Wilson, 1986). DGAT was initially solubilized from the microsomes with the detergent CHAPS and further purified by Sepharose CL4B chromatography and agarose gel electrophoresis. The enzyme was purified 3000-fold and was relatively stable for 3 months at -20°C. Further characterization of the purified DGAT revealed that the enzyme consisted of 3 subunits with molecular weights of 40.8, 28.7, and 24.5 kDa and occur in a molar ratio of 1:2:2 (Kwanyuen and Wilson, 1990). The 5 polypeptides formed a 153.1 kDa subcomponent and the native enzyme was proposed to consist of about 10 subcomponents. Kwanyuen and Wilson (1990) speculated that a greater proportion of the surface area of the 40.8 kDa subunit could be exposed to the cytosol whereas both the small subunits were more deeply embedded within the membrane. Recently, this DGAT preparation was reported to contain oil body proteins (Wilson *et al.*, 1993) suggesting that interpretations about native quaternary structure may be premature at this time.

Bernerth and Frentzen (1990) reported high DGAT activity in the 20 000 *g* and 100 000 *g* fractions from developing seeds of *B. napus* L.. High levels of DGAT activity have also been found to be associated with both the 10 000 *g* and the 100 000 *g* fractions of MD embryos of *B. napus* L. (Weselake *et al.*, 1991). MD embryos have been used in further purification of microsomal DGAT. The enzyme was dispersed

from the membrane of particulate fractions with the non-ionic detergent, octanoyl-N-methylglucamide (MEGA-8) (Weselake *et al.*, 1993a). Glycerol was also shown to stimulate DGAT activity. DGAT activity was further separated into different forms following Mono Q ion exchange chromatography. There was about 40% recovery of enzyme activity and 10- to 80- fold purification of DGAT. Gel filtration chromatography on Sepharose CL4B indicated DGAT may be a part of a large complex. Little *et al.* (1994) solubilized DGAT from microsomal fractions of MD embryo and cell suspension cultures of *B. napus*. DGAT was solubilized from the 1500 - 100 000 *g* particulate fraction with 1% (w/v) MEGA-8 and 2 M NaCl. There was about 80% solubilization of DGAT from membranes of MD embryos and 50% solubilization from membranes of the cell suspension. DGAT activity in the solubilized fractions from MD embryos was 2-fold greater than that of the particulate fraction. The solubilized DGAT was stimulated 4- to 5- fold with 3 to 4 mg BSA, and 2- to 3- fold and 1.4-fold with fluoride salts and NaCl, respectively. Following gel filtration chromatography, solubilized DGAT displayed an apparent M_r of 2 000 000. The pH optimum was 7.0 and the solubilized enzyme displayed a preference for 18:1-CoA and 16:0-CoA over 18:0-CoA.

Photoaffinity-labeling of DGAT in microsomes of MD embryos has also been attempted (Weselake *et al.*, 1994). 12-[(4-Azidosalicyl)amino]dodecanoyl-CoA (ASD-CoA) and 12-azidooleoyl-CoA substrate analogs were effective in the inhibition of DGAT activity. Photoaffinity-labeling of particulate and solubilized fractions with the substrate analogs suggested that these analogs may be useful as "probes" in the isolation of DGAT.

Mutants defective in TG biosynthesis may prove useful in both genetics and developing insights into the regulation of TG biosynthesis. Katavic *et al.* (1995) identified a mutant of *Arabidopsis thaliana* with reduced levels of very long chain fatty acids. The mutant also displayed decreased DGAT activity and a reduced TG

biosynthetic capacity. This was the first indication that a change in TG biosynthesis at the level of the Kennedy pathway could result in a dramatic change in extraplastidial fatty acid modification (Katavic *et al.*, 1995). As a result of the decrease in DGAT activity, 18:1 was readily available for desaturation to yield 18:3, whereas elongation of 18:1 to produce 20:1 did not occur.

Progress in the Purification and Characterization of PA Phosphatases and DGATs from other Organisms

Our knowledge of plant enzymes is often preceded by existing knowledge on equivalent enzymes found in mammalian and microbial systems (Weselake and Jain, 1992; Kocsis and Weselake, 1996). Thus, it is worthwhile to examine the literature dealing with the purification of PA phosphatase and DGAT in these systems.

In mammalian tissue, two different forms of PA phosphatase have been identified which perform separate functions (Jamal *et al.*, 1991; Brindley and Waggoner, 1996). The enzyme involved in glycerolipid synthesis has been referred to as PA phosphatase-1 (Jamal *et al.*, 1992). This PA phosphatase, which is Mg^{2+}-dependent and sensitive to the thiol-reactive reagent N-ethylmaleimide (NEM), is thought to accumulate within the cytosol and become functionally active in glycerolipid biosynthesis following translocation to the membrane of the ER (Brindley, 1984; Gomez-Muñoz *et al.*, 1992). A second form of PA phosphatase does not display a requirement for Mg^{2+} and is insensitive to NEM. This form of PA phosphatase, which is associated with the plasma membrane, is thought to play a role in signal transduction and has been referred to as PA phosphatase-2 (Jamal *et al.*, 1991; Brindley and Waggoner, 1996). In mammalian systems, NEM-sensitive PA phosphatase has been partially purified from rat liver (Butterwith *et al.*, 1984). PA phosphatase was purified 416-fold from the soluble fraction of rat liver following adsorption on calcium phosphate, chromatography on DE-52 DEAE cellulose, separation on Ultrogel AcA-34

and chromatography on CM Sepharose 6B. Two peaks of activity were observed following CM Sepharose 6B chromatography. Activity near the void volume corresponded to an apparent M_r of 2 000 000 whereas activity in the second peak corresponded to an apparent M_r between 12 000 and 68 000. In the presence of phospholipids, PA phosphatase displayed clearer separation into two distinct peaks following gel filtration. PA phosphatase was stimulated by Mg^{2+} and was inhibited 82% by 1 mM NEM. Tween 20 helped to stabilize the enzyme activity.

NEM-insensitive PA phosphatase has been purified to homogeneity from porcine thymus (Kanoh *et al.*, 1992) and rat liver (Fleming and Yeaman, 1995a; Waggoner *et al.*, 1995). PA phosphatase was purified about 2300-fold from the membrane of porcine thymus. Following solubilization with ß-octylglucoside and Triton X-100, PA phosphatase was purified by fractionation with ammonium sulfate, and chromatography on Sephacryl S-300, hydroxylapatite, heparin-Sepharose and Affi-Gel Blue. PA phosphatase displayed an apparent M_r of 83 000 and was Mg^{2+}-independent and insensitive to NEM. In a recent review, Brindley and Waggoner (1996) have indicated that the apparent M_r of 83 000 was an artifact and that the M_r of PA phosphatase may be 35 000.

Fleming and Yeaman (1995a) purified PA phosphatase-2 5900-fold from membrane fractions of rat liver using a procedure similar to that of Kanoh *et al.* (1992). Following chromatography on Sephacryl S-300, PA phosphatase was separated into two peaks of activity. PA phosphatase 2A was eluted in the void volume and contained approximately 35% of the enzyme activity whereas PA phosphatase 2B eluted with a V_e/V_o of approximately 1.30 and contained the remainder of the activity. The peaks were separately pooled for further purification. PA phosphatase 2A was purified approximately 2600-fold, but was not purified to homogeneity. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the PA phosphatase 2A fraction was found to contain 3 proteins with apparent M_rs of 83 000,

54 000, and 34 000. PA phosphatase 2B was purified to apparent homogeneity and displayed a 5900-fold increase in purification. It was suggested that PA phosphatase 2A and PA phosphatase 2B represent different isoenzymes of PA phosphatase-2. Both the rat liver and porcine thymus enzyme had similar properties. For instance, PA phosphatase displayed an apparent M_r of 83 000 and was insensitive to NEM and did not require Mg^{2+}. Both enzymes were inhibited by Mn^{2+}, Zn^{2+} and amphillic amines, and were not activated by fatty acids or phospholipids. The enzymes displayed differences in native M_rs and responded differently to Ca^{2+}, DG and MG. These differences between the PA phosphatases from the two tissues may have important implications in their regulation.

Waggoner *et al.* (1995) purified PA phosphatase-2 from the plasma membranes of rat liver and further separated cationic and anionic forms of the enzyme. The cationic form displayed an apparent M_r of 51 000 and an isoelectric point of 9 whereas the anionic form displayed an M_r of about 53 000 and an isoelectric point of < 4. The cationic form was purified following a series of chromatographies including hydroxylapatite, Quaternaryammonium Sepharose Fast Flow, Sulfonate Sepharose Fast Flow and Mono-Q. The cationic form was separated into two peaks of activity. The initial peak was purified to homogeneity following chromatography with immobilized wheat germ agglutinin and hydroxylapatite. The anionic form was also separated into two peaks of activity following Mono-Q chromatography and purified about 2700-fold. Polyclonal antibodies raised against the cationic and anionic forms of PA phosphatase were used to confirm the identity of these enzymes. The anionic form of PA phosphatase-2 could be converted to the cationic form by the addition of neuramidase. Thus, the two forms most likely represented different sialated states of the protein.

Recently, an immunological approach was used to identify another form of NEM-insensitive PA phosphatase from rat liver (Siess and Hofstetter, 1996). PA phosphatase was solubilized from rat liver membrane using sodium cholate and Thesit.

PA phosphatase was partially purified 600-fold by chromatography, immunoadsorption and glycerol gradient centrifugation. Antibodies developed against the partially purified PA phosphatase were further used to identify denatured PA phosphatase on immunoblots resulting from SDS-PAGE of partially purified enzyme according to the immunodetection method described by Muilerman *et al.* (1982). In this method, the denatured enzyme protein on nitrocellulose is incubated with excess polyclonal antibodies, to increase the possibility of generating complexes where only one of two antigen binding sites on a divalent antibody remain free. Upon subsequent incubation with nondenatured enzyme, a "sandwich" of denatured enzyme/antibody/nondenatured enzyme is formed which can be detected following incubation with enzyme substrate. Using this method, Siess and Hofstetter (1996) detected a PA phosphatase with an apparent M_r of about 31 000.

The various purified forms of NEM-insensitive PA phosphatase from rat liver membranes have displayed a number of different M_rs. This may be attributable to different isoenzymes. These isoenzymes may be subject to different modes of regulation.

Hosaka and Yamashita (1984) identified PA phosphatase activity in both membrane and soluble fractions of yeast *(Saccharomyces cerevisiae)*. PA phosphatase was partially purified from the soluble fraction following fractionation with ammonium sulfate and polyethylene glycol (PEG), and chromatography on DEAE Sepharose, Sephadex G-100 and Blue Sepharose. Two peaks of activity were observed following chromatography on Blue Sepharose. PA phosphatase was purified about 600-fold in the second peak eluted from the column. The two isoforms displayed similar characteristics, including an absolute requirement for Mg^{2+}. PA phosphatase obtained from peak 2 displayed an apparent M_r of about 75 000.

In yeast, a NEM-sensitive PA phosphatase was purified to homogeneity (Lin and Carman, 1989: Morlock *et al.*, 1991). PA phosphatase was purified 9833-fold

from the membrane following solubilization with sodium cholate and chromatography on DE-53, Affi-Gel Blue, hydroxylapatite, Mono-Q and Superose 12. Optimal enzyme activity was dependent on Mg^{2+}, Triton X-100 and a pH of 7.0. The apparent M_r of the enzyme was 91 000. Immunoblot analysis of yeast extracts using antibodies specific for the 91 000 PA phosphatase revealed a 45 000 form of the enzyme and a 104 000 form (Morlock *et al.*, 1991). The 91 000 Da form of PA phosphatase was determined to be a proteolytic product of the 104 000 Da form. The 45 000 Da and 104 000 Da forms were purified and were determined to be distinct from one another. Following subcellular fractionation and detection by antibodies, the 45 000 Da form was found within the mitochondrial fraction whereas both the 45 000 Da and 104 000 Da forms were present in the microsomal fraction. Both forms displayed sensitivity to NEM and a requirement for Mg^{2+} but displayed an opposite effect in regulation with the addition of inositol. The expression of the 45 000 Da form was induced with the addition of inositol whereas the 104 000 Da form was unaffected.

Characterization studies of DGAT from both mammalian and yeast tissues has been extensive. The enzyme has also been solubilized from those sources. In previous studies with rat liver microsomes, DGAT was partially purified following solubilization with Triton X-100 and separation by gel filtration chromatography and sucrose density gradient centrifugation (Hosaka *et al.*, 1977). This DGAT was highly sensitive to sulfhydryl-binding reagents and displayed a broad specificity to acyl-CoA substrates. Polokoff and Bell (1980) demonstrated partial purification of DGAT from rat liver microsomes following solubilization with cholate. DGAT was purified about 9-fold following chromatography on Sepharose 4B and separation by sucrose density gradient centrifugation. The partially purified enzyme was stimulated 5-fold by the addition of phospholipids and was strongly dependent on Mg^{2+}. DGAT was also purified about 8-fold following gel filtration chromatography of a solubilized acetone precipitate obtained from the membrane of *Mycobacterium smegmatis* (Akao and Kusaka, 1976).

Following gel filtration, the enzyme activity corresponded to an apparent M_r of about 50 000. This DGAT was also inhibited by sulfhydryl-binding reagents and, unlike the mammalian DGAT (Polokoff and Bell, 1980), the addition of $MgCl_2$ did not affect DGAT activity in bacterial cells (Nakagawa *et al.*, 1976).

The functional size, *in situ*, of rat liver DGAT has been investigated by radiation inactivation (Ozasa *et al.*, 1989). The loss of enzymatic activity from frozen rat liver microsomes was measured following periods of irradiation. The rate of loss was directly related to the mass of the molecules that were involved in the activity or function measured. It was determined that a single-sized unit of about 72 000 Da was required for expression of DGAT activity. Lehner and Kuksis (1995) isolated a triacylglycerol synthetase complex containing acyl-CoA ligase, acyl-CoA acyltransferase, monoacylglycerol acyltransferase and DGAT. The complex was purified following solubilization with CHAPS and separation on a Cibacon blue 3GA-agarose affinity column. Polypeptides ranging from 52 000 - 72 000 Da were observed following SDS-PAGE.

There is only one report, however, on the purification of mammalian DGAT to near homogeneity. DGAT was purified 415-fold from rat liver microsomes following solubilization with CHAPS, and further purification by gel filtration chromatography, Q-Sepharose chromatography, and immunoaffinity chromatography (Andersson *et al.*, 1994). A fraction of rat liver microsomes prepared by chromatography on Q-Sepharose and Sephacryl S-200 HR was enriched in proteins approximately 70 000 Da in size as determined by SDS-PAGE. This was similar to the size of DGAT which was determined by Ozasa *et al.* (1989) following radiation inactivation studies. Thus, the proteins were subsequently used for immunizations and development of monoclonal antibodies. Western blot analysis indicated that one clone reacted with the DGAT-containing fraction from gel filtration chromatography as well as with a 60 000 Da protein present in the solubilized microsomal fraction. The monoclonal antibody was

used to isolate DGAT by immunoaffinity chromatography. Following immunoaffinity chromatography, 60 000 Da and 77 000 Da proteins were identified by SDS-PAGE. The monoclonal antibody reacted with the 60 000 Da protein and not to the 77 000 Da protein as determined by Western blot analysis.

The purification of PA phosphatase and DGAT to homogeneity has not been reported in any plant system. Thus, further characterization of the microsomal and solubilized forms of these enzymes may be useful in designing purification strategies.

MATERIALS AND METHODS

Plant Material

MD embryo cultures of oilseed rape (*Brassica napus* L. cv Topas) were provided by Dr. M. K. Pomeroy of the Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, ON. Microspores were isolated and induced to form MD embryos according to Pomeroy *et al.* (1991). An 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 and Agri-Food Canada, Ottawa, ON. The culture was maintained according to Orr *et al.* (1986) with the exception that the cells were grown in media containing varying sucrose concentrations. Cells were grown at 2, 6, 10, 14, 18, and 22 % (w/v) sucrose in a growth chamber (Conviron Model E-15, Conviron, Winnipeg, MB) at 25°C with a constant photon flux rate of 22.4 μmol/s/m^2.

Chemicals and Chromatography Matrices

HPLC-grade solvents were purchased from BDH Chemicals Inc. (Toronto, Ontario). A DEAE-Sepharose Mono Q (HR 5/5) column was from Pharmacia LKB Biotechnology International AB (Uppsala, Sweden). Chemicals for electrophoresis and protein determination and the Macro-Prep 50Q Anion Exchange Support were obtained from Bio-Rad Laboratories (Richmond, California). All lipids, detergents and other biochemicals were from Sigma Chemical Co. (St. Louis, Missouri).

Preparation of PA and LPA

Preparation of Dierucoyl-PA and Erucoyl-LPA. Using a method modified from that of Kanda and Wells (1981), dierucoyl-PA and erucoyl-LPA were chemically synthesized by the acylation of *sn*-glycerol-3-phosphate catalyzed by trifluoroacetic acid (TFA) anhydride. In a 3 mL screw-capped reaction vial, 50 μmol *sn*-glycerol-3-

phosphate [di(monocyclohexylammonium)salt] was dissolved in 185 μL TFA. The mixture was combined with 200 μmol erucic acid dissolved in 56 μL TFA anhydride. The contents of the vial were allowed to react for 30 min at room temperature and the TFA was reduced to dryness under N_2. The resulting lipids were suspended in 200 μL chloroform:methanol (2:1, v/v) and 40 μL was applied to a 20 x 20 cm Silica gel 60 G Aluminum-backed TLC plate. As described by Bocckino *et al.* (1989), PA and LPA were separated using ethyl acetate:iso-octane:acetic acid (45:15:10, v/v/v) as the solvent system. The PA and LPA products were visualized by the charring of a section of the TLC plate, which was dipped in 10% (w/v) $CuSO_4$ and 8% (w/v) H_3PO_4, followed by heating at 195°C for 15 min. The PA and LPA were eluted from the silica using a modified solution from the method by Bligh and Dyer (1959). The PA and LPA lipids were extracted with 2 mL dichloromethane:methanol:1 M KCl in 0.2 M maleic acid (1:2:0.8, v/v/v) and the phases were separated by the addition of 2 mL dichloromethane followed by 2 mL 1 M KCl in 0.2 M maleic acid. The lower organic phase was removed with a pasteur pipette and the dichloromethane was reduced to dryness under N_2. The PA and LPA were weighed and stored under N_2 at -20°C.

Preparation of Particulate Fractions of MD Embryos

MD embryos at the EC to LC stage of development were rinsed with water over a nylon mesh (60 μm), blotted with paper toweling and the fresh weight determined. Membrane fractions (10 000 - 100 000 *g* and 1500 - 100 000 *g*) from MD embryos were prepared by differential centrifugation of embryo homogenate.

Preparation of Particulate Fractions Containing PA phosphatase. All procedures were performed at 0 - 4°C. Tissues were ground using a chilled mortar and pestle with 4 volumes of homogenizing buffer per g of fresh weight. The homogenizing buffer consisted of 62.5 mM Tris/62.5 mM Maleic acid-NaOH, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 0.5 M sucrose. Homogenate

was centrifuged at 10 000 *g* (Beckman J2-21M induction drive centrifuge with JA-20 34° fixed angle rotor using 50 mL polycarbonate centrifuge tubes) for 20 min and the resulting pellet discarded. The supernatant was filtered through glass wool and centrifuged at 100 000 *g* (Beckman model L3-50 ultracentrifuge with Type 65 23.5° fixed angle rotor using 16 mm x 76 mm Beckman Ultra-Clear tubes) for 1 h. The resulting pellet was washed by suspending the pellet in 10 mL of 62.5 mM Tris/62.5 mM Maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA and 2.5 mM $MgCl_2$ followed by recentrifugation at 100 000 *g* for 1 h. The final particulate fraction was resuspended to one-tenth the tissue weight in 62.5 mM Tris/62.5 mM Maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA and 2.5 mM $MgCl_2$ and flash frozen in small aliquots using liquid N_2. The aliquots were stored at -20°C. Particulate fractions for the study of Mg^{2+} requirement were prepared without $MgCl_2$ in the buffers.

Preparation of Particulate Fractions Containing DGAT. All procedures were performed at 0 - 4°C. Tissues were ground using a chilled mortar and pestle with 4 volumes of homogenizing buffer per g of fresh weight and a small amount of acid-washed silica. The homogenizing buffer consisted of 0.2 M Hepes-NaOH, pH 7.4, containing 0.5 M sucrose. Homogenate was centrifuged at 1500 *g* (Beckman J2-21M induction drive centrifuge with JA-20 34° fixed angle rotor using 50 mL polycarbonate centrifuge tubes) for 15 min and the resulting pellet discarded. The supernatant was filtered through glass wool and centrifuged at 100 000 *g* (Beckman model L3-50 ultracentrifuge with Type 65 23.5° fixed angle rotor using 16 mm x 76 mm Beckman Ultra-Clear tubes) for 2 h. The resulting pellet was washed by suspending the pellet in 10 mL of 10 mM Tris-HCl buffer, pH 8.0, containing 20% (w/v) glycerol, 1 mM DTT and 2 M NaCl and recentrifuging at 100 000 *g* for 2 h. The final particulate fraction was resuspended to one-tenth the tissue weight in 10 mM Tris-HCl buffer, pH 8.0,

containing 20% (w/v) glycerol, 1 mM DTT and 2 M NaCl and flash frozen in small aliquots using liquid N_2. The aliquots were stored at -20°C.

Preparation of Particulate Fractions from MD Cell Suspension Culture

Cells were harvested following a 2 week growth period, rinsed and prepared as described for MD embryos. Fresh weight (FW) and settled volume were also determined. A small amount of cells (0.5 g) was placed in an oven (Fisher isotemp oven, Fisher Scientific, Inc., Bohemia, New York) at 70°C for 24 h and dry weight (DW) was determined.

Preparation of Solubilized Fractions Containing PA Phosphatase Activity

Particulate fractions were thawed on ice and centrifuged for 1 h at 105 000 *g* (Beckman Optima TLX ultracentrifuge with TLA 100.3 30° fixed angle rotor using 13 mm x 51 mm Beckman polyallomer or polycarbonate centrifuge tubes). The resulting pellet was resuspended at a detergent to protein ratio of 1:1 (w/w) using solubilization buffer. The solubilization buffer consisted of 62.5 mM Tris/62.5 mM Maleic acid-NaOH, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 1% (w/v) Tween-20. The microcentrifuge tubes, containing the solubilization mixture, were secured on a shaker (Fisher Vortex Genie 2, Scientific Industries, Inc., Bohemia, New York) set up at 4°C. The tubes were shaken at setting 3 for 30 min and then centrifuged at 105 000 *g* for 1 h. The supernatant, or solubilized fraction, was assayed for PA phosphatase activity and protein. Solubilized fractions used in the studies of Mg^{2+} dependency were prepared without $MgCl_2$ in the buffers.

Preparation of Solubilized Fractions Containing DGAT

Particulate fractions were thawed on ice and centrifuged for 1 h at 105 000 *g*. The resulting pellet was resuspended at a detergent to protein ratio of 2:1 (w/w) using

DGAT solubilization buffer. The DGAT solubilization buffer consisted of 10 mM Tris-HCl, pH 8.0, containing 20% (w/v) glycerol, 1 mM DTT, 2 M NaCl and 1% (w/v) MEGA-8. The microcentrifuge tubes, containing the solubilization mixture, were secured on a shaker (Fisher Vortex Genie 2, Fisher Scientific Inc., Bohemia, New York) set up at 4°C. The tubes were shaken at setting 3 for 30 min and then centrifuged at 105 000 *g* for 1 h. The supernatant, or solubilized fraction, was assayed for DGAT activity and protein.

Enzyme Assays and Protein Determination

PA phosphatase activity was determined by monitoring the appearance of P_i according to the procedure described in Kocsis *et al.* (1996), which was based on the colorimetric method originally described by Chen *et al.* (1956) and modified by Ichihara *et al.* (1989). All assays were performed using glassware washed with phosphate-free Extran 300 concentrate (E. Merck, Darmstadt, Germany).

The standard reaction mixture contained 50 mM Tris/50 mM Maleic acid-NaOH buffer, pH 6.75, containing 0.1 mM EDTA, 2 mM $MgCl_2$, 1.0 mM PA, 0.65 mM PC and 1 mg BSA in a final volume of 1 mL. Reactions were initiated with enzyme fraction and allowed to proceed for 1 h at 30°C. The reactions were quenched with 1 mL 20% (w/v) trichloroacetic acid (TCA). The tubes were vortexed and then centrifuged for 10 min at 1500 *g* (Mistral 2000, MSE Scientific Instruments, Leicestershire, England). A 1.5 mL aliquot of the supernatant was mixed with 3 mL of color reagent C, which contained water/6 N H_2SO_4/2.5% (w/v) ammonium molybdate/10% (w/v) ascorbic acid at a 2:1:1:1 (v/v/v/v) ratio. The mixture was incubated at 37°C for 1.5-2 h and was allowed to cool to room temperature prior to measuring the absorbance at 820 nm using a spectrophotometer (Pharmacia LKB Novaspec II, Pharmacia LKB Biotechnology International AB, Uppsala, Sweden). The P_i concentration of the supernatant was calculated using KH_2PO_4 standards. Reactions

were corrected for the presence of endogenous P_i by subtracting absorbance values resulting from treatment of extracts with TCA prior to the addition of substrate.

GPAT activity was determined by monitoring the appearance of radiolabelled LPA. This assay was modified by Dr. D. M. Hodges (personal communication) from the methods described by Bertrams and Heinz (1981) and Frentzen *et al.* (1983). The standard reaction mixture contained 0.22 M [(3-N-morpholino)propane sulfonic acid] (MOPS)-NaOH buffer, pH 7.5, containing 0.8 mg BSA, 0.44 mM palmitoyl CoA and 1.78 mM *sn*-[^{3}H]glycerol-3-phosphate (0.232 μCurie/μmole) in a final volume of 90 μL. Reactions were initiated with enzyme fraction and allowed to proceed for 15 min at 24°C. The reactions were quenched with 2.5 mL chloroform:methanol (1:1, v/v). The chloroform layer was partitioned following the addition of 1.1 mL of 1 M KCl in 0.2 M H_3PO_4 and centrifugation at 1550 revolutions per min (rpm) (Precision micro-semi micro centricone, Precision Scientific Co., Chicago, IL) for 1 min. The lower organic layer was removed with a pasteur pipette and transferred to a screw-capped test tube. The upper aqueous phase was re-washed with 1 mL of chloroform. The organic layers were combined and the chloroform was reduced to dryness under N_2. The reaction products were resuspended in 50 μL of chloroform:methanol (2:1, v/v). Radiolabelled LPA was separated by thin layer chromatography (TLC) using 20 X 20 cm Silica gel 60 H preparative TLC plates and chloroform:methanol:acetic acid:water (85:15:10:4, v/v/v/v) as the solvent system. The radioactivity was determined using a liquid scintillation counter (PW4700 liquid scintillation counter, Philips Export B.V., Eindhoven, Netherlands). The position of radiolabelled LPA on the TLC plate was established based on the co-chromatography of a standard.

LPAAT activity was determined by measuring the appearance of radiolabelled phosphatidate. This assay was modified by Dr. D. M. Hodges (personal communication) from the methods described by Oo and Huang (1989) and Cao *et al.* (1990). The standard reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5,

containing 40 μM lysophosphatidic acid, 1 mM $MgCl_2$ and 20 μM [1-^{14}C]oleoyl-CoA (56 μCurie/μmole) in a final reaction volume of 80 μL. The reactions were initiated with enzyme extract and incubated at 30°C for 4 min. The reactions were quenched with 2.5 mL chloroform:methanol (1:1, v/v) and the chloroform layer was partitioned following the addition of 1.1 mL of 1 M KCl in 0.2 M H_3PO_4 and centrifugation at 1550 rpm (Precision micro-semi micro centricone, Precision Scientific Co., Chicago IL) for 5 min. The lower organic phase was removed using a pasteur pipette and transferred to a screw-capped test tube. The upper phase was re-washed with 1 mL chloroform. The organic layers were combined and the chloroform was reduced to dryness with N_2. The reaction products were resuspended in 50 μL chloroform:methanol (2:1, v/v). Radiolabelled PA was separated by TLC using a Silica gel 60 H preparative TLC plate and chloroform:methanol:acetic acid:water (85:15:10:4, v/v/v/v) as the solvent system. The radioactivity was determined using a scintillation counter. The position of radiolabelled PA on the TLC plate was established based on the co-chromatography of a standard.

DGAT activity was determined by measuring the appearance of radiolabelled TG according to the method described by Little *et al.* (1994). The standard reaction mixture contained 0.2 M Hepes-NaOH, pH 7.4, containing 3 mM $MgCl_2$, 1 mM ATP, 330 μM coenzyme A, 330 μM *sn*-1,2-diolein, 0.02% (w/v) Tween-20, 0.5% (w/v) BSA and 15 μM [1-^{14}C]oleoyl-CoA (56 μCurie/μmole) in a final reaction volume of 60 μL. Reactions were initiated with enzyme extracts and were incubated for 10 min at 30°C. The reactions were quenched with 10 μL 5% (w/v) sodium dodecyl sulfate (SDS) and radiolabelled TG was separated by TLC using a Silica gel 60 H preparative TLC plate and hexane:diethyl ether (80:20, v/v) as the solvent system. The radioactivity was determined using a scintillation counter. The position of radiolabelled TG on the TLC plate was established based on the co-chromatography of a standard.

The protein content was determined using the Bio-Rad protein microassay based on the method by Bradford (1976), with BSA as the standard.

Preparation of Solubilized Fractions Containing PA Phosphatase or DGAT Using Various Precipitating Agents

Solubilized fractions of MD cultures containing PA phosphatase or DGAT were precipitated with ammonium sulfate, polyethylene glycol 8000 (PEG), ethanol, or acetone. The various agents were added to the fractions and the suspensions were incubated on ice for 15 min. The suspensions were centrifuged at 15 900 *g* (Beckman microfuge E, Beckman Instruments Inc., Palo Alto, CA) for 10 min and the resulting pellets resuspended in appropriate buffer for determination of PA phosphatase or DGAT activity.

Affinity Chromatography of Solubilized DGAT

Affinity chromatography was performed using small, disposable, plastic columns (Bio-Rad) containing 0.2 mL palmitoyl-CoA-agarose or alkyl-CoA-sepharose equilibrated with 10 mM Tris-HCl, pH 8.0, containing 1% (w/v) MEGA-8, 2 M NaCl and 20% (w/v) glycerol. Samples of solubilized enzyme (1 mL) were applied to each column followed by 2 mL of equilibration buffer. Each column was eluted with 0.4 mL of 5 mM erucoyl-CoA in equilibration buffer and the eluted fractions were precipitated with 6% (w/v) polyethylene glycol 8000 (PEG). Solid PEG was added to each fraction and the fractions were vortexed and incubated on ice for 15 min. The fractions were centrifuged at 15 900 *g* (Beckman microfuge E, Beckman Instruments Inc., Palo Alto, CA) for 10 min and the pellets were resuspended in equilibration buffer without erucoyl-CoA. Solubilized, unbound and eluted fractions were assayed for DGAT activity.

Anion Exchange Chromatography of Solubilized PA Phosphatase

Ion exchange chromatography was performed using both a Mono Q column (HR 5/5, bed volume = 1 mL) and a Macro-prep 50Q column (bed volume = 4 mL) equilibrated with 10 mM Tris-HCl, pH 8.5, containing 1% (w/v) Tween 20. Samples of solubilized enzyme were further fractionated with ammonium sulfate (20-50% saturation) and 2 mL samples were applied to the ion exchange columns. Columns were washed with 10 mL 10 mM Tris-HCl, pH 8.5, containing 1% (w/v) Tween 20 and eluted with a 0-2 M NaCl gradient (total volume = 20 mL). Columns were operated at a flow rate of 30 mL/h with a Fast Performance Liquid Chromatography (FPLC) system (Pharmacia LKB Biotechnology International AB, Uppsala, Sweden). Column fractions of 1 mL were collected and assayed for PA phosphatase activity and protein content.

Gel Filtration Chromatography of Solubilized PA Phosphatase

Gel filtration chromatography was performed using a Superose 6 column (HR 10/30, bed volume = 23.6 mL) equilibrated with 60 mM Tris/60 mM maleic acid-NaOH buffer, pH 6.75, containing 2.5 mM $MgCl_2$, 0.125 mM EDTA, 0.1 M KCl and 0.2% (w/v) Tween 20. Samples (200 μL or 500 μL) of freshly prepared solubilized enzyme or those which had undergone one cycle of freezing and then thawing were applied to the column. Columns were operated at a flow rate of 12 mL/h with a FPLC system (Pharmacia LKB Biotechnology International, AB, Uppsala, Sweden). Column fractions of 1mL were collected and assayed for PA phosphatase activity. Molecular weight markers were used to calibrate the column in both the presence and absence of 0.2% (w/v) Tween 20.

SDS-Polyacrylamide Gel Electrophoresis

The following fractions were subjected to SDS-PAGE according to Laemmli (1970): (1) particulate, solubilized, ammonium sulfate, and ion exchange chromatography fractions containing PA phosphatase activity and (2) particulate, solubilized and PEG precipitated fractions containing DGAT activity. Proteins from the fractions were precipitated by incubating the fractions with 80% (v/v) acetone for 2 h at -20°C followed by centrifugation at 10 000 *g* for 20 min at -10°C. The proteins were suspended in 0.125 M Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.005% (w/v) bromophenol blue and were denatured by boiling for 5 min. Electrophoresis was conducted using the Bio-Rad Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA) and 1 mm thick polyacrylamide gels (12% total monomer concentration; 3% cross-linking monomer concentration). The total monomer concentration of the stacking gel was 4%. Following electrophoresis, the gel was stained for polypeptides using the Bio-Rad silver staining method based on the procedure of Merril *et al.* (1981).

Immunological Methods

Preparation of Monoclonal Antibodies Against a Solubilized Fraction Containing DGAT. DGAT was solubilized from a 10 000 - 100 000 *g* particulate fraction prepared from MD embryos of *B. napus* L. cv Topas using 1% (w/v) MEGA-8, 1mM DTT and 2 M NaCl. The solubilized fraction was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% (w/v) MEGA-8, and 20% (w/v) glycerol. The fraction was centrifuged at 105 000 *g* for 1 h and the pellet resuspended in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Mice (female BALB/c) were immunized with 50 - 100 μg protein mixed with Freund's complete adjuvant via intraperitoneal injections. The mice were subjected to two additional injections with Freund's incomplete adjuvant followed by a final intravenous injection without

Freund's adjuvant. The immunizations, animal care, and bleedings were carried out by Greg Tiffin of the Animal Diseases Research Institute, Agriculture and Agri-Food Canada, Lethbridge, AB. Following a final ocular bleed, monoclonal antibodies were prepared as described by Weselake (1994).

Immunoaffinity Chromatography of Solubilized DGAT. Immunoglobulin G (IgG) was purified from ascites fluid containing monoclonal antibodies (P_3A_7/NA_6A_7) raised against partially purified DGAT. Control IgG was purified from ascites fluid containing monoclonal antibodies which were raised against an unrelated antigen. The IgG was precipitated with ammonium sulfate (40% saturation) and isolated by DEAE Sepharose chromatography. In a small, disposable, plastic column (Bio-Rad), 2 mL of DEAE Sepharose were equilibrated with 10 mL 100 mM sodium phosphate-NaOH buffer, pH 7.0. The control IgG and the anti-DGAT IgG antibodies were applied to separate columns and eluted with 2 mL of equilibration buffer. The eluted fractions were dialyzed against 100 mM sodium borate-NaOH, pH 8.5. The IgG was coupled to epoxy-activated Sepharose 6B as outlined in the manual Affinity Chromatography - Principles and Methods, which was published by Pharmacia Fine Chemicals, Uppsala, Sweden. In a 10 mL screw-capped test tube, 0.6 mL epoxy-activated sepharose 6B was equilibrated with 100 mM sodium borate-NaOH, pH 8.5. For each IgG fraction, 1 mL IgG solution containing approximately 675 μg IgG was incubated for 16 h at 37°C with continuous shaking. In order to block unreacted epoxy groups, the Sepharose was washed twice with borate buffer and was incubated with 1 M ethanolamine, pH 8.5 for 4 h at 37°C with continuous shaking. The gels were washed with 5 mL sodium borate-NaOH buffer, pH 8.0 followed by 5 mL water, 5 mL sodium borate-NaOH buffer, pH 8.0, containing 0.5 M NaCl and 100 mM sodium acetate-HCl buffer, pH 4.0, containing 0.5 M NaCl. Each type of IgG-epoxy-sepharose 6B was used to prepare two columns of 0.3 mL in disposable Bio-Rad columns. The columns were equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 20% (w/v)

glycerol, 1% (w/v) MEGA-8 and 2 M NaCl at 4°C. A sample (500 μL) of solubilized DGAT was applied to each column followed by 1 mL of equilibration buffer. One set of columns was eluted with 0.6 mL of 1 M KNO_3 in equilibration buffer. The second set of columns was eluted with 0.6 mL of 0.1 M glycine-HCl buffer, pH 2.5, containing 2 M NaCl. The eluate following the elution with low pH was collected in test tubes containing 0.1 mL of 0.8 M Hepes-NaOH buffer, pH 7.8 to neutralize the eluted fraction. The solubilized, unbound, and eluted fractions were assayed for DGAT activity.

Preparation of Polyclonal Antibodies Against A Solubilized Fraction Containing PA Phosphatase and DGAT. PA phosphatase was solubilized from a 10 000 - 100 000 *g* particulate fraction prepared from MD embryos of *B. napus* L. cv Topas. The solubilized fraction was further fractionated using ammonium sulfate (20 - 50% saturation). The fraction was incubated with 20% (w/v) ammonium sulfate for 15 min on ice followed by centrifugation at 15 900 *g* for 10 min. The supernatant was incubated in a final concentration of 50% (w/v) ammonium sulfate for 15 min on ice followed by centrifugation at 15 900 *g* for 10 min. The resulting pellet was resuspended in 62.5 mM Tris/62.5 mM maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 1% (w/v) Tween 20. The protein was precipitated by incubating the fraction with 80% (v/v) acetone for 2 h at -20°C followed by centrifugation at 10 000 *g* for 20 min at -10°C. The final pellet was resuspended in 10 mM sodium phosphate-NaOH buffer, pH 7.4, containing 0.15 M NaCl for immunizations. DGAT was sequentially solubilized from the same microsome as PA phosphatase and the solubilized fraction was further fractionated using PEG 8000 (2 - 6% fractionation). The fraction was incubated with 2% (w/v) PEG on ice for 15 min followed by centrifugation at 15 900 *g* for 10 min. The supernatant was incubated with a final concentration of 6% (w/v) PEG on ice for 15 min followed by centrifugation at 15 900 *g* for 10 min. The resulting pellet was

resuspended in 10 mM Tris-HCl buffer, pH 8.0, containing 1% (w/v) MEGA-8 and 2 M NaCl. The protein was precipitated by incubating the fraction with 80% (v/v) acetone for 2 h at -20°C followed by centrifugation at 10 000 *g* for 20 min at -10°C. The final pellet was resuspended in 10 mM sodium phosphate-NaOH buffer, pH 7.4, containing 0.15 M NaCl for immunizations.

Four rabbits were immunized with three intraperitoneal injections using 200 μg protein mixed with Specol adjuvant (D. Busch v.d. Houten Institute for Animal Science and Health, Dellstad, The Netherlands). A fourth injection consisted of 100 μg protein mixed with Specol adjuvant. Rabbits 1 and 2 were immunized with partially purified PA phosphatase whereas rabbits 3 and 4 were immunized with partially purified DGAT. The rabbits were prebled prior to injections to obtain control sera. All immunizations, animal care and bleeds were done by Greg Tiffin of the Animal and Diseases Research Institute, Agriculture and Agri-Food Canada, Lethbridge, AB.

Samples (500 μL) of control serum and antiserum from rabbits 1 and 2 were dialyzed against 62.5 mM Tris/62.5 mM maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA, and 2.5 mM $MgCl_2$ for 16 h at 4°C. Antisera were tested for PA phosphatase inhibition by preincubating a 10 000 - 100 000 *g* particulate fraction from MD embryos with antiserum for 30 min at 30°C and then assaying for PA phosphatase activity.

Antisera from rabbits 3 and 4 were tested for DGAT inhibition by preincubation of the 10 000 - 100 000 *g* particulate fraction from MD embryos with antiserum for 30 min at 30°C and then assaying for DGAT activity. DGAT was assayed in the absence of ATP and CoA in the reaction mixture.

Preparation of Polyclonal Antibodies Against Partially Purified Soybean DGAT. DGAT was partially purified from germinating soybean (*Glycine max* L. Merr cv Marple Amber) cotyledons as described by Kwanyuen and Wilson (1986). The partially purified fraction was dialyzed against 10 mM sodium phosphate-NaOH buffer,

pH 7.4, containing 0.15 M NaCl for immunizations. Two female rabbits were immunized with 250 μg protein mixed with Freund's complete adjuvant via intraperitoneal injections followed by four injections of 250, 110, 720, and 300 μg protein without adjuvant. The immunizations, animal care, and bleedings were carried out by Greg Tiffin of the Animal Diseases Research Institute, Agriculture and Agri-Food Canada, Lethbridge, AB.

Immunodetection of DGAT Using Polyclonal Antibodies. DGAT was solubilized from the 10 000 - 100 000 *g* particulate fraction prepared from MD embryo of *B. napus* L. cv Topas. The solubilized fraction was further fractionated with PEG (2 - 6% fractionation). The fraction was incubated with 2% (w/v) PEG on ice for 15 min followed by centrifugation at 15 900 *g* for 10 min. The supernatant was incubated in a final concentration of 6% (w/v) PEG for 15 min on ice followed by centrifugation at 15 900 *g* for 10 min. The protein was resuspended in 0.5 M Tris-HCl buffer, pH 6.8, containing 1% (w/v) SDS and 5% (v/v) ß-mercaptoethanol followed by disruption by boiling for 5 min. Various concentrations of protein were applied to nitrocellulose membrane according to the procedure described in the instruction manual for the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Richmond, CA). The nitrocellulose was washed with 200 μL Tris buffered saline (TBS) buffer which consisted of 20 mM Tris-HCL, pH 7.5, containing 0.5 M NaCl. The protein on the nitrocellulose was probed for enzyme activity by a immunodetection method which was modified from that described by Muilerman *et al.* (1982). The nitrocellulose, with applied denatured enzyme, was cut into strips and each strip was placed into 15 mL conical centrifuge tubes with the protein-loaded sites directed to the interior of the tubes. All washing and incubation steps were performed by mechanical rolling of the tubes in a hybridization oven (Tek-Star hybridization oven, Bio/Can Scientific, Mississauga, ON). The nitrocellulose strips were incubated for 2 h at room temperature with 5 mL of "blotto" buffer which consisted of TBS buffer, pH 7.4,

containing 0.5% (w/v) Tween 20, and 5% (w/v) skim milk powder. The nitrocellulose was then incubated with 500 μL of a 5-fold dilution of anti-soybean DGAT polyclonal antibodies in "blotto" buffer for 16 h followed by three washes with TBS buffer containing 0.05% (v/v) Tween 20 for 5 min and three washes for 10 min with 10 mM Tris-HCl buffer, pH 8.0, containing 1% (w/v) MEGA-8 and 2 M NaCl. As a control, the nitrocellulose in one centrifuge tube was incubated with polyclonal antibodies prepared from an unrelated source of antigen. The nitrocellulose strips were incubated with 400 μL solubilized DGAT which was suspended in 10 mM Tris-HCl buffer, pH 8.0, containing 1% (w/v) MEGA-8 and 2 M NaCl for 4 h at room temperature followed by three washes for 5 min with 10 mM Tris-HCl buffer, pH 8.0, containing 1% (w/v) MEGA-8 and 2 M NaCl and three washes for 5 min with 0.2 M Hepes-NaOH buffer, pH 7.4. The individual dots of protein were cut from the nitrocellulose membrane using a hole punch and were placed into 3 mL disposable culture tubes with the protein-loaded side facing the interior of the tubes. The nitrocellulose pieces were assayed for DGAT activity.

RESULTS AND DISCUSSION

PA phosphatase may represent an important control point between TG and phospholipid biosynthesis (Kocsis and Weselake, 1996). DGAT catalyzes the committed step in TG biosynthesis (Stymne and Stobart, 1987) and may play an important regulatory role in this process (Kocsis and Weselake, 1996). The purification and characterization of these two enzymes from MD cultures of oilseed rape would help to increase our knowledge of seed oil formation and also provide useful tools for the genetic modification of oilseed metabolism. In this study, PA phosphatase and DGAT activities were characterized in both particulate and solubilized preparations of MD embryos and MD cell suspension cultures of *Brassica napus* L. These investigations have also helped in developing purification strategies for the enzymes.

Stability of PA Phosphatase and DGAT in Solubilized Fractions from MD Embryos of *B. napus* L.

Previous studies on PA phosphatase and DGAT from MD embryos have indicated that these enzymes are unstable during prolonged storage (Weselake *et al.*, 1993a; Kocsis *et al.*, 1996). Thus before purification studies were conducted, the stability of PA phosphatase and DGAT in solubilized preparations of MD embryos was further investigated. Less than 40% and 10% of solubilized PA phosphatase activity was retained following storage for 120 h at 4°C and 30°C, respectively (Figure 2). PA phosphatase activity sharply decreased during storage from 0 to 24 h and then remained stable following storage beyond 24 h. The stability of solubilized DGAT is shown at 4°C and 30°C in Figure 3. Following storage for 120 h at 4°C, about 70% of the initial activity of the solubilized DGAT was retained. A substantially greater degree of

Figure 2. Stability of solubilized PA phosphatase from the EC stage of MD embryos of *B. napus* L. cv Topas. The solubilized enzyme was prepared from a 10 000 - 100 000 *g* particulate fraction using 62.5 mM Tris/62.5 mM maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 1% (w/v) Tween 20 at a detergent to protein ratio of 1:1 (w/w). Protein concentration of the solubilized enzyme was 1.1 mg/mL. Enzyme fractions were stored at 4°C (●) and 30°C (○) and 50 μL aliquots were assayed for PA phosphatase activity at intervals over a 120 h period. The results of triplicate assays are shown.

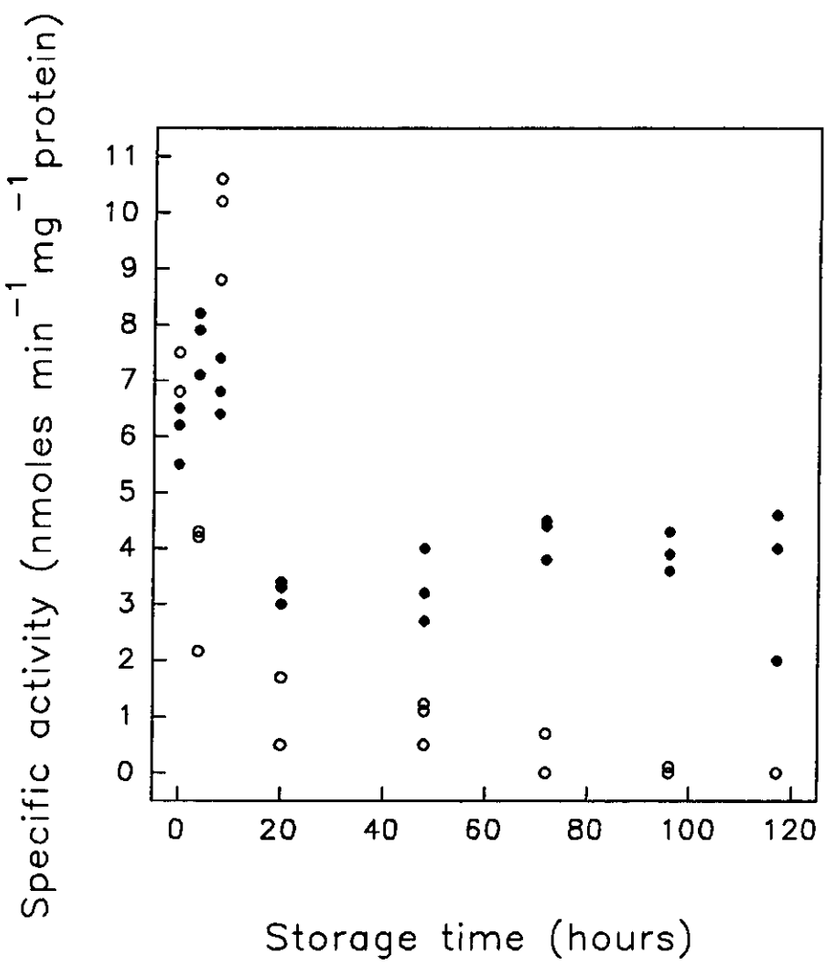
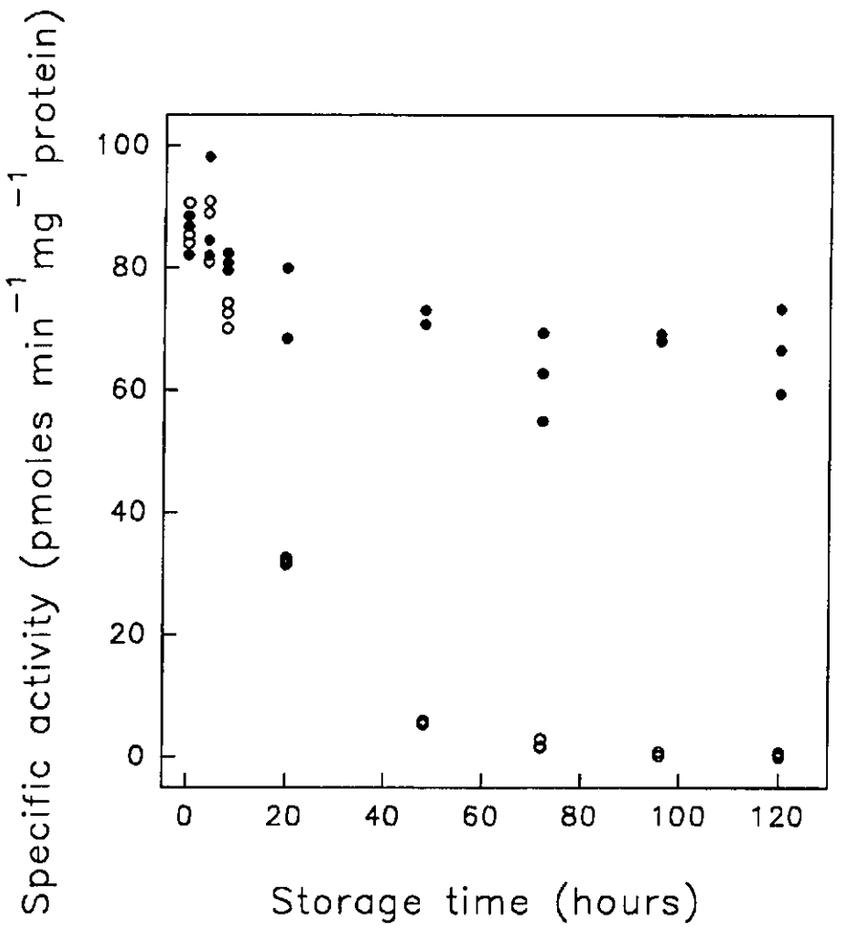

Specific activity (nmoles min^{-1} mg^{-1} protein)
0
1
2
3
4
5
6
7
8
9
10
11
0
20
40
60
80
100
120
Storage time (hours)

Figure 3. Stability of solubilized DGAT from the LC stage of MD embryos of *B. napus* L. cv Topas. The solubilized enzyme was prepared from a 10 000 - 100 000 *g* particulate fraction using 10 mM Tris-HCl buffer, pH 8.0, containing 2 M NaCl and 1% (w/v) MEGA-8 at a detergent to protein ratio of 2:1 (w/w). Protein concentration of the solubilized enzyme was 2.5 mg/mL. Enzyme fractions were stored at 4°C (●) and 30°C (○) and 15 μL aliquots were assayed for DGAT activity at intervals over a 120 h period. The results of triplicate assays are shown.


Specific activity (pmoles min^{-1} mg^{-1} protein)
100
80
60
40
20
0
0
20
40
60
80
100
120
Storage time (hours)

activity was lost following storage at 30°C. DGAT dispersed from particulate fractions of MD embryos of oilseed rape was previously shown to be unstable to a similar extent (Weselake *et al.*, 1993a). Solubilized DGAT from *Mycobacterium smegmatis* also showed substantial loss of enzyme activity following storage at 0°C for 4 days (Akao and Kusaka, 1976).

The effects of various concentrations of BSA in the storage media of PA phosphatase and DGAT are shown in Tables 1 and 2. The activity of both solubilized PA phosphatase and DGAT was stabilized to a large extent by the addition of 10% (w/v) BSA. In the presence of BSA, about 70% of the PA phosphatase activity was retained after 24 at 4°C (Table 1). Lower concentrations of BSA were less effective in stabilizing the enzyme activity. Ichihara *et al.* (1989) reported that BSA stimulated PA phosphatase activity. After 24 h at 4°C, over 90% of the initial solubilized DGAT activity was retained following storage in the presence of 10% (w/v) BSA (Table 2). BSA was most effective at a concentration of 10% (w/v) in preserving DGAT activity. BSA was previously shown to stimulate DGAT activity about 4- to 5-fold (Little *et al.*, 1994). BSA has commonly been used as an agent to stabilize proteins (North, 1989). Similar to detergents, BSA may act to stabilize PA phosphatase and DGAT by covering hydrophobic areas and thus help to maintain enzyme configuration and activity. BSA was not incorporated during purification steps due to problems with viscosity and protein determination at high concentrations of BSA. Both PA phosphatase and DGAT were stabilized at a high concentration of BSA but this made chromatographic procedures using BSA unfeasible. Other additives were also explored (Appendix Tables 3 and 4). Sucrose, sorbitol and trehalose had little effect in preserving the activity of the TG biosynthetic enzymes. Glycerol (10%, w/v) maintained about 60% of the initial solubilized PA phosphatase activity following storage for 24 h (Appendix Table 3).

Table 1. Effect of various concentrations of BSA on the stability of solubilized PA phosphatase. Enzymes were solubilized from particulate fractions prepared from the EC stage of MD embryos of *B. napus* L. cv Topas. Enzyme activity was assayed at 0 (initial activity), 24 and 48 h following storage at 4°C. The results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage. Results of another experiment are shown in Appendix Table 1.

BSA CONCENTRATION (%, w/v)	SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)		
	0 h	24 h	48 h
0	4.0	1.7 (42)	1.2 (31)
0.1	7.6	1.0 (13)	1.2 (16)
0.5	2.5	1.5 (59)	0.4 (15)
1.0	4.4	1.6 (37)	1.3 (29)
5.0	3.7	1.9 (52)	1.2 (32)
10.0	3.4	2.4 (72)	2.4 (70)

Table 2. Effect of various concentrations of BSA on the stability of solubilized DGAT. Enzymes were solubilized from particulate fractions prepared from the EC stage of MD embryos of *B. napus* L. cv Topas. Enzyme activity was assayed at 0 (initial activity), 24 and 48 h following storage at 4°C. The results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage. Results of another experiment are shown in Appendix Table 2.

BSA CONCENTRATION (%, w/v)	SPECIFIC ACTIVITY OF SOLUBILIZED DGAT (pmol/min/mg)		
	0 h	24 h	48 h
0	4.6	3.5 (77)	1.3 (29)
0.1	4.6	4.4 (95)	3.7 (80)
0.5	10.2	8.9 (87)	6.1 (60)
1.0	16.5	15.3 (93)	12.0 (73)
5.0	29.9	26.6 (89)	23.9 (80)
10.0	25.0	24.8 (99)	22.3 (89)

The presence of BSA in solubilized preparations suggested that this protein may serve to protect PA phosphatase or DGAT by providing proteases with an alternative source to act upon. In Tables 3 and 4, the effect of protease inhibitors on the stability of TG biosynthetic enzymes in particulate fractions from MD embryos at the LC stage are shown. The addition of a mixture of protease inhibitors (1 mM PMSF, 1 mM EDTA, and 1 mg/ml [w/v] leupeptin) did not affect the activities of solubilized PA phosphatase or DGAT (Tables 3 and 4, respectively). This suggested that proteases were not a major factor in the lability of these TG biosynthetic enzymes. Coleman and Bell (1978) reported that PA phosphatase in mammalian tissue was not affected by proteases. A number of studies on plant PA phosphatases, however, have routinely incorporated protease inhibitors during purification procedures. Malherbe *et al.* (1995) have incorporated protease inhibitors due to instability of PA phosphatase following hydroxyapatite chromatography.

It should be noted that during studies on enzyme stability it became apparent that a limited amount of particulate fraction could be prepared from EC MD embryos. Subsequently, LC stage of embryos were used in order to obtain more membranes. DGAT activity was previously shown to occur throughout the EC to LC stages of development but the highest specific activity was observed at the mid-cotyledonary (MC) stage of development (Weselake *et al.*, 1993b). EC embryos also showed higher specific PA phosphatase activity than the LC stage embryos (Appendix Tables 5 and 7). Thus, a balance between obtaining a larger amount of tissue to work with at the LC stage of development, and the loss of some enzyme activity, resulted in the compromise of adapting the use of embryos from the LC stage of development.

Table 3. Effect of protease inhibitors (1 mM PMSF, 1 mM EDTA, and 1 mg/mL [w/v] leupeptin) on the activity of solubilized PA phosphatase prepared from the LC stage of MD embryos of *B. napus* L. cv Topas. Assays were performed on solubilized preparations of PA phosphatase which were prepared from the 10 000 *g* - 100 000 *g* particulate fraction. Results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage. Results of additional experiments are shown in Appendix Table 5.

TREATMENT	SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)		
	0 hr	24 hr	48 hr
10 000-100 000 *g* (without inhibitors)	3.7	3.1 (84)	2.4 (65)
10 000-100 000 *g* (with PMSF, EDTA, leupeptin)	4.7	4.0 (85)	3.6 (77)

Table 4. Effect of protease inhibitors (1 mM PMSF, 1 mM EDTA, and 1 mg/mL [w/v] leupeptin) on the activity of solubilized DGAT prepared from the LC stage of MD embryos of *B. napus* L. cv Topas. Assays were performed on solubilized preparations of DGAT which were prepared from 1500 *g* - 100 000 *g* and 10 000 *g* - 100 000 *g* particulate fractions. Results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage. Results of additional experiments are shown in Appendix Table 6.

TREATMENT	SPECIFIC ACTIVITY OF SOLUBILIZED DGAT (pmol/min/mg)		
	0 hr	24 hr	48 hr
1500-100 000 *g* (without inhibitors)	72.5	60.0 (84)	56.6 (78)
1500-100 000 *g* (with PMSF, EDTA, leupeptin)	71.7	78.2 (108)	76.8 (107)
10 000-100 000 *g* (without inhibitors)	73.2	59.2 (81)	61.0 (83)
10 000-100 000 *g* (with PMSF, EDTA, leupeptin)	78.9	60.6 (77)	69.3 (88)

Contamination of Solubilized PA Phosphatase and DGAT by Other Kennedy Pathway Enzymes

Solubilized preparations containing PA phosphatase and DGAT were assayed for the presence of other Kennedy pathway enzymes. The solubilized preparation of PA phosphatase contained detectable amounts of GPAT, LPAAT and DGAT activity, but the preparation was relatively enriched in PA phosphatase activity (Table 5). The solubilized preparation of DGAT contained detectable amounts of LPAAT and PA phosphatase activity, but the preparation was relatively enriched in DGAT activity (Table 6). The enrichment of PA phosphatase and DGAT in their respective preparations, relative to other enzymes of the Kennedy pathway, suggested that the solubilized preparations may be used in studies of regulation with limited influence by the other Kennedy pathway enzymes. About 20% of PA phosphatase was solubilized from particulate fractions of MD embryos whereas Kocsis *et al.* (1996) reported solubilizations of more than 50% in the presence of 1% Tween 20. As well, about 36% of DGAT was solubilized from the particulate fractions whereas Little *et al.* (1994) reported solubilizations of about 80%. These differences may be due to batch to batch variations in the developing MD embryos. For example, slight changes in light, temperature, plating density and growth media have shown dramatic changes in enzyme activities (Holbrook *et al.*, 1992; Taylor *et al.*, 1992). In the study of PA phosphatase-1 and PA phosphatase-2 in brain tissue, Fleming and Yeaman (1995b) have reported that both the specific activities and activity ratios of these two isoforms varied greatly from tissue to tissue. The differences observed in the current study may also be due to the use of LC MD embryos instead of the EC MD embryos used by Kocsis *et al.* (1996) and Little *et al.* (1994).

Table 5. Determination of the activities of other Kennedy pathway enzymes in preparations of particulate fraction and solubilized PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. Enzymes were assayed as described in the MATERIALS AND METHODS. Enzyme activities from a 10 000 - 100 000 *g* particulate fraction are shown. Enzyme activities and the percentage of enzyme activity solubilized from the particulate fraction are also shown. Results represent the means of triplicate assays. Results of another experiment are shown in Appendix Table 8.

ENZYME	PARTICULATE FRACTION		SOLUBILIZED FRACTION		
	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	% solubilized
PA phosphatase	10.8	45.0	11.10	7.90	17.7
GPAT	0.1	0.4	0.04	0.03	8.2
DGAT	0.1	0.6	0.04	0.03	4.5
LPAAT	38.6	160.6	8.70	6.20	3.9

Table 6. Determination of the activities of other Kennedy pathway enzymes in preparations of particulate fraction and solubilized DGAT from the LC stage of MD embryos of *B. napus* L. cv Topas. Enzymes were assayed as described in the MATERIALS AND METHODS. Enzyme activities from a 10 000 - 100 000 *g* particulate fraction are shown. Enzyme activities and the percentage of enzyme activity solubilized from the particulate fraction are also shown. Results represent the means of triplicate assays. Results of another experiment are shown in Appendix Table 9.

ENZYME	PARTICULATE FRACTION		SOLUBILIZED FRACTION		
	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	% solubilized
DGAT	0.1	0.5	0.1	0.2	35.5
PA phosphatase	10.8	39.3	2.6	4.6	11.7
LPAAT	38.6	140.4	3.9	6.9	4.9
GPAT	0.1	0.3	0	0	0

Properties of Particulate and Solubilized PA Phosphatase from MD Embryos of *B. napus* L.

The substrate specificity of solubilized PA phosphatase from MD embryos of *B. napus* L. cv Topas was investigated using various types of PA and other phosphate-containing compounds (Table 7). The solubilized preparation containing PA phosphatase catalyzed the dephosphorylation of all the compounds tested. Solubilized PA phosphatase displayed a preference for dioleoyl-PA and dipalmitoyl-PA over the other forms of PA tested. Ichihara (1991) reported that PA phosphatase from developing safflower had both a broad specificity and broad selectivity which were specific and selective for unsaturated forms of PA over other forms of PA tested.

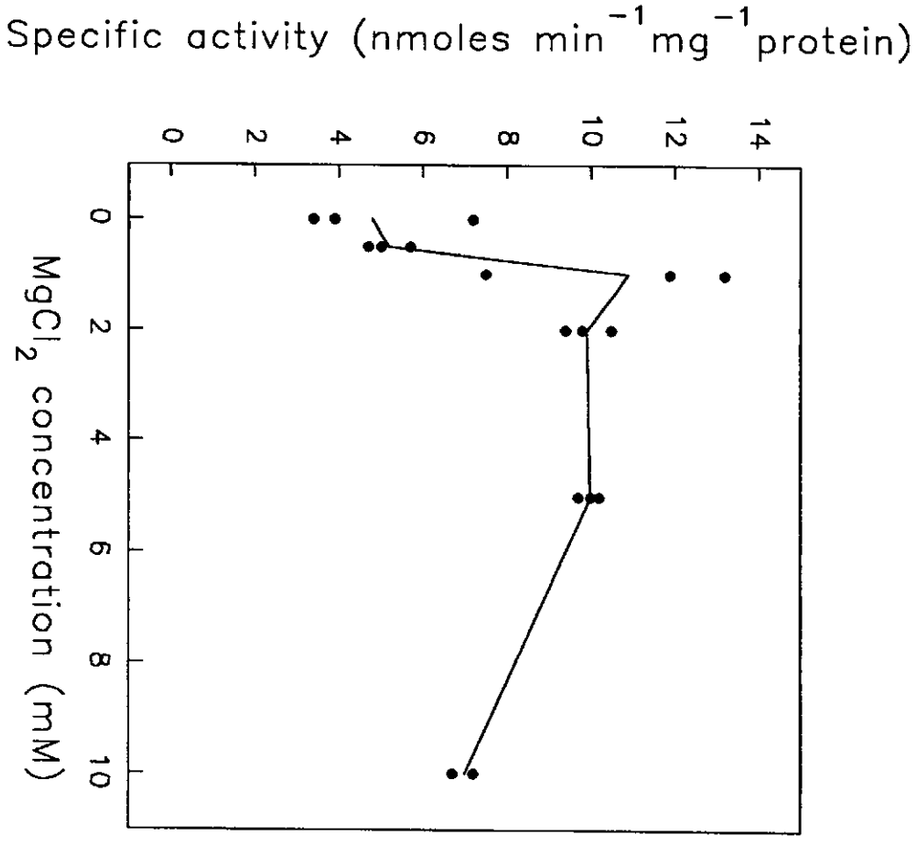
PA phosphatase from mammalian systems occurs in two separate forms which can be distinguished from one another by their sensitivity to inhibition by sulfhydryl-reagents, including NEM, and requirement for Mg^{2+} (Jamal *et al.*, 1991; Brindley and Waggoner, 1996). One form of PA phosphatase has been referred to as PA phosphatase-1 and is located in the cytosol. The enzyme has been shown to become highly active following translocation from the cytosol to the membrane of the ER in response to an increase in fatty acids. PA phosphatase-1 is both NEM-sensitive and Mg^{2+}-dependent. Mg^{2+} may improve the interaction between the enzyme and the highly negatively charged substrate (Joyard and Douce, 1979; Brindley and Sturton, 1982). PA phosphatase-1 is thought to function in glycerolipid synthesis. A second form of PA phosphatase is located in the plasma membrane and is often referred to as PA phosphatase-2. PA phosphatase-2 is NEM-insensitive and Mg^{2+}-independent. It is thought to have a role in signal transduction.

Based on the above knowledge of mammalian systems, particulate and solubilized PA phosphatase from microsomes of MD embryos of *B. napus* L. cv Topas were tested for Mg^{2+} dependence and NEM-sensitivity. Maximum activity of particulate PA phosphatase was obtained using 1 mM $MgCl_2$ (Figure 4). The activity

Table 7. Dephosphorylation of various compounds catalyzed by the solubilized fraction from the EC stage of MD embryos of *B. napus* L. cv Topas. The reaction mixture (1mL) contained 1 mM substrate with 0.5 mg PC/mL and 1 mg BSA/mL in the presence of 50mM Tris/50 mM maleic acid/NaOH buffer (pH 6.75) containing 0.1mM EDTA and 2 mM $MgCl_2$. Reactions were allowed to proceed for 60 min using 165 μg solubilized protein per reaction mixture. The results represent the means of duplicate assays. The specific activity of pNPP was 22.5 nmol/min/mg.

Substrate	Relative specific activity (% maximum)
PC/BSA	2.5
egg PA	7.2
dioleoyl-PA	11.2
dierucoyl-PA	4.9
distearoyl-PA	2.9
dipalmitoyl-PA	9.9
erucoyl-LPA	4.1
sn-glycerol 3-phosphate	21.6
adenosine 5'-monophosphate	12.3
phytate	6.2
*p*NPP	100.0

Figure 4. Effect of Mg^{2+} on microsomal PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. A 10 000 - 100 000 g particulate fraction was prepared in the absence of $MgCl_2$ then 20 μL aliquots containing 50 μg protein were assayed for PA phosphatase activity with varying concentrations of $MgCl_2$. The results of triplicate assays are shown with the plot line connecting the means of the replicate points. Results of additional experiments are shown in Appendix Table 10.


Specific activity (nmoles min^{-1} mg^{-1} protein)
0
2
4
6
8
10
12
14
$MgCl_2$ concentration (mM)
0
2
4
6
8
10

of particulate PA phosphatase in the absence of $MgCl_2$ was substantially less than the activity found in the presence of 1 mM $MgCl_2$. Thus, PA phosphatase activity appeared to be strongly dependent on Mg^{2+}. Concentrations of $MgCl_2$ beyond 1 mM resulted in decreased activity relative to that observed at 1 mM $MgCl_2$. These results suggested that there may be both Mg^{2+}-dependent and Mg^{2+}-independent forms of PA phosphatase present in membrane preparations from MD embryos. Similar results were found in solubilized preparations containing PA phosphatase activity (Appendix Table 11). There was some variation in the degree of $MgCl_2$ dependency between different batches of embryos. This variation may have been attributable to differences in the relative proportions of Mg^{2+}-dependent and Mg^{2+}-independent isoforms. This requirement for Mg^{2+} was not observed for chloroplast envelope membrane PA phosphatase, and solubilized chloroplast envelope membrane PA phosphatase, where Mg^{2+} inhibited PA phosphatase activity (Block *et al.*, 1983; Malherbe *et al.*, 1995). In some mammalian and yeast systems, Mg^{2+} was shown to have no effect on PA phosphatase activity (Hosaka and Yamashita, 1984; Kanoh *et al.*, 1992).

In both particulate and solubilized fractions of *B. napus* L. cv Topas, PA phosphatase activity was partially inactivated by NEM. Inactivation of the particulate enzyme following preincubation with varying concentrations of NEM is shown in Figure 5. PA phosphatase activity decreased rapidly between preincubation concentrations of 0 and 0.05 mM NEM. A maximum of 50% inactivation was observed at 0.5 mM NEM. The effect of 4 mM NEM on PA phosphatase activity in particulate and solubilized fractions is shown in Table 8. The solubilized enzyme was also partially inactivated by NEM. Inactivation of PA phosphatase by NEM indicated that there may be key sulfhydryl groups which are required for activity. As only partial inactivation of the enzyme activity was observed, the preparations of PA phosphatase from oilseed rape may also contain forms of PA phosphatase involved in glycerolipid synthesis and signal transduction. If this was the case, the PA phosphatase

Figure 5. Effect of NEM on microsomal PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. Aliquots of 25 μL of a 10 000 - 100 000 *g* particulate fraction, containing 148 μg protein, were preincubated with varying concentrations of NEM for 15 min at 30°C then assayed for PA phosphatase activity. The results of triplicate assays are shown with the plot line connecting the means of the replicate points.

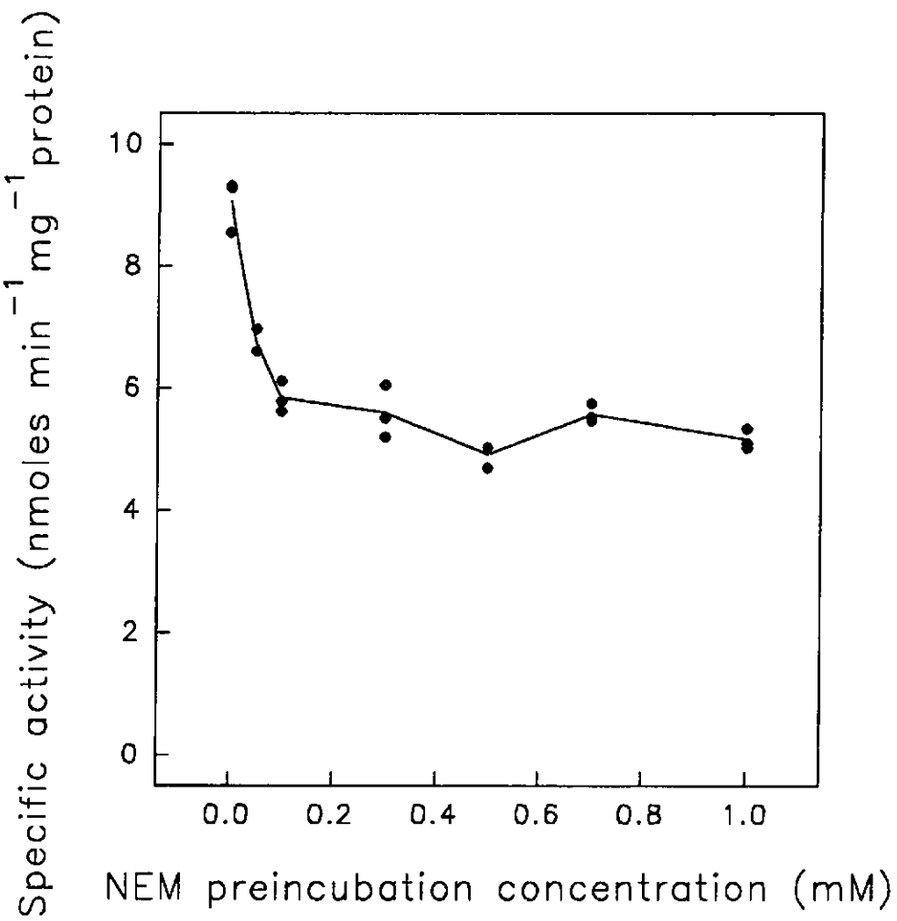

Specific activity (nmoles min^{-1} mg^{-1} protein)
10
8
6
4
2
0
0.0
0.2
0.4
0.6
0.8
1.0
NEM preincubation concentration (mM)

Table 8. Effect of NEM on the activity of particulate and solubilized PA phosphatase from the EC stage of MD embryos of *B. napus* L. cv Topas. Particulate and solubilized PA phosphatase were preincubated with the thioreactive reagent at a concentration of 4 mM for 15 min at 30°C prior to assaying for enzyme activity. The results represent the means of triplicate assays. Results of additional experiments are shown in Appendix Table 12.

TREATMENT	PA PHOSPHATASE ACTIVITY (nmol Pi/min/mg protein)
microsome	3.9
microsome + NEM	2.0
solubilized PA phosphatase	6.2
solubilized PA phosphatase + NEM	2.9

specificities shown in Table 7 could represent the combined effects of PA phosphatase isoforms. It is also possible that the microsomes may have contained PA phosphatase, involved in glycerolipid synthesis, which was only partially inactivated by NEM. Studies with mammalian systems, however, have shown that NEM-sensitive PA phosphatases are almost completely inactivated following preincubation with 5 mM NEM (Butterwith *et al.*, 1984; Jamal *et al.*, 1991; Martin *et al.*, 1991). Overall, the differential sensitivity of *B. napus* microsomal PA phosphatase to both NEM and Mg^{2+} suggested the presence of PA phosphatases involved in both glycerolipid synthesis and signal transduction.

Further Purification of Solubilized PA Phosphatase

Various protein precipitating agents were investigated in an attempt to further purify PA phosphatase (Table 9). PEG 8000, ammonium sulfate, acetone and ethanol were investigated as precipitating agents. PEG 8000, acetone and ethanol appeared to result in a considerable degree of enzyme deactivation following analyses of enzyme activities in the resulting supernatants and pellets. Fractionation with ammonium sulfate (80% saturation) resulted in complete recovery of enzyme activity in the resuspended pellet. The specific activity of PA phosphatase in the resuspended pellet, however, was similar to the specific activity for the enzyme in the initial solubilized fraction. Through further investigation, it was found that about 80% of the PA phosphatase activity in the solubilized fraction could be recovered by precipitation with ammonium sulfate (20 - 50% saturation) (Appendix Table 14).

Anion exchange chromatography was conducted in an attempt to further purify PA phosphatase and to separate NEM-sensitive and NEM-insensitive forms of the enzyme. PA phosphatase activity was resolved into two distinct peaks following chromatography at pH 8.5 with both Mono Q and Macro Prep 50Q ion exchange resins

Table 9. Precipitation of PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas by various agents. Solubilized PA phosphatase was incubated with either 80% (w/v) PEG 8000, 80% (saturation) ammonium sulfate, 80% (v/v) acetone or 80% (v/v) ethanol for 15 min on ice then spun in a microfuge at 15 900 *g* for 10 min. Treatments with the various agents were performed with solubilized enzyme from a different batch of MD embryos. The pellets were resuspended in 62.5 mM Tris/ 62.5 mM maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 1% (w/v) Tween 20 and 25 μL of the pellets and supernatants were assayed for PA phosphatase activity. The results represent the means of triplicate assays. Results of additional experiments are shown in Appendix Table 13.

TREATMENT	TOTAL ENZYME ACTIVITY (nmol/min)	SPECIFIC ACTIVITY (nmol/min/mg)
Acetone		
Initial Extract	3.4	10.2
Supernatant	0	0
Pellet	0.2	1.1
Ethanol		
Initial Extract	3.1	9.9
Supernatant	0	0
Pellet	0.1	0.5
Ammonium Sulfate		
Initial Extract	4.6	11.4
Supernatant	0.2	2.7
Pellet	5.3	10.7
PEG 8000		
Initial Extract	2.8	8.9
Supernatant	0.1	1.3
Pellet	0.8	2.5

(Figures 6 and 7). The solubilized enzyme was precipitated with 20-50% saturation ammonium sulfate prior to chromatography on the Macro Prep 50Q ion exchange resin. Following chromatography using both ion exchange resins, one component of the activity was unretained by the column and a second component was eluted from the column using an NaCl gradient. The retained peak was eluted with about 0.4 M NaCl. The total recovery of activity was about 10% of that applied to the column. Various amounts of PA phosphatase, representing different protein and activity levels, were applied to the Macro Prep 50Q ion exchange column and the two distinct peaks of PA phosphatase activity were consistently obtained (Appendix Figures 1 and 2). Thus, the resulting peaks were not due to column overloading and may represent two isoforms of PA phosphatase. Macro Prep 50Q ion exchange chromatography, however, failed to resolve NEM-sensitive and NEM-insensitive PA phosphatase (Figure 8). Figure 8 represents the same chromatographic experiment described in Figure 7. All fractions contained both NEM-sensitive and NEM-insensitive activities. The specific activities of PA phosphatase in fractions from peaks I and II containing maximum enzyme activity were 2.2 and 1.8 nmol/min/mg, respectively. These specific activities were lower than that of the post ammonium sulfate precipitation fraction (5.8 nmol/min/mg). This loss of activity and the lability of the enzyme prevented further purification of PA phosphatase. The results of ion exchange chromatography indicated that there may be forms of both NEM-sensitive and NEM-insensitive PA phosphatase with different isoelectric points. There may have also been artifacts due to protein-protein interactions. The relative proportion of NEM-sensitive and NEM-insensitive PA phosphatase in peaks I and II varied with each batch of MD embryos which suggested that slight changes in the developmental stage of the tissue may have resulted in changes in the relative proportions of the two forms of PA phosphatase. The relative proportions of PA phosphatase isoforms may be related to the developmental status of

Figure 6. Mono Q anion-exchange chromatography of solubilized PA phosphatase from the EC stage of MD embryos of *B. napus* L. cv Topas. The column (HR 5/5, total bed volume = 1 mL) was equilibrated with 10 mM Tris-HCl buffer, pH 8.5, containing 1% (w/v) Tween 20. Two mL of solubilized protein (4 mg) were applied to the column followed by 10 mL equilibration buffer then the column was eluted with a linear gradient of 0 - 2 M NaCl (total volume = 20 mL). The column was run at a flow rate of 30 mL/h using an FPLC system. Fractions of 1 mL were collected and 200 μL aliquots were assayed for PA phosphatase activity.

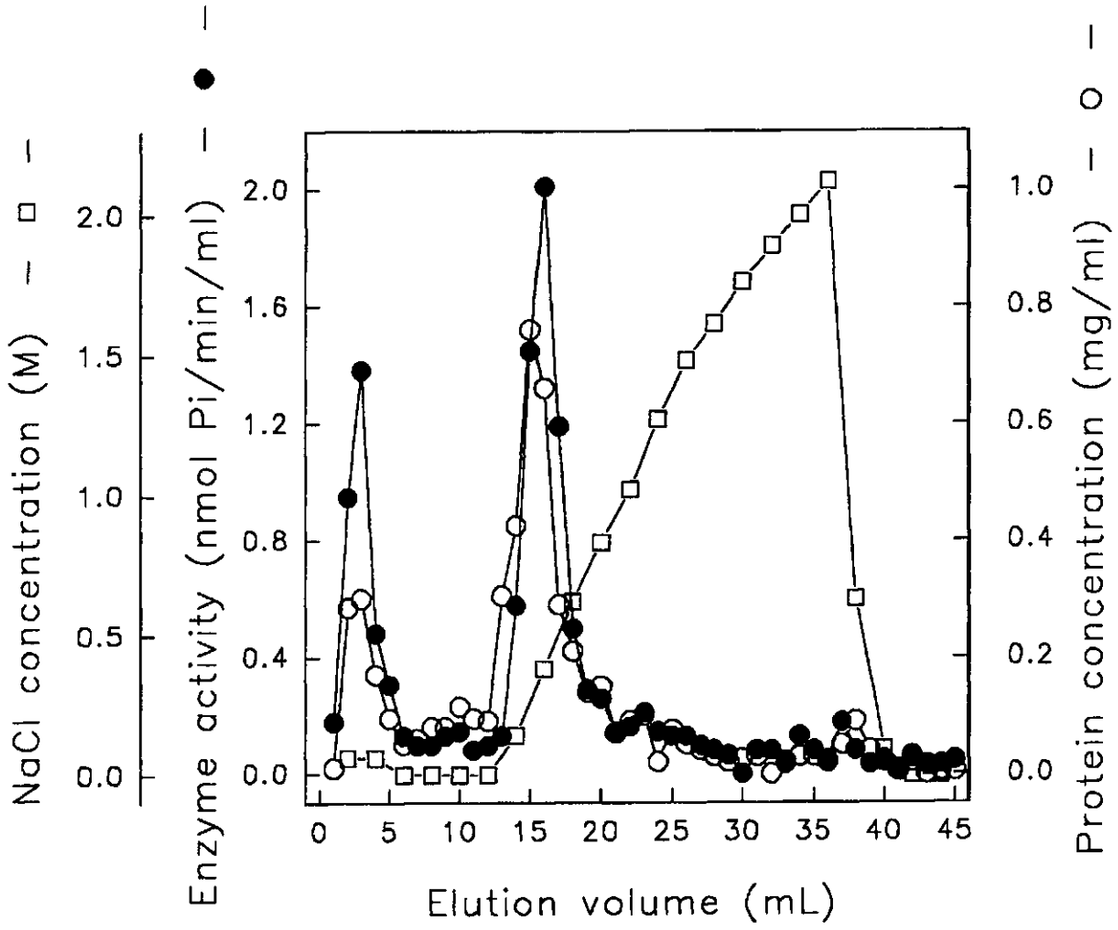

NaCl concentration (M) – □ –
Enzyme activity (nmol Pi/min/ml) – ● –
Protein concentration (mg/ml) – ○ –
0.0
0.5
1.0
1.5
2.0
0.4
0.8
1.2
1.6
0.2
0.6
0
5
10
15
20
25
30
35
40
45
Elution volume (mL)

Figure 7. Macro Prep 50Q anion-exchange chromatography of solubilized PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. The column (BioRad resin, total bed volume = 4 mL) was equilibrated with 10 mM Tris-HCl buffer, pH 8.5 containing 1% (w/v) Tween 20. Two mL of solubilized protein was fractionated with ammonium sulfate (20 - 50% saturation). The post ammonium sulfate fraction (6.1 mg) in 2 mL was applied to the column followed by 10 mL equilibration buffer. The column was eluted with a linear gradient of 0 - 2 M NaCl (total volume = 20 mL). The column was run at a flow rate of 30 mL/h using an FPLC system. Fractions of 1 mL were collected and 100 μL aliquots were assayed for PA phosphatase activity.

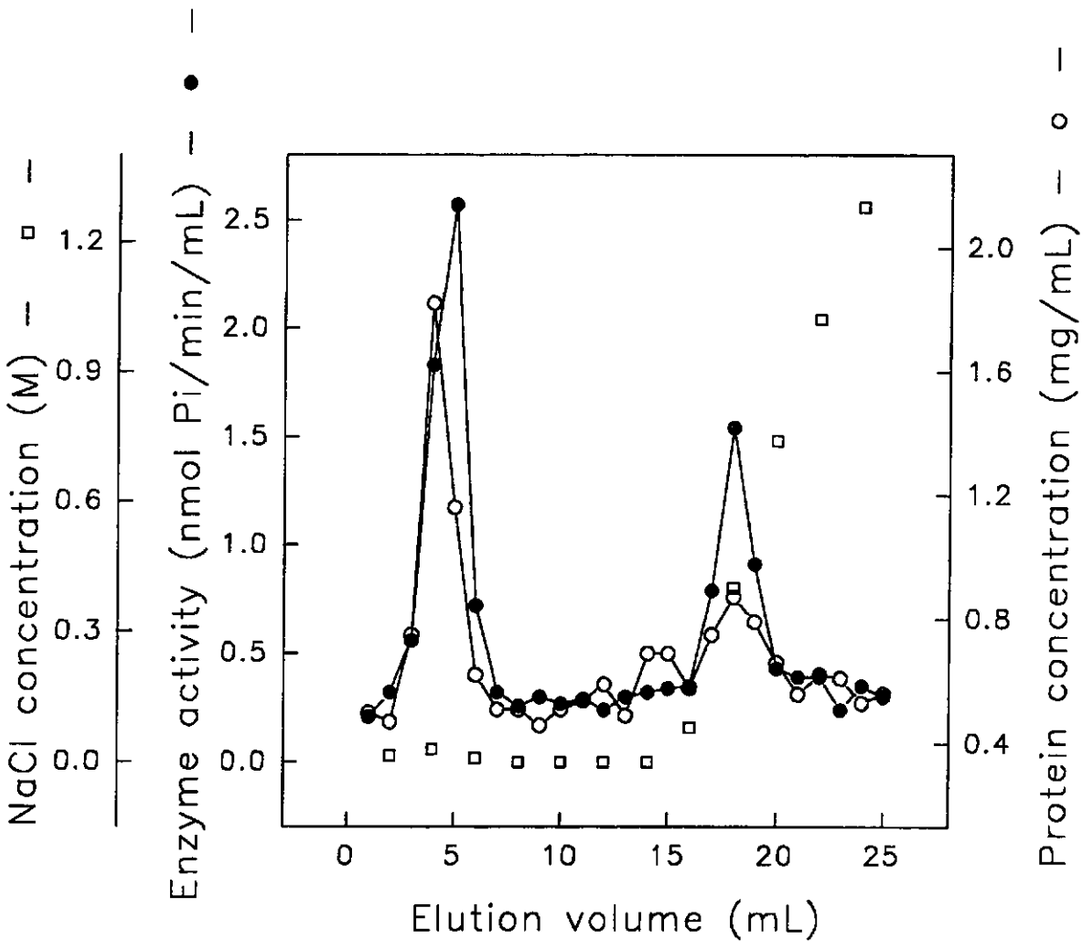
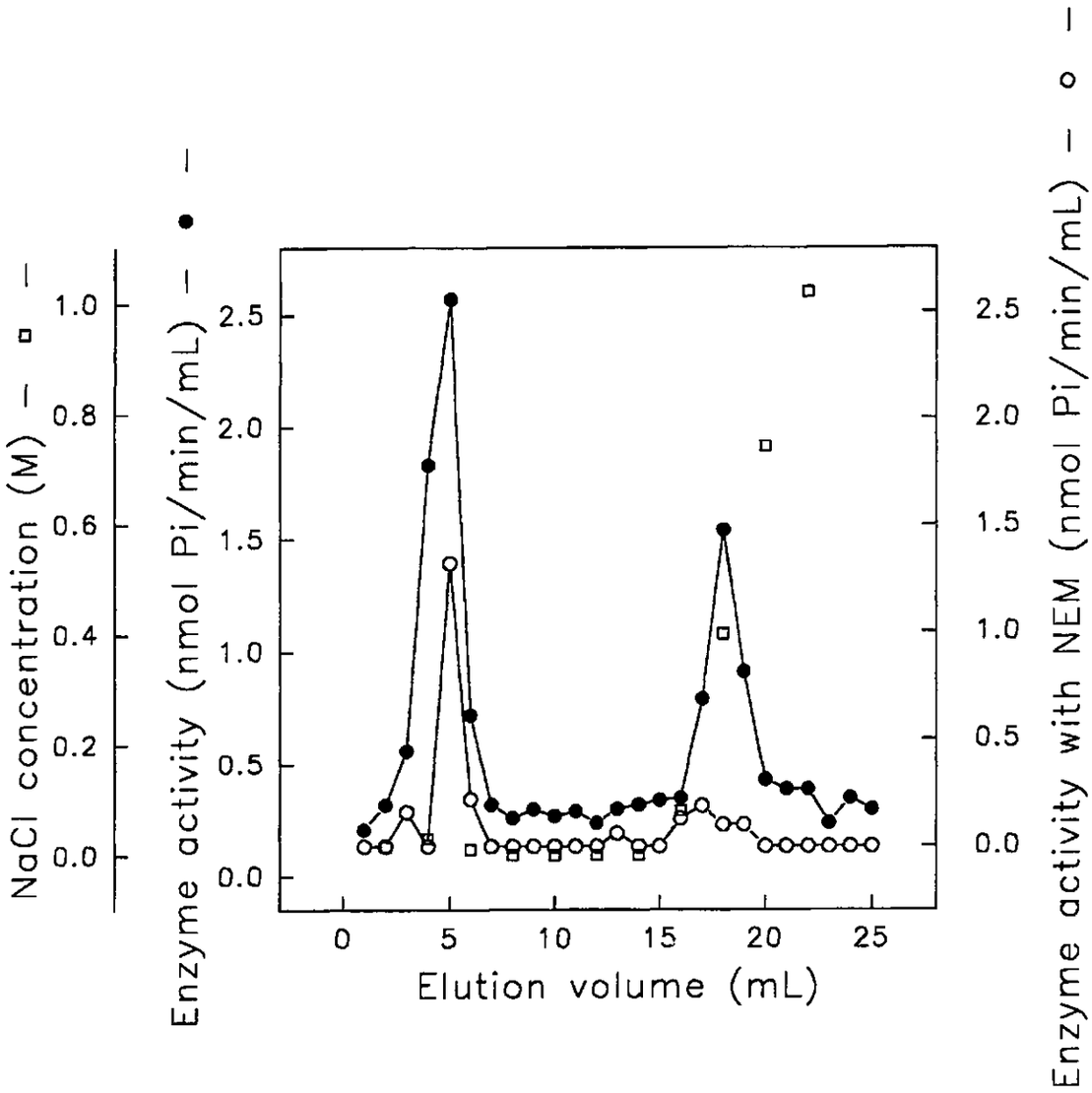

NaCl concentration (M) — □ —
Enzyme activity (nmol Pi/min/mL) — ● —
Protein concentration (mg/mL) — o —
0.0
0.3
0.6
0.9
1.2
0.0
0.5
1.0
1.5
2.0
2.5
0.4
0.8
1.2
1.6
2.0
0
5
10
15
20
25
Elution volume (mL)

Figure 8. Detection of NEM-sensitive and NEM-insensitive forms of PA phosphatase following Macro Prep 50Q anion-exchange chromatography of solubilized PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. The column (BioRad resin, total bed volume = 4 mL) was equilibrated with 10 mM Tris-HCl buffer, pH 8.5 containing 1% (w/v) Tween 20. Two mL of solubilized protein were fractionated with ammonium sulfate (20 - 50% saturation). The post-ammonium sulfate fraction (6.1 mg) in 2 mL was applied to the column followed by 10 mL equilibration buffer. The column was eluted with a linear gradient of 0 - 2 M NaCl (total volume = 20 mL). The column was run at a flow rate of 30 mL/h using an FPLC system. Fractions of 1 mL were collected and 100 μL aliquots were preincubated with 4 mM NEM for 15 min at 30°C. The fractions were then assayed for PA phosphatase activity.


NaCl concentration (M) — □ —
Enzyme activity (nmol Pi/min/mL) — ● —
Enzyme activity with NEM (nmol Pi/min/mL) — ○ —
Elution volume (mL)
0.0
0.2
0.4
0.6
0.8
1.0
0.5
1.5
2.0
2.5
0
5
10
15
20
25

the tissue. This may lead to the development of molecular probes which may be useful in studying embryogenesis.

A number of different forms of PA phosphatase-2 may be present in rat liver (Kocsis and Weselake, 1996). For example, Fleming and Yeaman (1995a) have reported the separation of PA phosphatase-2 into two distinct peaks of enzyme activity following Sephacryl S-300 gel filtration. The peaks were determined to be separate enzymes and were further purified. Waggoner *et al.* (1995) also observed the separation of an anionic and cationic form of PA phosphatase-2 into two peaks of activity following Mono Q chromatography.

SDS-PAGE of the protein from the microsomal fraction, solubilized fraction, post ammonium sulfate fraction and the two column peaks (shown in Figures 7 and 8) are shown in Figure 9. The ammonium sulfate fraction (lane 4) displayed fewer polypeptides than the particulate (lane 2) and solubilized (lane 3) fractions. The ammonium sulfate fraction appeared to be relatively enriched in a polypeptide with an apparent M_r of 36 000. Furthermore, fewer polypeptide bands were observed for the two column peaks (lanes 5 and 6) in comparison to the ammonium sulfate fraction. Peak I from Macro Prep 50 Q appeared to be relatively enriched in a polypeptide with an apparent M_r of about 42 000. Although PA phosphatase was not homogeneous, as determined by SDS-PAGE, a decrease in the number of polypeptides in the ammonium sulfate fraction suggested that the preparation may be a more purified plant PA phosphatase. A corresponding increase in specific activity may not have been observed due to the instability of the enzyme. Further attempts at purification following ion exchange chromatography were unsuccessful due to the instability of the enzyme.

Gel filtration chromatography was also attempted in the purification of PA phosphatase. Superose 6 gel filtration chromatography of solubilized PA phosphatase was conducted in the presence of 0.2% (w/v) Tween 20. The results of four chromatographic runs are depicted in Figure 10. PA phosphatase was solubilized from

Figure 9. SDS-PAGE of fractions containing PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. The following samples were applied to the gel: lane 1, M_r markers from top to bottom were BSA (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 200) (4 μg protein per band); lane 2, 10 000 - 100 000 *g* particulate fraction; lane 3, solubilized PA phosphatase; lane 4, ammonium sulfate fraction (20 - 50% saturation) of solubilized PA phosphatase; lane 5, peak I following Macro Prep 50Q anion-exchange chromatography of solubilized PA phosphatase; and lane 6, peak II from Macro Prep 50Q anion-exchange chromatography of solubilized PA phosphatase. About 20 μg of protein were applied in each lane from lanes 2 to 6. Gels were stained with the silver staining method by Merril *et al.* (1983).

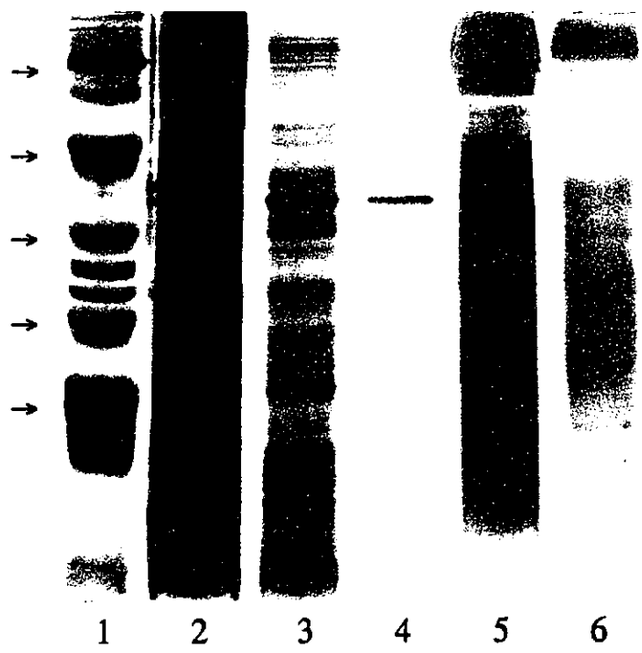
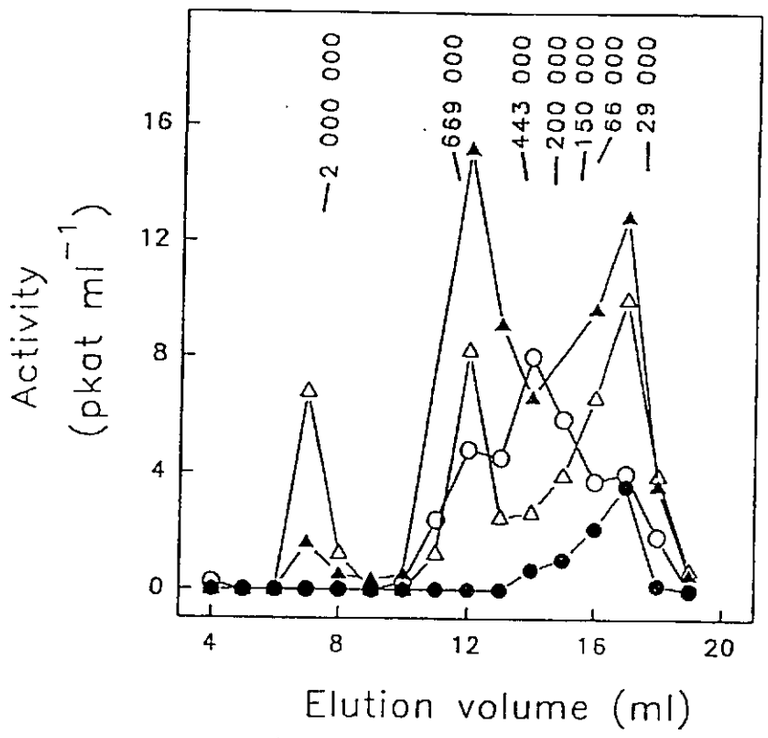

1
2
3
4
5
6

Figure 10. Gel filtration chromatography of solubilized PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas on a Superose 6 column (HR 10/30, total bed volume = 23.6 mL). The column was equilibrated with 0.2% (w/v) Tween 20 in 60 mM Tris-60 mM maleic acid-NaOH buffer, pH 6.75, containing 0.1 M KCl, 0.12 mM EDTA and 2.5 mM $MgCl_2$. Samples of 200 or 500 μL were injected onto the column which was run at a flow rate of 12 mL/h using an FPLC system. The following samples were applied to the column: batch #1 - MD embryos, 426 μg solubilized enzyme exposed to one cycle of freezing/thawing (○); batch #1 - embryos, 426 μg solubilized enzyme exposed to two cycles of freezing/thawing (●); batch #2 - embryos, 720 μg freshly solubilized enzyme (△); batch #2 - embryos, 1800 μg solubilized enzyme exposed to one cycle of freezing/thawing (▲). Fractions of 1 mL were collected and 300 μL aliquots were assayed for PA phosphatase activity. M_r markers were run as above in the absence of 0.2% (w/v) Tween 20. M_r markers were as follows : blue dextran (2 000 000), thyroglobulin (669 000), apoferritin (443 000), ß-amylase (200 000), alcohol dehydrogenase (150 000), bovine serum albumin (66 000), and carbonic anhydrase (29 000).


2 000 000
669 000
443 000
200 000
150 000
66 000
29 000
16
12
8
4
0
4
8
12
16
20
Activity
(pkat ml^{-1})
Elution volume (ml)

two different batches of MD embryos and between column runs, each solubilized preparation had undergone one cycle of freezing and thawing. The elution positions of various protein standards are also shown in Figure 10. The elution volumes of M_r standards remained the same in the presence or absence of 0.2% (w/v) Tween 20. PA phosphatase was eluted in a number of fractions within the sieving range of the gel. This confirmed that the enzyme was solubilized. A minimum apparent M_r of about 40 000 was observed for both preparations of solubilized PA phosphatase. The range of M_r species suggested that PA phosphatase may have aggregated or different forms of the enzyme may have been resolved. These results are perhaps not unexpected when one considers the observed differences in Mg^{2+} sensitivity, NEM sensitivity and behavior of the enzyme during ion exchange chromatography. This may be further complicated by possible differences from batch to batch of MD embryos.

Gel filtration chromatography studies with PA phosphatases from other sources have resulted in a range of M_rs. PA phosphatase-2, purified to apparent homogeneity from porcine thymus, displayed a M_r of about 218 000 following gel filtration chromatography on a Superose 12 column in the presence of 1% (w/v) Triton X-100 (Kanoh *et al.*, 1992). Fleming and Yeaman (1995a) separated PA phosphatase-2 from rat liver into two distinct peaks following gel filtration on Sephacryl S-300. Following further purification and gel filtration chromatography on a Superose 6 column, peak 2A displayed an apparent M_r between 290 000 - 300 000 whereas peak 2B exhibited an apparent M_r of 265 000. Lin and Carman (1989) purified yeast PA phosphatase to homogeneity and reported a M_r of 93 000 as a result of gel filtration chromatography in the presence of 1% (w/v) sodium cholate.

In the current study, PA phosphatase activity from MD embryos decayed rapidly following various types of chromatography. In the future, it may be worthwhile to explore the use of [^{32}P]PA as enzyme substrate in order to increase the sensitivity of PA phosphatase detection. This type of assay, combined with the use of

enzyme extracts prepared from larger amounts of tissue may allow us to monitor PA phosphatase activity through a number of chromatographic steps.

Development of an Immunochemical Probe for PA Phosphatase

Antibodies recognizing the PA phosphatase polypeptide might be prepared without the need for pure enzyme in immunizations. In an attempt to develop these antibodies, rabbits were immunized with partially purified and delipidated PA phosphatase from microsomes of MD embryos of oilseed rape. The strategy was to use the resulting polyspecific antibodies to isolate a sub-class of monospecific antibodies recognizing PA phosphatase. The monospecific polyclonal antibodies recognizing PA phosphatase would be potentially useful for screening cDNA expression libraries in order to isolate cDNA encoding PA phosphatase.

Microsomal PA phosphatase was inhibited about 36% and 8%, respectively, following preincubation of microsomes with antisera from two immunized rabbits (Table 10). The inhibition data suggested that the antisera contained antibodies which recognized PA phosphatase. In the future, the sub-class of antibodies recognizing PA phosphatase may be isolated using the procedure of Olmsted (1981) or Muilerman *et al.* (1982). In both these techniques crude preparations of enzyme would be subjected to SDS-PAGE followed by transfer of the separated polypeptide to nitrocellulose. A polypeptide(s) representing PA phosphatase would then be identified based on its ability to capture specific antibodies which either inhibited or captured native PA phosphatase. The approach of Muilerman *et al.* (1982) has been applied to the identification of a PA phosphatase-2 polypeptide from rat liver membranes (Siess and Hofstetter, 1996).

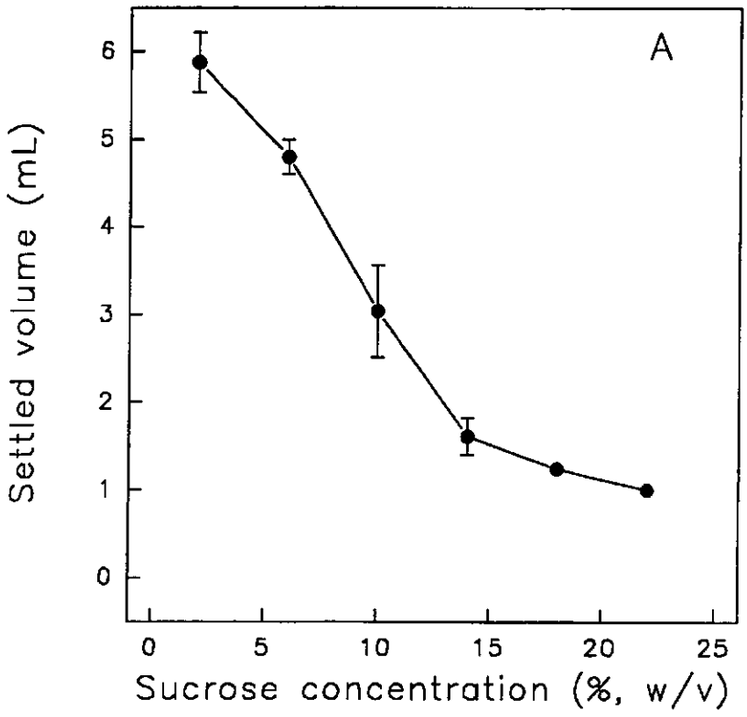
Table 10. Inhibition of microsomal PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas by rabbit polyclonal antisera. A 10 000 - 100 000 *g* particulate fraction (10 μL containing 84 μg protein) was preincubated for 30 min at 30°C with 10 μL antisera obtained as outlined in MATERIALS AND METHODS, and then assayed for PA phosphatase activity. Antisera from two rabbits and preimmune sera from each rabbit were dialyzed against Tris-maleic acid-NaOH buffer, pH 6.75, to decrease the levels of endogenous P_i. The net absorbance at 820 nm and the percentage of activity as compared to the control are shown. The results represent the means of triplicate preincubations and assays. Results of another experiment are shown in Appendix Table 15.

ANTISERUM	NET ABSORBANCE at 820 nm	PA PHOSPHATASE ACTIVITY (% of control)
Control 1	0.315	100
Antiserum 1	0.199	63
Control 2	0.312	100
Antiserum 2	0.287	92

Properties of PA Phosphatase from a MD Cell Suspension Culture of *B. napus* L.

PA phosphatase activity was also investigated in MD cell suspension cultures of *B. napus* L. cv Jet Neuf that were grown under varying sucrose concentrations. Weselake *et al.* (1993, 1997) have demonstrated that MD cell suspension cultures of *B. napus* L. cv Jet Neuf may be potentially useful in studies of the enzymology and molecular biology of TG formation in oilseed rape. The cells have been shown to accumulate oil more effectively as sucrose concentration increased from 2 - 14% (w/v) (Weselake *et al.*, 1997). In the current study, PA phosphatase activity was observed in cells grown in media containing sucrose which ranged in concentration from 2-22%. The largest amounts of cells were harvested at 2 - 6% sucrose as determined by settled volume and fresh weight (FW) data (Figures 11 A and B, respectively). Maximum dry weights (DW) were obtained when cells were cultured in 2 - 6% sucrose (Figure 12). The DW/FW ratio increased in a nearly linear fashion between 2 and 18% sucrose (Figure 13). The cell suspension system was further used to assess the relative proportions of NEM-sensitive and NEM-insensitive PA phosphatase activity as a function of sucrose concentration. The results of enzyme assays conducted with and without preincubation with 10 mM NEM are shown in Figure 14. In the presence or absence of NEM, PA phosphatase activity was greatest when cells were grown in 10% sucrose and decreased as the sucrose concentration was increased to 22%. PA phosphatase was partially inactivated in the presence of NEM but the enzyme from the microsomes of the cell suspension was not as sensitive to NEM as the enzyme from the microsomes of MD embryos. Thus, forms of PA phosphatase involved in glycerolipid synthesis and signal transduction, respectively, may also be present in the cell suspension system.

Figure 11. Settled volume (A) and fresh weight (B) of cell suspension cultures of *B. napus* L. cv Jet Neuf which were cultured for two weeks in media that contained sucrose concentrations ranging from 2 - 22% (w/v). Each data point (± standard error) represents the means for three independent cultures.


A
Settled volume (mL)
0
1
2
3
4
5
6
0
5
10
15
20
25
Sucrose concentration (%, w/v)

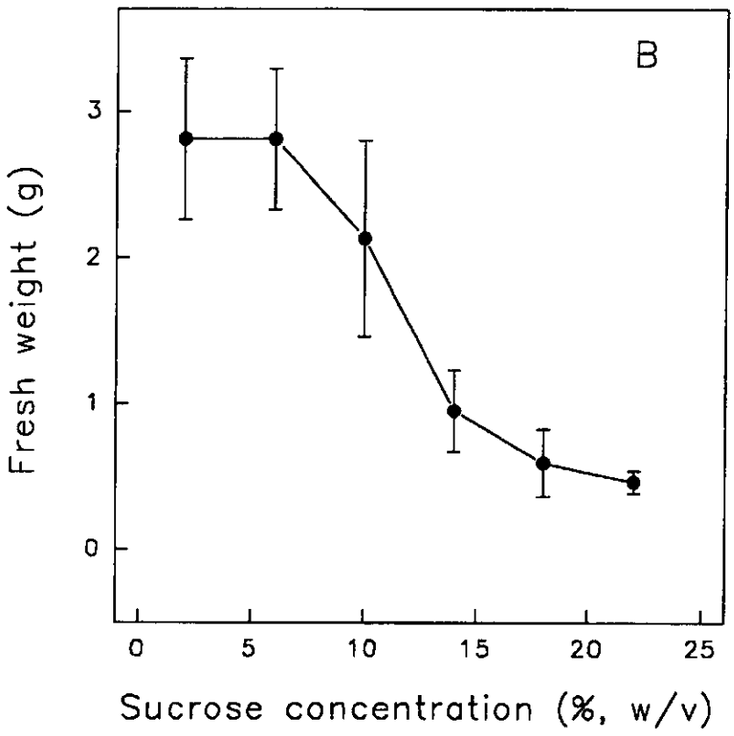

B
Fresh weight (g)
0
1
2
3
0
5
10
15
20
25
Sucrose concentration (%, w/v)

Figure 12. Dry weight of cell suspension cultures of *B. napus* L. cv Jet Neuf which were cultured for two weeks in media that contained sucrose concentrations ranging from 2 - 22% (w/v). Each data point (± standard error) represents the means for three independent cultures.

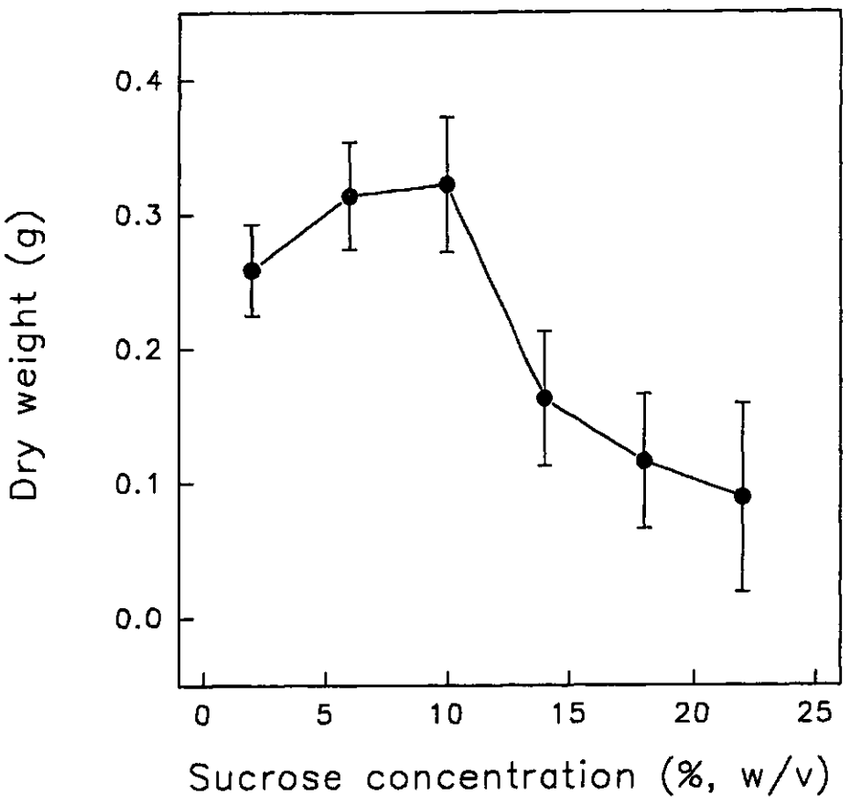

Dry weight (g)
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
Sucrose concentration (%, w/v)

Figure 13. Ratio of dry weight to fresh weight of cell suspension cultures of *B. napus* L. cv Jet Neuf which were cultured for two weeks in media that contained sucrose concentrations ranging from 2 - 22% (w/v). Each data point represents the means for three independent cultures.

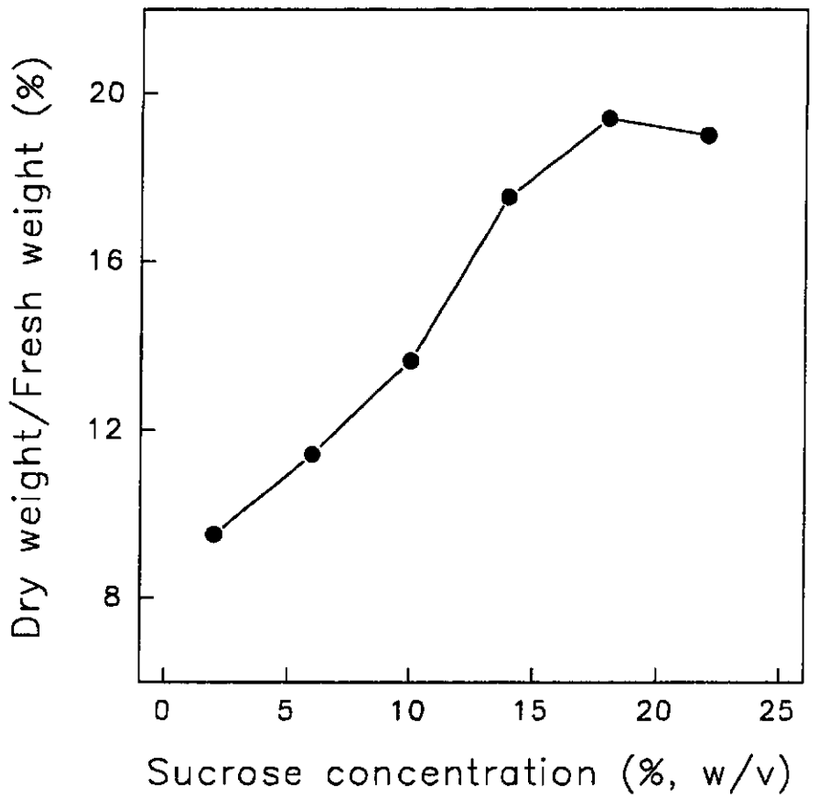
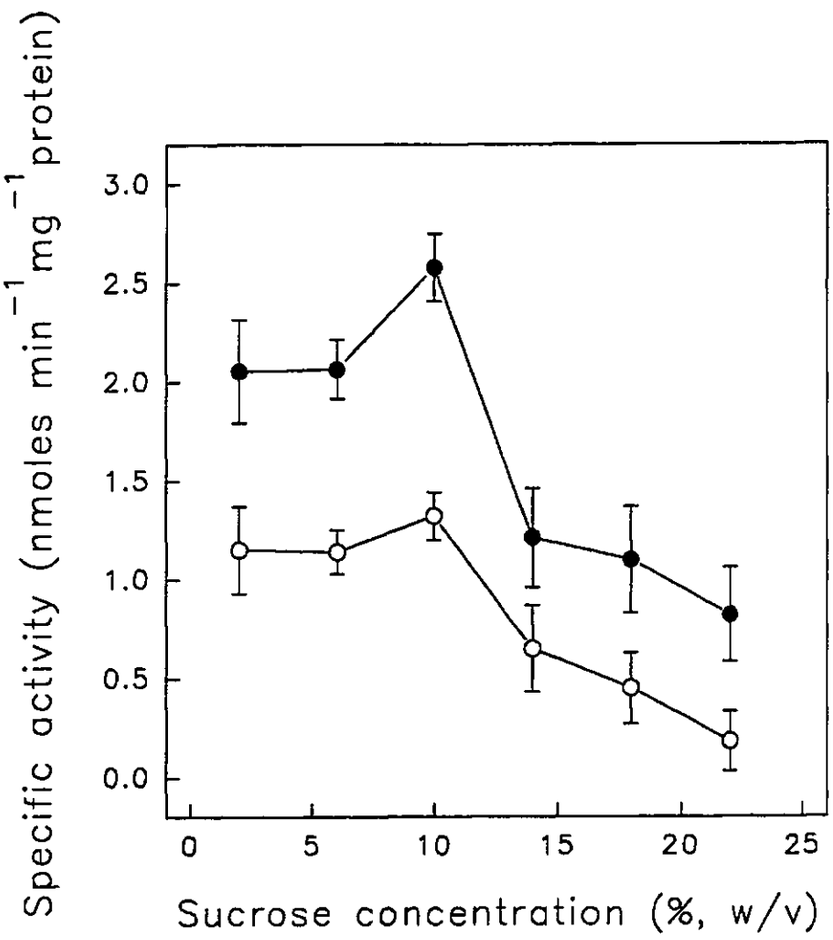

Dry weight/Fresh weight (%)
20
16
12
8
0
5
10
15
20
25
Sucrose concentration (%, w/v)

Figure 14. Activity of microsomal PA phosphatase of cell suspension cultures of *B. napus* L. cv Jet Neuf which were cultured for two weeks in media that contained sucrose concentrations ranging from 2 - 22% (w/v). Aliquots of 25 μL of 10 000 - 100 000 *g* particulate fractions of each sucrose concentration were preincubated in the presence (○) and absence (●) of 4 mM NEM for 15 min at 30°C then assayed for PA phosphatase activity. The amount of microsomal protein assayed for cells cultured in 2, 6, 10, 14, 18 and 22% (w/v) sucrose was 92.5, 63.8, 35, 17.5, 12.5 and 17.5 μg protein, respectively. The results (± standard error) represent the means for triplicate assays of three independent cultures.


Specific activity (nmoles min^{-1} mg^{-1} protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Sucrose concentration (%, w/v)

Further Purification of DGAT

Affinity chromatography of DGAT was investigated using alkyl-CoA Sepharose and palmitoyl-CoA agarose columns. The alkyl-CoA Sepharose resin contains a thioether linkage between the alkyl- group and CoA whereas the palmitoyl-CoA agarose resin contains a thioester linkage between the palmitoyl- moiety and CoA. The thioether linkage is not a biologically relevant linkage and is less likely to be attacked by thioesterases, which are commonly found in biological systems. Preparations containing solubilized DGAT were applied to each column and were eluted with a high concentration of erucoyl-CoA. Eluted proteins were precipitated with PEG 8000 (6%, w/v) or a PEG 8000 (6%, w/v)/BSA (1%, w/v) mixture in an attempt to concentrate eluted enzyme. For both affinity columns, enzyme activities of the solubilized DGAT, unbound fraction, eluted fraction, and fractions containing proteins precipitated with PEG are shown in Table 11. Following affinity chromatography, about 20% of the initial activity was recovered in the unbound fractions from each affinity column. This suggested that a large amount of activity may have bound to the affinity columns. Elution of the columns with erucoyl-CoA, however, resulted in very low levels of activity in the eluted fractions. The loss of enzyme activity may have resulted from strong interaction with the column matrix or the instability of enzyme.

Immunoaffinity chromatography of DGAT was performed using immobilized monoclonal antibodies derived from mice which were immunized with partially purified DGAT from MD embryos of oilseed rape. These antibodies were shown to inhibit microsomal DGAT from MD embryos of oilseed rape (Weselake, 1995). Solubilized DGAT was applied to the column and eluted with either 1 M KNO_3 or 0.1 M glycine, pH 2.5. DGAT activities from the solubilized fraction, and the unbound and eluted fractions from both control and ascites columns are shown in Table 12. About 10% of the applied enzyme activity was found in the unbound fractions. Following elution from the columns, no enzyme activity was detected. The elution conditions may have

Table 11. Affinity chromatography of solubilized DGAT from the EC stage of MD embryos of *B. napus* L. cv Topas using alkyl-CoA Sepharose and palmitoyl-CoA agarose. Solubilized DGAT was subjected to affinity chromatography as described in MATERIALS AND METHODS and eluted with 0.5 mM erucoyl-CoA. Fractions were collected and precipitated with either 6% (w/v) PEG 8000 or 6% (w/v) PEG 8000 with 1% (w/v) BSA. Samples of 10 μL were assayed for DGAT activity. The results represent the means of triplicate assays. Results of additional experiments are shown in Appendix Table 16.

Fraction	Total Volume (mL)	Total Enzyme Activity (pmol/min)
Solubilized enzyme	1.0	213.4
ALKYL-CoA		
Unbound	3.0	82.0
Eluted	0.4	1.0
Post PEG ppt.	0.05	0.1
Post PEG ppt. +BSA	0.05	0.1
PALMITOYL-CoA		
Unbound	3.0	75.0
Eluted	0.4	0.1
Post PEG ppt.	0.05	0
Post PEG ppt. +BSA	0.05	0.1

Table 12. Immunoaffinity chromatography of solubilized DGAT from the EC stage of MD embryos of *B. napus* L. cv Topas. Antibodies from mice were prepared from a native preparation of DGAT from MD embryos of *B. napus* L. cv Topas, as outlined in MATERIALS AND METHODS, and were coupled to epoxy-activated Sepharose 6B. Control antibodies, from mice without exposure to the DGAT antigen, were also coupled to affinity columns. Columns were eluted with either 1 M KNO_3 or 0.1 M glycine, pH 2.5. Fractions were collected and 10 μL were assayed for DGAT activity. The results represent the means of triplicate assays. Results of another experiment are shown in Appendix Table 17.

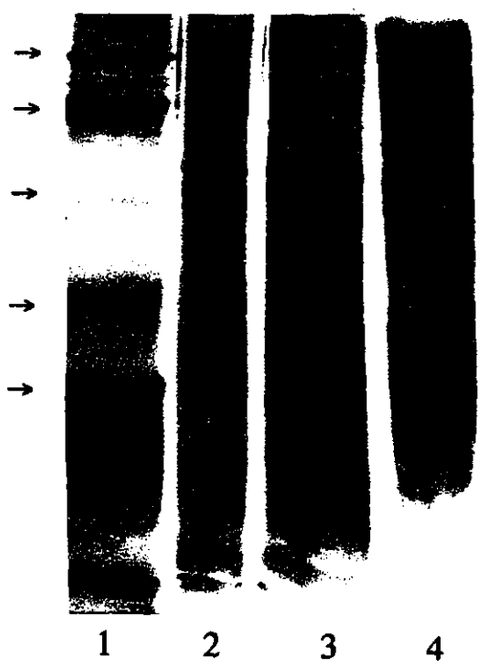
Fraction	Total Volume (mL)	Total Enzyme Activity (pmol/min)
solubilized enzyme	0.5	81.5
ELUTION WITH KNO_3		
unbound control IgG	1.0	9.7
unbound ascites IgG	1.0	7.9
control IgG	0.6	0
ascites IgG	0.6	0.1
ELUTION WITH LOW PH		
unbound control IgG	1.0	6.3
unbound ascites IgG	1.0	7.6
control IgG	0.7	0.4
ascites IgG	0.7	0.4

been too harsh for the enzyme or the enzyme lost activity following its interaction with the column.

Development of an Immunochemical Probe for DGAT

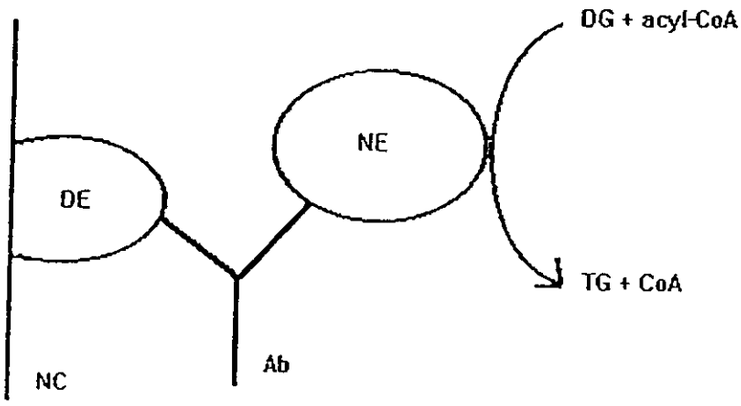
In the preparation of the antibodies from MD embryos of oilseed rape, PA phosphatase and DGAT were sequentially solubilized from the same microsomes using Tween 20 and MEGA-8, respectively. The purity of DGAT increased about 4- to 7-fold over that of the solubilized enzyme following precipitation with 2 - 6% PEG 8000 (w/v) (Appendix Table 18). SDS-PAGE of the protein from the microsomal fraction, solubilized fraction and post PEG fraction are shown in Figure 15. The PEG-precipitated fraction (lane 4) displayed few polypeptides than the particulate (lane 2) and solubilized (lane 3) fractions. The PEG-precipitated fraction containing DGAT was used for the development of polyspecific polyclonal antibodies and these antibodies were further used to investigate an alternative approach to identifying the polypeptide DGAT. Using an immunodetection method similar to that described by Muilerman *et al.* (1982), antibodies developed in rabbits against a partially purified preparation of MD embryos of oilseed rape were used as "immunochemical probes" to detect DGAT activity. Polypeptides from particulate fractions containing DGAT from MD embryos were separated by SDS-PAGE and transferred to nitrocellulose. The polypeptides were incubated with anti-DGAT antibodies followed by a second incubation with solubilized DGAT from MD embryos of oilseed rape. Sections of the nitrocellulose were subsequently assayed for DGAT activity. In theory, DGAT protein which was bound to the nitrocellulose membrane would be detected by the anti-DGAT antibodies. The antibodies would bind to the protein with some antibodies binding to only one antigen binding site of a bivalent antibody, leaving the other antigen binding site available for further binding. Following the second incubation with "active" solubilized DGAT, the

Figure 15. SDS-PAGE of fractions containing DGAT from the LC stage of MD embryos of *B. napus* L. cv Topas. The following samples were applied to the gel: lane 1, M_r markers from top to bottom were BSA (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 200) (4 μg protein per band); lane 2, 10 000 - 100 000 *g* particulate fraction; lane 3, solubilized DGAT; and lane 4, PEG 8000 fractionation (2 - 6%, w/v fractionation) of solubilized DGAT. About 20 μg of protein were applied to each lane in lanes 2 to 4. Gels were stained by the silver staining method by Merril *et al.* (1983).


1
2
3
4

free arm of the antibody would bind to the "active" DGAT. The proposed binding of "active" DGAT to anti-DGAT antibodies/denatured enzyme complex is illustrated in Figure 16. Initial results, using a dot blot apparatus to apply denatured microsomal protein from MD embryos of oilseed rape, have indicated that DGAT activity can be detected following incubation with anti-DGAT antibodies followed by incubation with "active" solubilized DGAT (Appendix Table 19). Thus, the polyclonal antibodies may be useful, as immunochemical probes, in the purification of DGAT.

Figure 16. Schematic representation of a proposed immunodetection method for the purification of DGAT from MD embryos of *B. napus* L. cv Topas using anti-DGAT antibodies. In theory, following transfer of the denatured DGAT enzyme (DE) to nitrocellulose (NC) and incubation with antibodies (Ab) derived from partially purifed DGAT from MD embryos of *B. napus* L. cv Topas, the anti-DGAT antibodies may bind to the denatured enzyme with only one antigen binding site with the remaining antigen binding site available for binding to native DGAT enzyme (NE). Enzyme activity may be determined by the detection of radiolabelled TG following incubation of the complex in the presence of radiolabelled oleoyl-CoA as the donor substrate.


DG + acyl-CoA
NE
DE
TG + CoA
Ab
NC

SUMMARY AND FUTURE DIRECTIONS

In an attempt to purify the TG biosynthetic enzymes of oilseed rape, PA phosphatase and DGAT from solubilized preparations from MD embryos of *B. napus* L. cv Topas have been further characterized. Both enzymes displayed a loss of enzyme activity following solubilization and storage for prolonged periods of time. BSA increased the stability of both PA phosphatase and DGAT. The solubilized preparations may be useful in studies of regulation with limited influence by the other Kennedy pathway enzymes.

Microsomal fractions from MD embryos and MD cell suspension cultures of oilseed rape contained both NEM-sensitive and NEM-insensitive PA phosphatase activity. Microsomal PA phosphatase was also partially dependent on Mg^{2+}. The partial sensitivity of PA phosphatase activity to both NEM and Mg^{2+} suggests that, similar to mammalian systems, MD embryos of oilseed rape may contain forms of PA phosphatase involved in both glycerolipid synthesis and signal transduction. The form of PA phosphatase involved in glycerolipid synthesis is thought to occur in the cytosol and in response to an increase in fatty acids, translocates to the membrane of the ER where it becomes highly active in TG biosynthesis. The exact location of the putative isoforms of PA phosphatase will depend on future analysis of enzyme activity in membrane fractions prepared by sucrose gradient fractionation and immunocytological studies. The cell suspension cultures may be useful in examining the possible regulating role of PA phosphatase in glycerolipid synthesis in plants.

In the purification of PA phosphatase, ammonium sulfate fractionation was shown to maintain over 80% of the activity of solubilized PA phosphatase with a reduced number of polypeptides compared to the initial solubilized fraction. This maintenance of activity with subsequent removal of other proteins was beneficial for further purification of PA phosphatase. PA phosphatase was resolved into two distinct

peaks of activity following anion exchange chromatography. Both peaks contained NEM-sensitive and NEM-insensitive PA phosphatase activity. PA phosphatase activity eluted over a broad range of fractions following gel filtration chromatography, but displayed a minimum apparent M_r of 40 000. Further investigation will be required to determine whether there are isoforms of PA phosphatase differing in M_rs or that the same enzyme displayed different states of aggregation. In the preparation of antibodies, PA phosphatase and DGAT were sequentially solubilized from the same microsomes using Tween 20 and MEGA-8, respectively. Using various detergents to sequentially solubilize other membrane-bound proteins from the same microsome from which PA phosphatase was solubilized, may result in a more purified form of DGAT when this enzyme is eventually solubilized from the same microsome.

Further purification of PA phosphatase was hampered by the rapid loss of enzyme activity. Further purification strategies, where the detection of enzyme activity was required in order to determine enzyme purity, were unsuccessful. The use of radiolabelled substrate may provide for a more sensitive assay system to detect low levels of PA phosphatase activity following chromatography. An alternate route to the purification of PA phosphatase may be the development of antibodies specific to PA phosphatase to be used in immunoaffinity chromatography and probing of cDNA expression libraries.

In the purification of DGAT, little or no enzyme activity was recovered following elution from both affinity and immunoaffinity columns. It is possible that the elution conditions may have been too harsh for the enzyme or the enzyme lost activity following its interaction with the columns. Alternate elution conditions may result in the recovery of detectable enzyme activity. As BSA was previously shown to stabilize both PA phosphatase and DGAT activity, the incorporation of BSA following elution from the columns may also result in increased recovery of enzyme activity. The development of antibodies specific to DGAT have shown promise in the detection

of DGAT by a technique which involves the "capture" of native enzyme to antibodies bound to denatured enzyme immobilized on nitrocellulose. The antibodies bind to the denatured enzyme with only one antigen binding site and the other binding site is available for the "capture" of native DGAT. Enzyme activity could be detected by enzyme assays, followed by the detection of the corresponding polypeptides from the SDS-polyacrylamide gels.

This thesis has described some characteristics of PA phosphatase and DGAT which may be useful in developing further purification schemes for these enzymes. The purification of PA phosphatase and DGAT will lead to the identification of the genes which encode these enzymes. Genetic manipulations which result in an increase in the levels of PA phosphatase and/or DGAT may subsequently result in increased oil accumulation in the seed. Similarly, genetic manipulations may result in changes to the acyl composition of TG which may improve oil quality for both edible and industrial uses. For example, it was shown that PA phosphatase had a preference for PA substrates containing dioleoyl- and dipalmitoyl- moieties. As well, DGAT has previously been shown to have a preference for acyl-CoA substrates containing oleoyl- and palmitoyl- moieties. Selectivity studies will be required to determine which alterations in the acyl composition of TG will be economically advantageous. These studies will depend on our knowledge of the fatty acid composition of PAs, DGs, and acyl-CoAs in the developing seed. PA phosphatase and/or DGAT may also be regulated by phosphorylation/dephosphorylation mechanisms and/or by allosteric effectors. A detailed biochemical characterization of TG biosynthetic enzymes is necessary in developing effective approaches in molecular breeding of oilseeds.

LITERATURE CITED

Akao T., and Kusaka T., 1976. Solubilization of diglyceride acyltransferase from the membrane of *Mycobacterium smegmatis*. J. Biochem., 80, 723-728.

Andersson M., Wettesten M., Borén J., Magnusson A., Sjöberg A., Rustaeus S., and Olofsson S.-O., 1994. Purification of diacylglycerol:acyltransferase from rat liver to near homogeneity. J. Lipid Res., 35, 535-545.

Andrews J., Ohlrogge J. B., and Keegstra K., 1985. Final step of phosphatidic acid synthesis in pea chloroplasts occurs in the inner envelope membrane. Plant Physiol., 78, 459-465.

Anonymous, 1996. Canola Council of Canada. Winnipeg, MB Canada.

Battey J. F., and Ohlrogge J. B., 1989. A comparison of the metabolic fate of fatty acids of different chain lengths in developing oilseeds. Plant Physiol., 90, 835-840.

Battey J. F., Schmid K. M., and Ohlrogge J. B., 1989. Genetic engineering for plant oils: potential and limitations. Tib. Tech., 7, 122-125.

Bell R. M., and Coleman R. A., 1983. Enzymes of triacylglycerol formation in mammals. In: The Enzymes, 16, pp. 87-111, Boyer P. D., ed. Academic Press, New York.

Bernerth R., and Frentzen M., 1990. Utilization of erucoyl-CoA by acyltransferases from developing seeds of *Brassica napus* (L.) involved in triacylglycerol biosynthesis. Plant Science, 67, 21-28.

Bertrams M., and Heinz E., 1981. Positional specificity and fatty acid selectivity of purified sn-glycerol-3-phosphate acyltransferase from chloroplasts. Plant Physiol., 68, 653-657.

Bligh E. G., and Dyer W. J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37, 911-917.

Block M. A., Dorne A.-J., Joyard J., and Douce R., 1983. The phosphatidic acid phosphatase of the chloroplast envelope is located on the inner envelope membrane. FEBS Lett., 164, 111-115.

Bocckino S. B., Wilson P., and Exton J. H., 1989. An enzymatic assay for picomole levels of phosphatidate. Anal. Biochem., 180, 24-27.

Bradford M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254.

Brindley D. N., 1984. Intracellular translocation of phosphatidate phosphohydrolase and its possible role in the control of glycerolipid synthesis. Prog. Lipid Res., 23, 115-133.

Brindley D. N., and Sturton R. G., 1982. In: Phospholipids. New Comp. Biochem., 4, pp. 179-213, Hawthorne J. N., and Ansell G. B., eds. Elsevier Biomedical Press, Amsterdam.

Brindley D. N., and Waggoner D. W., 1996. Phosphatidate phosphohydrolase and signal transduction. Chemistry and Physics of Lipids, 80, 45-57.

Butterwith S. C., Hopewell R., and Brindley D. N., 1984. Partial purification and characterization of the soluble phosphatidate phosphohydrolase of rat liver. Biochem. J., 220, 825-833.

Cao Y.-Z., and Huang A. H. C., 1987. Acyl coenzyme A preference of diacylglycerol acyltransferase from the maturing seeds of *Cuphea*, maize, rapeseed and canola. Plant Physiol., 84, 762-765.

Cao Y.-Z., and Huang A. H. C., 1986. Diacylglycerol acyltransferase in maturing oilseeds of maize and other species. Plant Physiol., 82, 813-820.

Cao Y.-Z., Oo K.-C., and Huang A. H. C., 1990. Lysophosphatidate acyltransferase in the microsome from maturing seeds of meadowfoam (*Limnanthes alba*). Plant Physiol., 94, 1199-1206.

Chen P. S. Jr., Toribara T. Y., and Warner H., 1956. Microdetermination of phosphorus. Anal. Chem., 28, 1756-1758.

Chew Y. H., and Oo K. C., 1989. Diacylglycerol acyltransferase in subcellular fractions of oil palm mesocarp. Proceedings of the Malaysian Biochemical Society Conference, 14, 190-193.

Coleman R., and Bell R. M., 1978. Evidence that biosynthesis of phosphatidylethanolamine, phosphatidylcholine and triacylglycerol occurs on the cytoplasmic side of microsomal vesicles. J. Cell Biol., 76, 245-253.

Davies H. M., Hawkins D. J., and Nelsen J. S., 1995. Lysophosphatidic acid acyltransferase from immature coconut endosperm having medium chain length substrate specificity. Phytochemistry, 39, 989-996.

Eccleston V. S., and Harwood J. L., 1995. Solubilization, partial purification and properties of acyl-CoA: glycerol-3-phosphate acyltransferase from avocado (*Persea americana*) fruit mesocarp. Biochim. Biophys. Acta., 1257, 1-10.

Fleming I. N., and Yeaman S. J., 1995a. Purification and characterization of n-ethylmaleimide-insensitive phosphatidic acid phosphatase phosphohydrolase (PAP2) from rat liver. Biochem. J., 308, 983-989.

Fleming I. N., and Yeaman S. J., 1995b. Subcellular distribution of N-ethylmaleimide-sensitive and -insensitive phosphatidic acid phosphohydrolase in rat brain. Biochim. Biophys. Acta, 1254, 161-168.

Frentzen M., 1993. Acyltransferases and triacylglycerols. In: Lipid Metabolism in Plants, pp. 195-230, Moore T. S. Jr., ed. CRC Press, Boca Raton.

Frentzen M., Heinz E., McKeon T. A., and Stumpf P. K., 1983. Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from peas and spinach chloroplasts. Eur. J. Biochem., 129, 629-636.

Gardiner S. E., and Roughan P. G., 1983. Relationship between fatty-acyl composition of diacylgalactosylglycerol and turnover of chloroplast phosphatidate. Biochem. J., 210, 949-952.

Gardiner S. E., Roughan P. G., and Browse J., 1984. Glycerolipid labelling kinetics in isolated intact chloroplasts. Biochem J., 224, 637-643.

Gomez-Muñoz A., Hatch G. M., Martin A., Jamal Z., Vance D. E., and Brindley D. N., 1992. Effects of okadoic acid on the activities of two distinct phosphatidate phosphohydrolases in rat hepatocytes. FEBS Lett. 301, 103-106.

Griffiths G., Stobart A. K., and Stymne S., 1985. The acylation of *sn*-glycerol 3-phosphate and the metabolism of phosphatidate in microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius* L.) seed. Biochem. J., 230, 379-388.

Gurr M. I., 1980. The biosynthesis of triacylglycerols. In: The Biochemistry of Plants, 4: Lipids, structure and function, pp. 205-248, Stumpf P. K., ed. Academic Press, New York.

Gurr M. I., and Harwood J. L., 1991. Lipid Biochemistry: An Introduction, 4th edn. Chapman & Hall, London.

Harwood J., 1989. Lipid metabolism in plants. Crit. Rev. Plant Sci., 8, 1-43.

Harwood J. L., 1980. Plant acyl lipids: structure, distribution and analysis. In: The Biochemistry of Plants, 4, Lipids, structure and function, pp. 1-55, Stumpf P. K., ed. Academic Press, New York.

Heinz E., and Roughan P. G., 1983. Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol., 72, 273-279.

Herman E. M., and Chrispeels M. J., 1980. Characteristics and subcellular localization of phospholipase D and phosphatidic acid phosphatase in mung bean cotyledons. Plant Physiol., 66, 1001-1007.

Holbrook L. A., Magus J. R., and Taylor D.C., 1992. Abscisic induction of elongase activity, biosynthesis and accumulation of very long chain monounsaturated fatty acids and oil body proteins in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Science, 84, 99-115.

Hosaka K., Schiele U., and Numa S., 1977. Diacylglycerol acyltransferase from rat liver microsomes. Eur. J. Biochem., 76, 113-118.

Hosaka K., and Yamashita S., 1984. Partial purification and properties of phosphatidate phosphatase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta, 796, 102-109.

Huang A. H. C., 1992. Oil bodies and oleosins. Annual Review of Plant Physiol. and Mol. Biol., 43, 177-200.

Ichihara K., 1991. The action of phosphatidate phosphatase on the fatty-acid composition of safflower triacylglycerol and spinach glycerolipids. Planta, 183, 353-358.

Ichihara K., Murota N., and Fujii S., 1990. Intracellular translocation of phosphatidate phosphatase in maturing safflower seeds: a possible mechanism of feedforward control of triacylglycerol synthesis by fatty acids. Biochim. Biophys. Acta, 1043, 227-234.

Ichihara K., and Noda M., 1982. Some properties of diacylglycerol acyltransferase in a particulate fraction from maturing safflower seeds. Phytochemistry, 21, 1895-1901.

Ichihara K., and Noda M., 1980. Fatty acid composition and lipid synthesis in developing safflower seeds. Phytochemistry, 19, 49-54.

Ichihara K., Norikura S., and Fujii S., 1989. Microsomal phosphatidate phosphatase in maturing safflower seeds. Plant Physiol., 90, 413-419.

Ichihara K., Takahashi T., and Fujii S., 1988. Diacylglycerol acyltransferase in maturing safflower seeds: its influences on the fatty acid composition of triacylglycerol and on the rate of triacylglycerol synthesis. Biochim. Biophys. Acta, 958, 125-129.

Jamal Z., Martin A., Gomez-Muñoz A., and Brindley D. N., 1991. Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol. J. Biol. Chem., 266, 2988-2996.

Jamal Z., Martin A., Gomez-Muñoz A., Hales P., Chang E., Russell J. C., and Brindley D. N., 1992. Phosphatidate phosphohydrolases in liver, heart and adipose tissue of the JCR:LA corpulent rat and the lean genotypes: implications for glycerolipid synthesis and signal transduction. International Journal of Obesity, 16, 789-799.

Joyard J. and Douce R., 1987. Galactolipid synthesis. In: The Biochemistry of Plants, 4, Lipids, structure and function, pp. 215-274, Stumpf P. K., ed. Academic Press, New York.

Joyard J., and Douce R., 1979. Characterization of phosphatidate phosphohydrolase activity associated with chloroplast envelope membranes. FEBS Lett., 102, 147-150.

Joyard J., and Douce R., 1977. Site of synthesis of phosphatidic acid and diacylglycerol in spinach chloroplasts. Biochim. Biophys. Acta, 486, 273-285.

Kanda P., and Wells M. A., 1981. Facile acylation of glycerophosphocholine catalyzed by trifluoroacetic anhydride. J. of Lipid Res., 22, 877-879.

Kanoh H., Imai S., Yamada K., and Sakane F., 1992. Purification and properties of phosphatidic acid phosphatase from porcine thymus membranes. J. Biol. Chem., 267, 25309-25314.

Katavic V., Reed D. W., Taylor D. C., Giblin M., Barton D. L., Zou J., MacKenzie S. L., Covello P. S., and Kunst L., 1995. Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. Plant Physiol., 108, 399-409.

Kates M., 1955. Hydrolysis of lecithin by plant plastid enzymes. Can. J. Biochem. Physiol., 33, 575-589.

Kennedy E. P., 1961. Biosynthesis of complex lipids. Fed. Proc. Am. Soc. Exp. Biol., 20, 934-940.

Kennedy E. P., 1957. Biosynthesis of phospholipides. Fed. Proc. Am. Soc. Exp., 16, 847-853.

Knutzon D. S., Lardizabal K. D., Nelsen J. S., Bleibaum J. L., Davies H. M., and Metz J. G., 1995. Cloning of a coconut endosperm cDNA encoding a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase that accepts medium-chain-length substrates. Plant Physiol., 109, 999-1006.

Kocsis M. G., and Weselake R. J., 1996. Phosphatidate phosphatases of mammals, yeast, and higher plants. Lipids, 31, 785-801.

Kocsis M. G., Weselake R. J., Eng J. A., Furukawa-Stoffer T. L., and Pomeroy M. K., 1996. Phosphatidate phosphatase from developing seeds and microspore-derived cultures of *Brassica napus*. Phytochemistry, 41, 353-363.

Königs B., and Heinz E., 1974. Investigation of some enzymatic activities contributing to the biosynthesis of galactolipid precursors in *Vicia faba*. Planta, 118, 159-169.

Kwanyuen P., and Wilson R. F., 1990. Subunit and amino acid composition of diacylglycerol acyltransferase from germinating soybean cotyledons. Biochim. Biophys. Acta, 1039, 67-72.

Kwanyuen P., and Wilson R. F., 1986. Isolation and purification of diacylglycerol acyltransferase from germinating soybean cotyledons. Biochim. Biophys. Acta, 877, 238-245.

Laemmli U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lassner M. W., Levering C. K., Davies H. M., and Knutzon D. S., 1995. Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the *sn*-2 position of triacylglycerol in transgenic rapeseed oil. Plant Physiol., 109, 1389-1394.

Lehner R., and Kuksis A., 1995. Triacylglycerol synthesis by purified triacylglycerol synthetase of rat intestinal mucosa. J. Biol. Chem., 270, 13 630-13 636.

Lehner R., Kuksis A., and Itabashi Y., 1993. Stereospecificity of monoacylglycerol and diacylglycerol acyltransferases from rat intestine as determined by chiral phase high-performance liquid chromatography. Lipids, 28, 29-34.

Lin Y. P., and Carman G. M., 1989. Purification and characterization of phosphatidate phosphatase from *Saccharomyces cerevisiae*. J. Biol. Chem., 264, 8641-8645.

Little D., Weselake R., Pomeroy K., Furukawa-Stoffer T., and Bagu J., 1994. Solubilization and characterization of diacylglycerol acyltransferase from microspore-derived cultures of oilseed rape. Biochem. J., 304, 951-958.

Malherbe A., Block M. A., Douce R., and Joyard J., 1995. Solubilization and biochemical properties of phosphatidate phosphatase from spinach chloroplast envelope membranes. Plant Physiol. Biochem., 33, 149-161.

Malherbe A., Block M. A., Joyard J., and Douce R., 1992. Feedback inhibition of phosphatidate phosphatase from spinach chloroplast envelope membranes by diacylglycerol. J. Biol. Chem., 267, 23 546-23 553.

Martin A., Gomez-Muñoz A., Jamal Z., and Brindley D. N., 1991. Characterization and assay of phosphatidate phosphatase. Meth. in Enzymol., 197, 553-563.

Martin-Sanz P., Hopewell R., and Brindley D. N., 1984. Long-chain fatty acids and their acyl-CoA esters cause the translocation of phosphatidate phosphohydrolase from the cytosolic to the microsomal fraction of rat liver. FEBS Lett., 175, 284-288.

Martin B. A., and Wilson R. F., 1985. Subcellular compartmentation of fatty acid and triacylglycerol synthesis in developing soybean seed. Agrochimica, 26, 89-96.

Martin B. A., and Wilson R. F., 1984. Subcellular localization of triacylglycerol synthesis in spinach leaves. Lipids, 19, 117-121.

Martin B. A., and Wilson R. F., 1983. Properties of diacylglycerol acyltransferase from spinach leaves. Lipids, 18, 1-6.

Merril C. R., Goldman D., Sedman S. A., and Ebert M. H., 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variance in cerebrospinal fluid proteins. Science, 211, 1437-1438.

Moore T. S., Lord J. M., Kagawa T., and Beevers H., 1973. Enzymes of phospholipid metabolism in the endoplasmic reticulum of castor bean endosperm. Plant Physiol., 52, 50-53.

Moore T. S. Jr., and Sexton J. C., 1978. Phosphatidate phosphatase of castor bean endosperm. Suppl. Plant Physiol., 61, 69 (abstract 382).

Morlock K. R., Lin Y. P., and Carman G. M., 1988. Regulation of phosphatidate phosphatase activity by inositol in *Saccharomyces cerevisiae*. J. Bacteriol., 170, 3561-3566.

Morlock K. R., McLaughlin J. J., Lin Y. P., and Carman G. M., 1991. Phosphatidate phosphatase from *Saccharomyces cerevisiae*: Isolation of 45- and 104 kDa forms of the enzyme that are differently regulated by inositol. J. Biol. Chem., 266, 3586-3593.

Muilerman H. G., Ter Hart H. G. J., and Van Dijk W., 1982. Specific detection of inactive enzyme protein after polyacrylamide gel electrophoresis by a new enzyme-immunoassay method using unspecific antiserum and partially purified active enzyme: application to rat liver phosphodiesterase I. Anal. Biochem., 120, 46-51.

Murphy D. J., 1993. Structure, function and biogenesis of storage lipid bodies and oleosins in plants. Prog. Lipid Res., 32, 247-280.

Murphy D. J., 1988. A highly active soluble diacylglycerol synthesizing system from developing rapeseed, *Brassica napus* L. Lipids, 23, 157-163.

Murphy D. J., and Mukherjee K. D., 1987. Acyltransferases in subcellular fractions of developing seeds of rape (*Brassica napus* L.). Lipids, 22, 293-298.

Nakagawa H., Kashiwabara Y., and Matsuki G., 1976. Metabolism of triacylglycerol in *Mycobacterium smegmatis*. J. Biochem., 80, 923-928.

North M. J., 1989. Prevention of unwanted proteolysis. In Proteolytic enzymes: A practical approach, 6, pp. 105-124. Beynon R. J. and Bond J. S., eds. IRL Press, Oxford.

Olmsted J. B., 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. J. Biol. Chem., 256, 11 955-11 957.

Ohlrogge J., and Browse J., 1995. Lipid biosynthesis. The Plant Cell, 7, 957-970.

Oo K.-C., and Chew Y.-H., 1992. Diacylglycerol acyltransferase in microsomes and oil bodies of oil palm mesocarp. Plant Cell Physiol., 33, 189-195.

Oo K.-C., and Huang A. H. C., 1989. Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. Plant Physiol., 96, 1288-1295.

Orr W., Keller W. A., and Singh J., 1986. Induction of freezing tolerance in an embryogenic cell suspension culture of *Brassica napus* by Abscisic acid at room temperature. J. Plant Physiol., 126, 23-32.

Ozasa S., Kempner E. S., and Erickson S. K., 1989. Functional size of acyl coenzyme A:diacylglycerol acyltransferase by radiation inactivation. J. Lipid Res., 30, 1759-1762.

Paliyath G., Lynch D. V., and Thompson J. E., 1987. Regulation of membrane phospholipid catabolism in senescing carnation flowers. Physiol. Plant., 71, 503-511.

Paliyath G., and Thompson J. E., 1987. Calcium- and calmodulin-regulated breakdown of phospholipid by microsomal membranes from bean cotyledons. Plant Physiol., 83, 63-68.

Perry H. J., and Harwood J. L., 1993a. Changes in the lipid content of developing seeds of *Brassica napus*. Phytochemistry, 32, 1411-1415.

Perry H. J., and Harwood J. L., 1993b. Radiolabelling studies of acyl lipids in developing seeds of *Brassica napus*: use of [1-^{14}C]acetate precursor. Phytochemistry, 33, 329-333.

Polokoff M. A., and Bell R. M., 1980. Solubilization, partial purification and characterization of rat liver microsomal diacylglycerol acyltransferase. Biochim. et Biophys. Acta, 618, 129-142.

Pomeroy M. K., Kramer J. K. G., Hunt D. J., and Keller W. A., 1991. Fatty acid changes during development of zygotic and microspore-derived embryos of *Brassica napus*. Physiol. Plant., 81, 447-454.

Rajasekharan R., and Sastry P. S., 1990. Effect of thiocarbamate, urea, and uracil herbicides on lipid metabolism in groundnut (*Arachis hypogea*) leaves. Biochem. Cell Biol., 68, 567-573.

Rajasekharan R., and Sastry P. S., 1989. Effect of phenoxy acids and their derivatives on lipid metabolism in groundnut (*Arachis hypogea*) leaves. Pesticide Biochemistry and Physiology, 33, 26-36.

Roughan P. G., and Slack C. R., 1982. Cellular organization of glycerolipid metabolism. Ann. Rev. Plant Physiol., 33, 97-132.

Sauer A., and Heiss K. P., 1983. On the light dependence of fatty acid synthesis in spinach chloroplasts. Plant Physiol., 73, 11-15.

Settlage S. B., Wilson R. F., and Kwanyuen P., 1995. Localization of diacylglycerol acyltransferase to oil body associated endoplasmic reticulum. Plant Physiol., 33, 399-407.

Siess E. A., and Hofstetter M. M., 1996. Identification of phosphatidate phosphatase purified from rat liver membranes on sds-polyacrylamide gel electrophoresis. FEBS Lett., 381, 169-173.

Simmonds D. H., Long N. E., and Keller W. A., 1991. High plating efficiency and plant regeneration frequency in low density protoplast cultures derived from an embryogenic *Brassica napus* cell suspension. Plant Cell, Tissue and Organ Culture, 27, 231-241.

Sonntag N. O. V., 1991. Erucic, behenic: feedstocks of the 21st century. INFORM, 2, 449-463.

Sparace S. A., and Kleppinger-Sparace K. F., 1993. Metabolism in nonphotosynthetic, nonoilseed tissues. In Lipid Metabolism in Plants, pp. 569-589, Moore T. S., ed. CRC Press, Boca Raton.

Stobart A. K., and Stymne S., 1990. Triacylglycerol biosynthesis. In: Methods in Plant Biochemsitry, 4, Lipids, membranes and aspects of photobiology, pp. 19-46, Harwood J. L., and Bowyer J. R., eds. Academic Press, London.

Stymne S., and Stobart A. K., 1987. Triacylglycerol biosynthesis. In: The Biochemistry of Plants, 9, pp. 175-214, Stumpf P. K., ed. Academic Press, New York.

Sukumar V., and Sastry P. S., 1987. Triacylglycerol synthesis in developing seeds of groundnut (*Arachis hypogaea*): studies on phosphatidic acid phosphatase and diacylglycerol acyltransferase during seed maturation. Biochem. Int., 14, 1153-1158.

Taylor D. C., and Weber N., 1994. Microspore-derived embryos of the *Brassicaceae*-Model system for studies of storage lipid bioassembly and its regulation. Fat Sci. Technol., 96, 228-235.

Taylor D. C., Magus J. R., Bhella R., Zou J., MacKenzie S. L., Giblin E. M., Pass E. W., and Crosby W. L., 1993. Biosynthesis of triacylglycerols in *Brassica napus* L. cv Reston; target: trierucin. In Seed Oils for the Future, pp. 77-102, MacKenzie S. L., and Taylor D. C., eds. AOCS Press, Champaign.

Taylor D. C., Barton D. L., Rioux K. P., MacKenzie S. L., Reed D. W., Underhill E. W., Pomeroy M. K., and Weber N., 1992. Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Physiol., 99, 1609-1618.

Taylor D. C., Weber N., Barton D. L., Underhill E. W., Hogge L. R., Weselake R. J., and Pomeroy M. K., 1991. Triacylglycerol bioassembly in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Physiol., 97, 65-79.

Taylor D. C., Weber N., Underhill E. W., Pomeroy M. K., Keller W. A., Scowcroft W. R., Wilen R. W., Moloney M. M., and Holbrook L. A., 1990. Storage-protein regulation and lipid accumulation in microspore embryos of *Brassica napus* L. Planta, 181, 18-26.

Tijburg L. B. M., Geelen M. J. H., and van Golde L. M. G., 1989. Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. *Biocim. Biophys. Acta*, 1004, 1-19.

Tzen J. T. C., Cao Y.-Z., Laurent P., Ratnayake C., and Huang A. H. C., 1993. Lipids, proteins, and structure of seed oil bodies from diverse species. Plant Physiol., 102, 565-571.

Vogel G., and Browse J., 1996. Cholinephosphotransferase and diacylglycerol acyltransferase: Substrate specificities at a key branch point in seed lipid metabolism. Plant Physiol., 110, 923-931.

Waggoner D. W., Martin A., Dewald J., Gomez-Muñoz A., and Brindley D. N., 1995. Purification and characterization of a novel plasma membrane phosphatidate phosphohydrolase from rat liver. J. Biol. Chem., 270, 19422-19429.

Wells G. N., Osborne L. J., and Jamdar S. C., 1986. Isolation and characterization of multiple forms of Mg^{2+}-dependent phosphatidate phosphohydrolase from rat adipose cytosol. Biochim. Biophys. Acta, 878, 225-237.

Weselake R. J., 1995. A molecular approach for increasing oil content in canola. Project #920031. Final report, 90 pages.

Weselake R. J., 1994. A target enzyme for increasing oil content in oilseed rape. Project #AARI92M406. Final report, 58 pages.

Weselake R. J., Davoren J. M., Byers S. D., Laroche A., Hodges D. M., Pomeroy M. K., and Furukawa-Stoffer T. L., 1997. Differential display of mRNA from oil-forming cell suspension cultures of *Brassica napus*. In: Physiology, Biochemistry and Molecular Biology of Plant Lipids, pp. 357-359, Williams J. P., Khan M. U., and Lem N. W., eds. Kluwer Academic Publishers, Dordrecht.

Weselake R. J., and Jain J. C., 1992. Strategies in the purification of plant proteins. Physiol. Plant., 84, 301-309.

Weselake R., Pomeroy K., Furukawa-Stoffer T., Little D., and Rajasekharan R., 1994. Interaction of photoreactive substrate analogs with diacylglycerol acyltransferase from microspore-derived embryos of oilseed rape. In Plant Lipid Metabolism, pp. 518-520, Kader J. C., and Mazliak P., eds. Kluwer Academic Publishers, Dordrecht.

Weselake R. J., Pomeroy M. K., Furukawa T. L., and Oishi R. L., 1993a. Partial purification and characterization of diacylglycerol acyltransferase from microspore-derived embryos of oilseed rape. In: Seed Oils for the Future, pp. 103-115, MacKenzie S. L., and Taylor D. C., eds. AOCS Press, Champaign.

Weselake R. J., Pomeroy M. K., Furukawa T. L., Golden J. L., Little D. B., and Laroche A., 1993b. Developmental profile of diacylglycerol acyltransferase in maturing seeds of oilseed rape and safflower and microspore-derived cultures of oilseed rape. Plant Physiol., 102, 565-571.

Weselake R. J., Taylor D. C., Pomeroy M. K., Lawson S. L., and Underhill E. W., 1991. Properties of diacylglycerol acyltransferase from microspore-derived embryos of *Brassica napus*. Phytochemistry, 30, 3533-3538.

Wilson R. F., and Kwanyuen P., 1986. Triacylglycerol synthesis and metabolism in germinating soybean cotyledons. Biochim. Biophys. Acta, 877, 231-237.

Wilson R. F., Kwanyuen P., Dewey R. E., and Settlage S. B., 1993. Recent developments in the molecular biochemistry and genetics of diacylglycerol acyltransferase from soybean. In Seed Oils for the Future, pp. 116-135, MacKenzie S. L., and Taylor D. C., eds. AOCS Press, Champaign.

Xue L., McCune L. M., Kleppinger-Sparace K. F., Brown M. J., Pomeroy M. K., and Sparace S. A., 1996. Characterization of the glycerolipid composition and biosynthetic capacity of pea root plastids. Plant Physiol., In Press.

APPENDIX

Appendix Table 1. Effect of various concentrations of BSA on the stability of solubilized PA phosphatase. Enzymes were solubilized from particulate fractions prepared from the EC stage of MD embryos of *B. napus* L. cv Topas. Enzyme activity was assayed at 0 (initial activity), 24 and 48 h following storage at 4°C. The results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage.

BSA CONCENTRATION (%, w/v)	SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)		
	0 h	24 h	48 h
0	1.9	1.5 (81)	1.5 (79)
0.05	2.1	2.1 (98)	1.2 (55)
0.1	2.2	1.9 (88)	0.7 (33)
1.0	2.7	1.8 (65)	1.0 (37)
5.0	2.8	1.8 (66)	3.2 (116)
10.0	2.8	3.8 (135)	2.3 (82)

Appendix Table 2. Effect of various concentrations of BSA on the stability of solubilized DGAT. Enzymes were solubilized from particulate fractions prepared from the EC stage of MD embryos of *B. napus* L. cv Topas. Enzyme activity was assayed at 0 (initial activity), 24 and 48 h following storage at 4°C. The results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage.

BSA CONCENTRATION (%, w/v)	SPECIFIC ACTIVITY OF SOLUBILIZED DGAT (pmol/min/mg)		
	0 h	24 h	48 h
0	12.4	11.8 (95)	4.3 (35)
0.05	14.2	10.4 (73)	2.3 (16)
0.1	12.0	9.0 (75)	0 (0)
1.0	29.6	26.3 (89)	8.0 (27)
5.0	30.4	30.1 (99)	0 (0)
10.0	23.5	29.4 (125)	0 (0)

Appendix Table 3. Effect of various agents (10%, w/v) on the stability of solubilized PA phosphatase. Enzymes were solubilized from particulate fractions prepared from the EC stage of MD embryos of *B. napus* L. cv Topas. Enzyme activity was assayed at 0 (initial activity), 24 and 48 h following storage at 4°C. The results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage.

TREATMENT (10%, w/v)	SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)		
	0 h	24 h	48 h
control	8.2	2.4 (29)	2.0 (24)
Trehalose	7.9	2.8 (35)	1.9 (24)
Sorbitol	7.7	3.7 (48)	2.8 (36)
Sucrose	7.4	2.9 (39)	2.1 (29)
BSA	10.0	8.1 (81)	5.5 (55)
Glycerol	6.6	3.8 (57)	2.6 (40)

Appendix Table 4. Effect of various agents (10%, w/v) on the stability of solubilized DGAT. Enzymes were solubilized from particulate fractions prepared from the EC stage of MD embryos of *B. napus* L. cv Topas. Enzyme activity was assayed at 0 (initial activity), 24 and 48 h following storage at 4°C. The results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage.

TREATMENT (10%, w/v)	SPECIFIC ACTIVITY OF SOLUBILIZED DGAT (pmol/min/mg)		
	0 h	24 h	48 h
control	9.0	2.0 (22)	1.4 (15)
Trehalose	10.6	1.0 (9)	1.3 (12)
Sorbitol	12.8	3.7 (29)	1.4 (11)
Sucrose	12.9	3.6 (28)	0.5 (4)
BSA	8.7	7.0 (80)	4.9 (56)
Glycerol	11.8	1.7 (14)	1.2 (10)

Appendix Table 5. Effect of protease inhibitors (1 mM PMSF, 1 mM EDTA, and 1 mg/mL [w/v] leupeptin) on the activity of solubilized PA phosphatase prepared from the EC and LC stages of MD embryos of *B. napus* L. cv Topas. Assays were performed on solubilized preparations of PA phosphatase which were prepared from the 10 000 *g* - 100 000 *g* particulate fraction. Results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage.

TREATMENT	SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)		
	0 hr	24 hr	48 hr
EC 10 000-100 000 *g* (without inhibitors)	2.6	1.5 (58)	0.8 (31)
EC 10 000-100 000 *g* (with PMSF, EDTA, leupeptin)	2.8	1.4 (50)	3.7 (124)
LC 10 000-100 000 *g* (without inhibitors)	1.8	0.2 (11)	0.3 (17)
LC 10 000-100 000 *g* (with PMSF, EDTA, leupeptin)	1.9	0.3 (16)	0.4 (21)

Appendix Table 6. Effect of protease inhibitors (1 mM PMSF, 1 mM EDTA, and 1 mg/mL [w/v] leupeptin) on the activity of solubilized DGAT prepared from the EC and LC stages of MD embryos of *B. napus* L. cv Topas. Assays were performed on solubilized preparations of DGAT which were prepared from the 10 000 *g* - 100 000 *g* particulate fraction. Results represent the means of triplicate assays. Numbers in parentheses represent the percent of initial activity remaining following storage.

TREATMENT	SPECIFIC ACTIVITY OF SOLUBILIZED DGAT (pmol/min/mg)		
	0 hr	24 hr	48 hr
EC 10 000-100 000 *g* (without inhibitors)	41.2	29.5 (72)	31.4 (76)
EC 10 000-100 000 *g* (with PMSF, EDTA, leupeptin)	40.0	40.5 (102)	38.8 (97)
LC 10 000-100 000 *g* (without inhibitors)	39.5	35.2 (89)	28.3 (72)
LC 10 000-100 000 *g* (with PMSF, EDTA, leupeptin)	37.8	35.7 (94)	32.7 (87)

Appendix Table 7. Enzyme activities of PA phosphatase solubilized from the particulate fractions of EC and LC stages of MD embryos of *B. napus* L. cv Topas. In each experiment, the results represent the means of triplicate assays.

STAGE OF MD EMBRYO		SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)
Experiment #1	EC	3.46
	LC	2.03
Experiment #2	EC	2.62
	LC	1.82

Appendix Table 8. Determination of the activities of other Kennedy pathway enzymes in preparations of particulate fraction and solubilized PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. Enzymes were assayed as described in the MATERIALS AND METHODS. Enzyme activities from a 10 000 - 100 000 *g* particulate fraction are shown. Enzyme activities and the percentage of enzyme activity solubilized from the particulate fraction are also shown. Results represent the means of triplicate assays.

ENZYME	PARTICULATE FRACTION		SOLUBILIZED FRACTION		
	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	% solubilized
PA phosphatase	3.7	86.5	1.75	14.8	17.1
DGAT	0.1	3.3	0.04	0.3	10.1
GPAT	0.3	6.2	0.06	0.5	7.9
LPAAT	14.8	347.9	0.08	0.8	0.2

Appendix Table 9. Determination of the activities of other Kennedy pathway enzymes in preparations of particulate fraction and solubilized DGAT from the LC stage of MD embryos of *B. napus* L. cv Topas. Enzymes were assayed as described in the MATERIALS AND METHODS. Enzyme activities from a 10 000 - 100 000 *g* particulate fraction are shown. Enzyme activities and the percentage of enzyme activity solubilized from the particulate fraction are also shown. Results represent the means of triplicate assays.

ENZYME	PARTICULATE FRACTION		SOLUBILIZED FRACTION		
	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)	% solubilized
DGAT	0.1	1.3	0.08	0.5	40.9
PA phosphatase	3.7	34.6	1.03	7.3	21.1
LPAAT	14.8	139.2	0.26	1.8	1.3
GPAT	0.3	2.5	0	0	0

Appendix Table 10. Effect of Mg^{2+} on microsomal PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. A 10 000 - 100 000 *g* particulate fraction was prepared in the absence of $MgCl_2$ then 20 μL aliquots containing 50 - 126 μg protein were assayed for PA phosphatase activity with varying concentrations of $MgCl_2$. In experiments #1-3, the results represent the means of triplicate assays and in experiment #4, the results represent the means of duplicate assays.

$MgCl_2$ CONCENTRATION (mM)	SPECIFIC ACTIVITY OF PA PHOSPHATASE (nmol/min/mg)			
	Experiment #1	Experiment #2	Experiment #3	Experiment #4
0	2.8	5.2	1.1	1.7
0.5	5.3	-	-	-
1	5.5	10.2	9.1	9.5
2	5.2	4.5	10.1	10.3
5	3.7	4.5	9.1	8.3
10	4.4	7.1	6.0	4.9

Appendix Table 11. Effect of Mg^{2+} on solubilized PA phosphatase from the EC stage of MD embryos of *B. napus* L. cv Topas. A 10 000 - 100 000 *g* particulate fraction was prepared in the absence of $MgCl_2$ then PA phosphatase was solubilized with the use of 1% (w/v) Tween 20. Aliquots containing 50 μg protein were assayed for PA phosphatase activity in the absence and presence of 2.0 mM $MgCl_2$ in the final reaction mixture. In each experiment, the results represent the means of duplicate assays.

TREATMENT	SPECIFIC ACTIVITY OF SOLUBILIZED PA PHOSPHATASE (nmol/min/mg)
Experiment #1	
+ $MgCl_2$	8.09
- $MgCl_2$	4.64
Experiment #2	
+ $MgCl_2$	7.40
- $MgCl_2$	4.19
Experiment #3	
+ $MgCl_2$	6.09
- $MgCl_2$	1.43

Appendix Table 12. Effect of NEM on the activity of solubilized PA phosphatase from the EC stage of MD embryos of *B. napus* L. cv Topas. Solubilized PA phosphatase was preincubated with varying concentrations of the thioreactive reagent for 15 min at 30°C prior to assaying for enzyme activity. For each experiment, the results represent the means of triplicate assays. The numbers in parentheses represent the percent inhibition of enzyme activity.

NEM CONCENTRATION (mM)	PA PHOSPHATASE ACTIVITY (nmol Pi/min/mg protein)	
	Experiment #1	Experiment #2
0	11.0	8.5
0.5	6.7 (39)	4.5 (47)
1	6.6 (40)	4.6 (46)
4	5.8 (47)	3.7 (57)
10	5.8 (47)	4.6 (46)

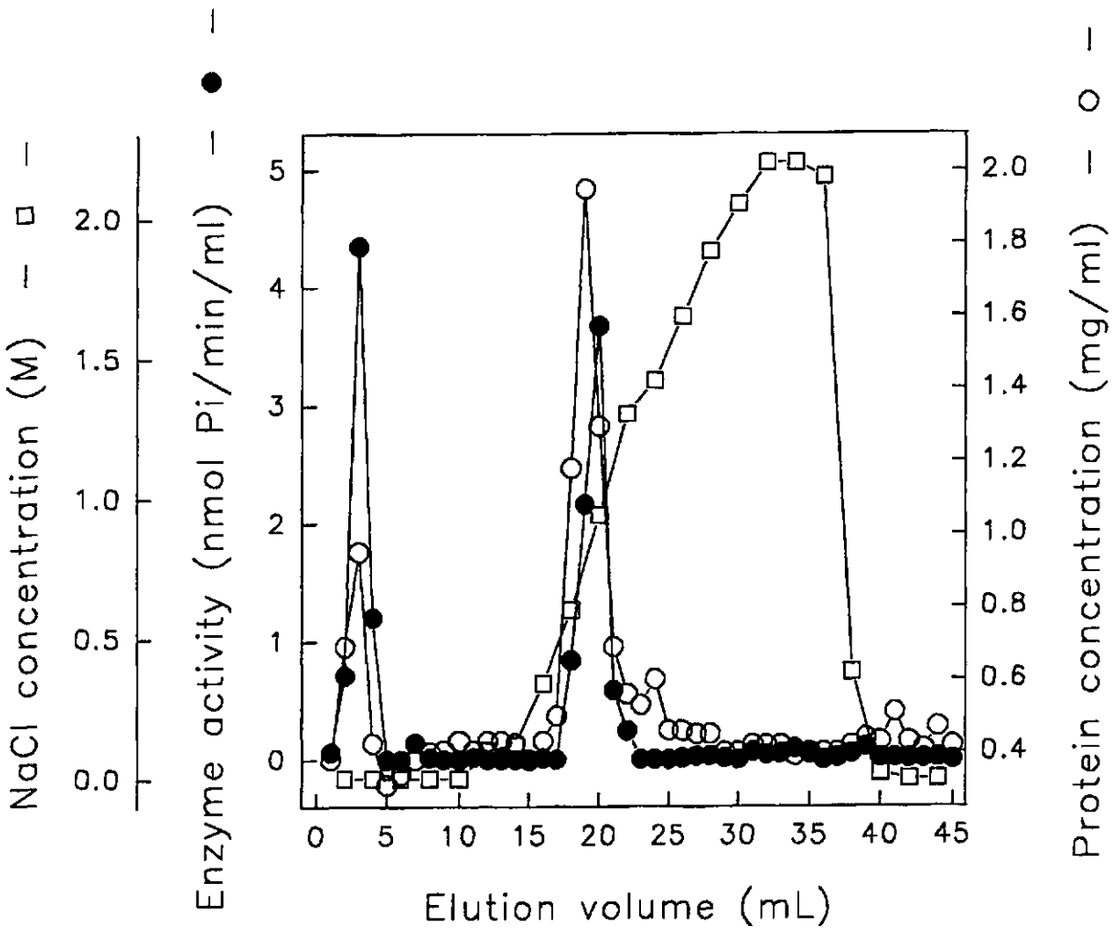
Appendix Table 13. Precipitation of PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas by various agents. Solubilized PA phosphatase was incubated with either 80% (w/v) PEG 8000, 80% (saturation) ammonium sulfate, or 80% (v/v) ethanol for 15 min on ice then spun in a microfuge at 15 900 *g* for 10 min. Treatments with the various agents were performed with solubilized enzyme from a different batch of MD embryos. The pellets were resuspended in 62.5 mM Tris/ 62.5 mM maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 1% (w/v) Tween 20 and 25 μL of the pellets and supernatants were assayed for PA phosphatase activity. The results represent the means of triplicate assays.

TREATMENT	TOTAL ENZYME ACTIVITY (nmol/min)	SPECIFIC ACTIVITY (nmol/min/mg)
Ethanol		
Initial Extract	6.9	11.0
Supernatant	0	0
Pellet	0	1.3
Ammonium Sulfate		
Initial Extract	2.8	17.1
Supernatant	0	0
Pellet	3.3	12.7
PEG 8000		
Initial Extract	1.7	12.1
Supernatant	0.2	3.1
Pellet	1.2	7.0

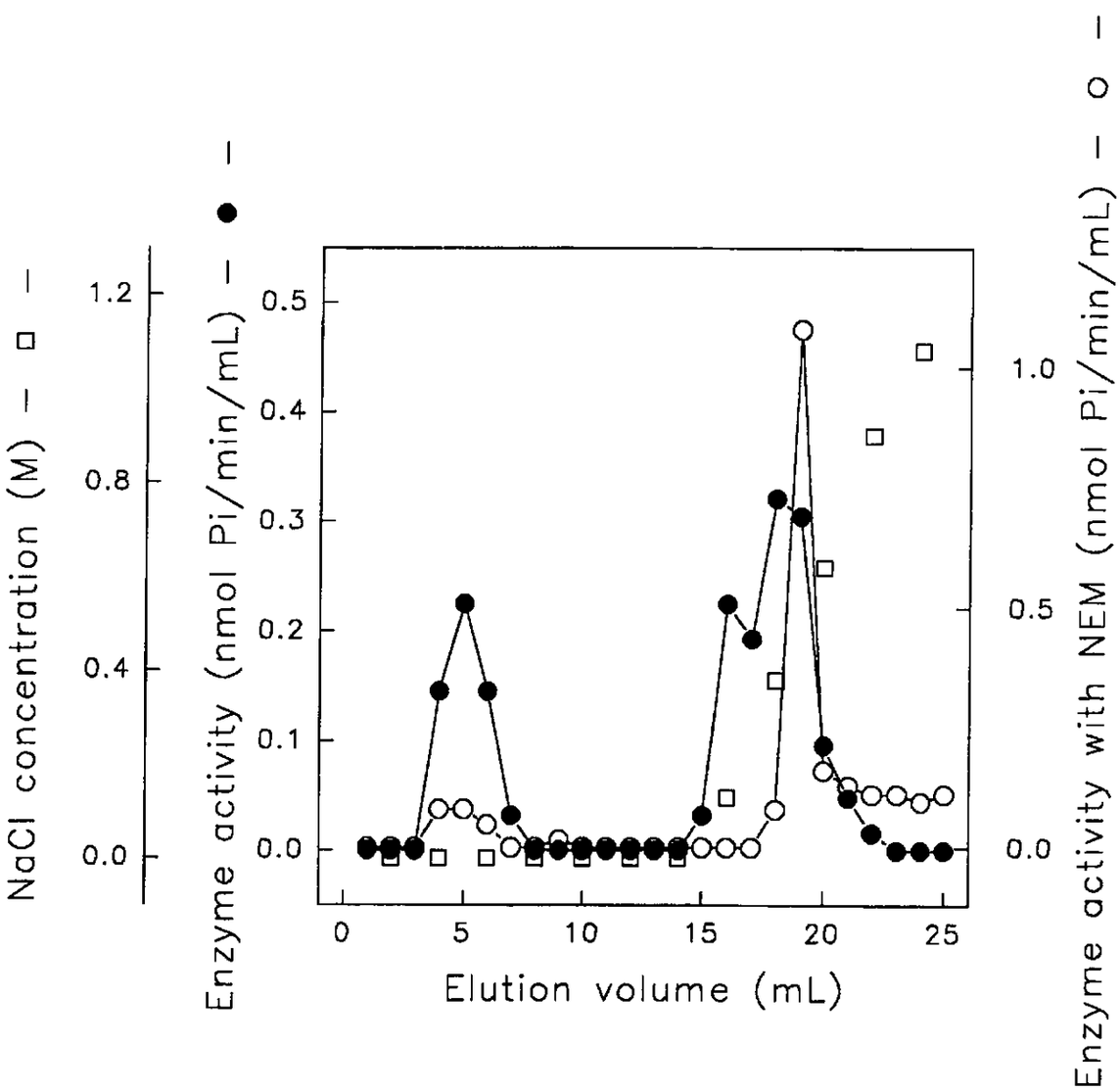
Appendix Table 14. Precipitation of PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas with 20 - 50% (saturation) ammonium sulfate. Solubilized PA phosphatase was incubated for 15 min on ice with 20% (saturation) ammonium sulfate then spun in a microfuge at 15 900 *g* for 10 min. The resulting supernatant was further incubated with 50% (saturation) ammonium sulfate for 15 min on ice then spun for 10 min. The pellets were resuspended in 62.5 mM Tris/62.5 mM maleic acid-NaOH buffer, pH 6.75, containing 0.125 mM EDTA, 2.5 mM $MgCl_2$ and 1% (w/v) Tween 20 and 25 μL of the pellets and supernatants were assayed for PA phosphatase activity. For each experiment, the results represent the means of triplicate assays.

TREATMENT	TOTAL ENZYME ACTIVITY (nmol/min)	SPECIFIC ACTIVITY (nmol/min/mg)
Ammonium Sulfate Experiment #1		
Initial Extract	6.9	11.0
20% pellet	0.1	0.3
50% supernatant	0.3	2.5
50% pellet	9.5	9.8
Ammonium Sulfate Experiment #2		
Initial Extract	73.6	25.0
20 - 50% saturation	26.2	23.0
Ammonium Sulfate Experiment #3		
Initial Extract	16.7	7.4
20 - 50% saturation	13.2	6.0

Appendix Figure 1. Mono Q anion-exchange chromatography of solubilized PA phosphatase from the EC stage of MD embryos of *B. napus* L. cv Topas. The column (HR 5/5, total bed volume = 1 mL) was equilibrated with 10 mM Tris-HCl buffer, pH 8.5, containing 1% (w/v) Tween 20. Two mL of solubilized protein (7 mg) were applied to the column followed by 10 mL equilibration buffer then the column was eluted with a linear gradient of 0 - 2 M NaCl (total volume = 20 mL). The column was run at a flow rate of 30 mL/h using an FPLC system. Fractions of 1 mL were collected and 200 μL aliquots were assayed for PA phosphatase activity.


NaCl concentration (M) — □ —
Enzyme activity (nmol Pi/min/ml) — ● —
Protein concentration (mg/ml) — ○ —
Elution volume (mL)
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
5
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
0
5
10
15
20
25
30
35
40
45

Appendix Figure 2. Detection of NEM-sensitive and NEM-insensitive forms of PA phosphatase following Macro Prep 50Q anion-exchange chromatography of solubilized PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas. The column (BioRad resin, total bed volume = 4 mL) was equilibrated with 10 mM Tris-HCl buffer, pH 8.5 containing 1% (w/v) Tween 20. Two mL of solubilized protein were fractionated with ammonium sulfate (20 - 50% saturation). The post-ammonium sulfate fraction (7.2 mg) in 2 mL was applied to the column followed by 10 mL equilibration buffer. The column was eluted with a linear gradient of 0 - 2 M NaCl (total volume = 20 mL). The column was run at a flow rate of 30 mL/h using an FPLC system. Fractions of 1 mL were collected and 100 μL aliquots were preincubated with 4 mM NEM for 15 min at 30°C. The fractions were then assayed for PA phosphatase activity.


NaCl concentration (M) — □ —
Enzyme activity (nmol Pi/min/mL) — ● —
Enzyme activity with NEM (nmol Pi/min/mL) — ○ —
Elution volume (mL)
0.0
0.4
0.8
1.2
0.1
0.2
0.3
0.4
0.5
0.5
1.0
0
5
10
15
20
25

Appendix Table 15. Inhibition of microsomal PA phosphatase from the LC stage of MD embryos of *B. napus* L. cv Topas by rabbit polyclonal antisera. A 10 000 - 100 000 *g* particulate fraction (10 μL containing 84 μg protein) was preincubated for 30 min at 30°C with 12.5 μL antisera obtained as outlined in MATERIALS AND METHODS, and then assayed for PA phosphatase activity. Antisera and preimmune sera from rabbit #1 were dialyzed against Tris-maleic acid-NaOH buffer, pH 6.75, to decrease the levels of endogenous P_i. The net absorbance at 820 nm and the percentage of activity as compared to the control are shown. The results represent the means of triplicate preincubations and assays.

ANTISERUM	NET ABSORBANCE at 820 nm	PA PHOSPHATASE ACTIVITY (% of control)
Control 1	0.059	100
Antiserum 1	0.044	25

Appendix Table 16. Affinity chromatography of solubilized DGAT from the EC stage of MD embryos of *B. napus* L. cv Topas using alkyl-CoA Sepharose and palmitoyl-CoA agarose. Solubilized DGAT was subjected to affinity chromatography as described in MATERIALS AND METHODS and eluted with 0.5 mM erucoyl-CoA. Fractions were collected and precipitated with 6% (w/v) PEG 8000. Samples of 10 μL were assayed for DGAT activity. The results represent the means of triplicate assays.

Fraction	Total Volume (mL)	Total Enzyme Activity (pmol/min)
Solubilized enzyme	1.0	219.6
ALKYL-CoA		
Unbound	3.0	129.5
Post PEG ppt.	0.05	0
PALMITOYL-CoA		
Unbound	3.0	54.5
Post PEG ppt.	0.05	0

Appendix Table 17. Immunoaffinity chromatography of solubilized DGAT from the EC stage of MD embryos of *B. napus* L. cv Topas. Antibodies from mice were prepared from a native preparation of DGAT from MD embryos of *B. napus* L. cv Topas, as outlined in MATERIALS AND METHODS, and were coupled to epoxy-activated Sepharose 6B. Control antibodies, from mice without exposure to the DGAT antigen, were also coupled to affinity columns. Columns were eluted with two 0.5 mL washes with 3 M sodium thiocyanate. Fractions were collected and 15 μL were assayed for DGAT activity. The results represent the means of triplicate assays.

Fraction	Total Volume (mL)	Total Enzyme Activity (pmol/min)
solubilized enzyme	0.5	415.4
ELUTION WITH SODIUM THIOCYANATE		
unbound control IgG	1.0	150.8
unbound ascites IgG	1.0	97.1
control IgG	0.6	0
ascites IgG	0.6	0

Appendix Table 18. Partial purification of DGAT from the EC stage of MD embryos of *B. napus* L. cv Topas. DGAT was solubilized from a 1500 - 100 000 *g* particulate fraction, from which PA phosphatase had previously been solubilized, as outlined in MATERIALS AND METHODS. Solubilized DGAT was precipitated with 2 - 6% (w/v) PEG 8000. Solubilized DGAT was incubated for 15 min on ice with 2% (w/v) PEG 8000 then spun in a microfuge at 15 900 *g* for 10 min. The resulting supernatant was further incubated with 6% (w/v) PEG for 15 min on ice then spun for 10 min. The pellets were resuspended in 10 mM Tris-HCl buffer, pH 8.0, containing 2 M NaCl and 1% (w/v) MEGA-8. For each experiment, 10 μL of fractions containing solubilized DGAT and PEG precipitated DGAT were assayed for activity. Results represent the means of triplicate assays.

Fraction	Specific Activity (pmol/min/mg)
Experiment #1	
Solubilized DGAT	148.2
PEG precipitated DGAT	521.5
Experiment #2	
Solubilized DGAT	55.0
PEG precipitated DGAT	229.0
Experiment #3	
Solubilized DGAT	42.0
PEG precipitated DGAT	546.0

Appendix Table 19. Immunodetection of solubilized DGAT from the LC stage of MD embryos of *Brassica napus* L. cv Topas using polyclonal antibodies specific to partially purified soybean DGAT. Various amounts of solubilized and denatured protein from MD embryos were applied to nitrocellulose using a Dot Blot apparatus and incubated with anti-soybean DGAT polyclonal antibodies as outlined in MATERIALS AND METHODS. The nitrocellulose was further incubated with 400 μL solubilized DGAT (2 μg/μL), then individual dots of nitrocellulose were assayed for DGAT activity.

Solubilized Protein (μg)	DGAT Activity (pmol/min)
0	0.035
20	0.040
50	0.078
70	0.011
100	0.001