

CHARACTERISTICS OF PHOSPHATIDATE PHOSPHATASE
FROM DEVELOPING SEEDS AND MICROSPORE-DERIVED CULTURES
OF OILSEED RAPE

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(Bachelor of Science, University of Lethbridge, 1991)

A Thesis Submitted
in Partial Fulfillment for
Requirements for the Degree

MASTER OF SCIENCE

UNIVERSITY OF LETHBRIDGE
LETHBRIDGE, ALBERTA, CANADA

November 22, 1994

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**TO MY PARENTS,
WILLIAM AND ROSEMARIE SHUMAKER**

ABSTRACT

Phosphatidate phosphatase (PAP, EC 3.1.3.4) was characterized from developing seeds and microspore-derived (MD) cultures of oilseed rape. In studies with homogenate from developing seeds (*Brassica napus* L. cv Westar) the time course for release of inorganic phosphate from phosphatidate was linear for at least 60 min and the enzyme was stable to at least three cycles of freezing and thawing. Differential centrifugation studies were conducted with homogenate prepared from developing seeds (*B. napus* L. cv Westar), MD embryos (*B. napus* L. cv Reston), and an embryogenic MD cell suspension culture (*B. napus* L. cv Jet Neuf). Among the three tissue types, the level of microsomal PAP ranged from 11% to 17% of the total recovered PAP activity whereas soluble PAP ranged from 25% to 61% of the total activity recovered. Microsomal PAP displayed optimal activity in the pH range of 6 to 7 whereas soluble PAP had a pH optimum of 5. Microsomal and soluble PAP exhibited temperature reaction optima of 40°C and 50°C, respectively, with activation energies of 15.6 kcal/mol and 9.4 kcal/mol. Assays with *p*-nitrophenyl phosphate as a substrate at pH 6.75 and pH 5 indicated that the overall character of phosphatase activity in the microsomal fraction was different from the enzyme in the soluble fraction. A number of detergents were screened for their ability to solubilize microsomal PAP from MD embryos of *B. napus* L. cv Topas. Tween 20 solubilized PAP effectively with concomitant maintenance of enzyme activity. The most effective solubilization of enzyme occurred at a concentration of 0.4% (w/v) Tween 20 at a detergent to protein ratio of 1:1 (w/w). The pH optimum (pH 6-7) of solubilized PAP was similar to that of the particulate enzyme and the assay of the solubilized enzyme was free from interference by phospholipase action. Solubilized microsomal PAP had an apparent M_r of about 300,000 based on gel filtration chromatography on a column of Superose 6. Polyclonal antibodies raised in mice against a crude extract from microsomes of MD embryos inhibited microsomal PAP activity.

ACKNOWLEDGEMENTS

I would like to thank my graduate committee members, Dr. Stewart B. Rood, Dr. Peter W. Dibble, and Dr. Robert E. Blackshaw, for their constructive criticisms, suggestions, and support, and Dr. Job Kuijt for introducing me, as an undergraduate student, to the interesting world of plants.

I would also like to thank my external thesis examiner, Dr. John Browse, for his time and valuable insights.

A special thank you to Tara Furukawa-Stoffer for her excellent technical assistance and advice, Dawn Little for her assistance in the laboratory and the time she spent helping to create Figure 1, Charlene Nykiforuk for her help with experiments leading to Figure 3, and John Eng for his help with experiments leading to Figure 21. I also thank Doug Bray for photographing the TLC plate shown in Figure 21.

Additionally, I am grateful for the assistance I received from Dr. Don Frantz with computer operations, and for enlightening conversations with Dr. John Bain on the morphology and taxonomy of oleaceous plants.

I would also like to give a word of thanks to Professors Andrew Hakin, René Boéré, and Douglas Dolman for stimulating discussions involving topics ranging from analytical techniques for the microdetermination of phosphorus in biological tissues, to the chemical interactions responsible for color change in certain aqueous solutions.

Most of all, I thank my supervisor, Dr. Randall J. Weselake, for his unending support and encouragement. From him I have learned one of the most valuable lessons of life. He has taught me that there is one thing even more important than working hard and trying to excel at your profession, and this is to be a kind and compassionate person. I only hope that some aspect of his character has permanently affected the nature of mine.

Also, I thank my wife, Connie, for her continuous support and encouragement, and for confidently and enthusiastically enduring with me the emotional roller coaster rides which are inherent to studies of this kind.

Finally, I would like to thank the Alberta Agricultural Research Institute (Farming for the Future) and Natural Sciences and Engineering Research Council of Canada for supporting this research.

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
BSA	bovine serum albumin
CDP-DG	cytidine diphosphate-diacylglycerol
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CHAPSO	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate
CMC	critical micelle concentration
CoA	coenzyme A
CTAB	cetyltrimethylammonium bromide
DG	diacylglycerol
DGAT	diacylglycerol acyltransferase (EC 2.3.1.20)
DGDG	digalactosyldiacylglycerol
DHAP	dihydroxyacetone phosphate
DOC	deoxycholate
DPG	diphosphatidylglycerol
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetate
ER	endoplasmic reticulum
G3P	glycerol-3-phosphate
GPAT	glycerophosphate acyltransferase (EC 2.3.1.15)
IP ₃	inositol-1,4,5-triphosphate
LP	lysophosphatidate
LPAT	lysophosphatidate acyltransferase (EC 2.3.1.51)
LPC	lysophosphatidylcholine
LPCAT	lysophosphatidylcholine acyltransferase (EC 2.3.1.23)
LTP	lipid transfer protein
MD	microspore-derived

MEGA-8	octanoyl-N-methylglucamide
MG	monoacylglycerol
MGDG	monogalactosyldiacylglycerol
<i>p</i> NPP	<i>p</i> -nitrophenyl phosphate
P _i	inorganic orthophosphate
PA	phosphatidate
PAP	phosphatidate phosphatase (EC 3.1.3.4)
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PG	phosphatidylglycerol
PGP	phosphatidylglycerol-3-phosphate
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PS	phosphatidylserine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<i>sn</i>	stereochemical numbering
SQDG	sulfoquinovosyldiacylglycerol
TCA	trichloroacetic acid
TG	triacylglycerol
TLC	thin layer chromatography

INTRODUCTION

Vegetable oils are derived from the storage lipids laid down in organs such as fruits and, in particular, oleaceous seeds. Currently, world trade in vegetable oils is about 65 million tonnes per year (Murphy, 1994). This figure may only represent a fraction of total global production, however, since most vegetable oils are used locally. Even so, seed oils and their products are an increasingly valuable commodity in world trade, with global production having increased by approximately 30% during the past decade (Pomeroy and Sparace, 1992). Also, with oilseed sales representing one-quarter of crop production in Canada (Scarth *et al.*, 1992), it is clear that oilseeds are an important part of Canada's agricultural economy. Much of this can be attributed to the production of low erucic acid, low glucosinolate rapeseed cultivars (canola). In the past, plant breeding has brought major improvements in the yield and quality of oilseed rape (*Brassica napus* L.). The small round seeds of this crop contain over 40% oil (Stymne and Stobart, 1987) and, after oil extraction, yield a meal containing high quality protein used as an animal feed supplement. Additionally, because *B. napus* can grow at relatively low temperatures, it is one of the very few sources of edible oil that can be successfully produced in the environmental extremes of the temperate regions (Downey, 1983).

In almost all plants, storage lipids are in the form of triacylglycerol (TG). The economic importance of seed oil for food and industrial uses has resulted in an accumulation of information about the fatty acid composition of TG from the seeds of many species (Gurr, 1980; Roughan and Slack, 1982). On the other hand, in relation to the level of understanding of TG biosynthesis in animals, there is a disparaging shortfall in the number of studies on the biosynthesis of seed oil lipids. Seeds accumulate lipid in a discontinuous manner (Harwood, 1989) and, therefore, it has been suggested that the lack of research on seed oil formation may have partly arisen

because of problems in obtaining sufficient working material at a well-defined stage of development (Gurr, 1980).

The formation of storage TG is catalyzed by the enzymes of the Kennedy pathway (Kennedy, 1961; Stobart and Stymne, 1990). The enzymes are primarily located in the endoplasmic reticulum (ER) and act by the sequential acylation of the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol-3-phosphate (G3P), with the removal of the phosphate (P_i) group occurring just prior to the final acylation step. In oilseed rape, the cotyledons of the embryo are the major site of TG accumulation (Murphy, 1993). In order to facilitate the study of seed oil biosynthesis a number of *in vitro* embryonic systems, with well-defined developmental stages, have been developed (Murphy *et al.*, 1993). One of the best characterized of these is the microspore-derived (MD) embryo system in *B. napus* (Taylor *et al.*, 1990; Pomeroy *et al.*, 1991). MD embryos are capable of forming TG in a manner similar to that of zygotic embryos (Weselake *et al.*, 1991; Taylor *et al.*, 1992a, 1992c). Furthermore, the TG which accumulates in MD embryos is similar in composition to the seed storage lipid (Taylor *et al.*, 1991, 1992b). Cell suspension cultures of various oilseeds have also been shown to contain TG (Radwan and Mangold, 1976), and culture conditions have been manipulated to alter lipid content and composition (Radwan and Mangold, 1976; Theimer *et al.*, 1991). An embryogenic MD cell-suspension culture of *B. napus* (cv Jet Neuf), originally described by Orr *et al.* (1986), has been shown to contain a low but significant amount of TG which suggests that this nondifferentiating system may be useful in the characterization and purification of TG bioassembly enzymes (Weselake *et al.*, 1993).

Although high yields are desirable, the composition, or quality, of seed oil is at least as important as the quantity of oil that can be extracted (Downey, 1983). The fatty acid composition of seed oil determines its physical and chemical properties and, therefore, its potential applications (Mancha *et al.*, 1994). In the main oil crops,

including oilseed rape, this composition is less than optimal to meet all the requirements for food and industry. The modification of the fatty acid composition of the reserve lipids of *B. napus* could be accomplished by altering the activity or the specificity and selectivity characteristics of the enzymes involved in their synthesis, and also by transferring new genes from other species for new enzyme activities (Ohlrogge *et al.*, 1991). In many cases, relatively large alterations can be made in the fatty acid composition of the storage TG without exerting any obvious deleterious effects on the growth and development of the plant. Although expectations are high, a thorough understanding of the biosynthesis of TG in plants, and its regulation, is essential for success in attempts at acyl manipulation through the current technologies (Stobart and Stymne, 1990). It appears that the most efficient way to attain further gains in crop improvement will be to combine classical plant breeding practices with rapidly emerging biotechnological approaches solidly underpinned with fundamental research (Pomeroy and Sparace, 1992).

Identification of the genes encoding Kennedy pathway enzymes could contribute to the improvement of oilseed crops, but molecular genetic studies have been impeded by the difficulties experienced in the solubilization and purification of these proteins. Phosphatidate phosphatase (PAP, EC 3.1.3.4) is the membrane-bound, hydrolytic enzyme responsible for the penultimate step involving the removal of P_i from phosphatidate (PA). PAP is a particularly important enzyme in glycerolipid metabolism because the diacylglycerol (DG) produced from the hydrolysis of PA is not only a direct precursor of TG, but also a substrate for the synthesis of membrane lipids (Stobart and Stymne, 1985a, 1990). Also, PAP appears to be an ubiquitous enzyme (Ichihara *et al.*, 1990). That is, the enzyme exists in different compartments of the cell and its intracellular translocation may help to regulate lipid metabolism (Wilson, 1980; Brindley, 1984). Genetic alteration of PAP abundance and/or selectivity in maturing oilseed rape may contribute to the development of cultivars with greater seed oil

content or improved oil quality. Before any type of altered expression system can be implemented, however, the gene(s) encoding the enzyme must be isolated through the study and characterization of the enzyme itself. This thesis has examined the properties of PAP from maturing seeds, MD embryos, and an embryogenic MD cell suspension culture of oilseed rape. Phosphatase activities were characterized in both the microsomal and soluble fractions in an attempt to elucidate the fundamental physiological properties of microsomal PAP versus a putative, cytosolic form of the enzyme. Additionally, the enzyme was solubilized from the microsomal fraction of MD embryos and further characterized.

LITERATURE REVIEW

Triacylglycerol Biosynthesis in Developing Oilseeds

Although some plant species store large quantities of oil in the mesocarp or pericarp of the fruit, the seed is the most important organ for the storage of reserve lipid (Gurr and Harwood, 1991). In oilseeds, storage lipid provides the respiratory fuel for seedling growth and is initially hydrolyzed by lipase in a manner analogous to the α -amylase degradation of starch in cereals (Imeson *et al.*, 1993). There are several hundred varieties of plants known to have oil bearing seeds, but only a handful are commercially significant. A few, such as canola for example, are important sources of edible oil for human consumption. Others are used in animal feeds or as industrial feedstocks. Triacylglycerol (TG) makes up the major lipid store in plants, sometimes representing as much as 80% of the dry weight of seeds (Gurr and Harwood, 1991). The only known exception to this is the jojoba seed, in which the fatty acids are esterified to long-chain alcohols to form waxes (Stymne and Stobart, 1987). It is interesting that droplets of TG are also present in the seeds of plants that store mainly starch as the primary source of fuel for seed germination. These include cereals like wheat or barley and legumes such as peas or beans (Gurr, 1980).

A triacylglycerol molecule is an ester of glycerol in which each of the three hydroxyl groups is esterified with a fatty acid, or acyl group (Gurr, 1980). A partial glyceride, having only one or two hydroxyl groups esterified, is respectively referred to as a monoacylglycerol (MG) or diacylglycerol (DG). All the carbon atoms of glycerol are readily distinguishable from one another because the molecule does not exhibit rotational symmetry (Stymne and Stobart, 1987). The carbon atoms are classified by stereochemical numbering (*sn*) and are referred to as the *sn*-1, *sn*-2, and *sn*-3 positions according to Brockerhoff (1971). Although the number of different fatty acids detected in plant tissues is in the hundreds (Harwood, 1980), with the more recent study of less common sources of lipid, such as blue-green algae of marine origin (Murata and

Nishida, 1987), it is now known that the number of naturally occurring fatty acids exceeds 1000 (Gunstone, 1990). In spite of the fact that many of them may be of significant industrial value, the number of different fatty acids found in the oleaceous tissues of the major agricultural oil crops is restricted to about 10 (Stobart and Stymne, 1990). These common fatty acids are all monocarboxylic acids with unbranched, even-numbered carbon chains. They include the saturated fatty acids, capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0), and stearic (18:0), and the unsaturated fatty acids, oleic (18:1 delta-9), linoleic (18:2 delta-9,12), α -linolenic (18:3 delta-9,12,15), gamma-linolenic (18:3 delta-6,9,12), and erucic (22:1 delta-13). (By convention, the number before the colon indicates the carbon chain length, and the figure immediately after corresponds to the number of double bonds. Numbers preceded by "delta" indicate points of unsaturation when numbering from the carboxylic end.) In fact, eight of these, excluding capric and gamma-linolenic, account for 97% of the fatty acids present in commercial vegetable oils (Stymne and Stobart, 1987).

The major pathway for the formation of triacylglycerol was first elucidated, using animal tissues, through the work of Kennedy (1953, 1957, 1961), Kornberg and Pricer (1953), and others (Smith *et al.*, 1957; Rossiter *et al.*, 1957). The first indication that plant tissues could synthesize triacylglycerols by a route resembling the mammalian pathway came in 1962 when Barron and Stumpf demonstrated that a particulate fraction, isolated from an acetone powder of avocado mesocarp by high-speed centrifugation, could synthesize radiolabelled TG from ^{14}C -glycerol phosphate. The formation of TG, from *sn*-glycerol-3-phosphate and activated fatty acid (fatty acyl-CoA) precursors, is known as the glycerol-3-phosphate (G3P) or Kennedy pathway (Figure 1). The glycerol backbone of the TG molecule is derived from G3P. The G3P dehydrogenase catalyzed reduction of dihydroxyacetone phosphate (DHAP), from either the glycolytic or pentose phosphate pathways, gives rise to G3P (Stymne and Stobart, 1987). To a lesser extent, G3P is also formed by the direct phosphorylation of

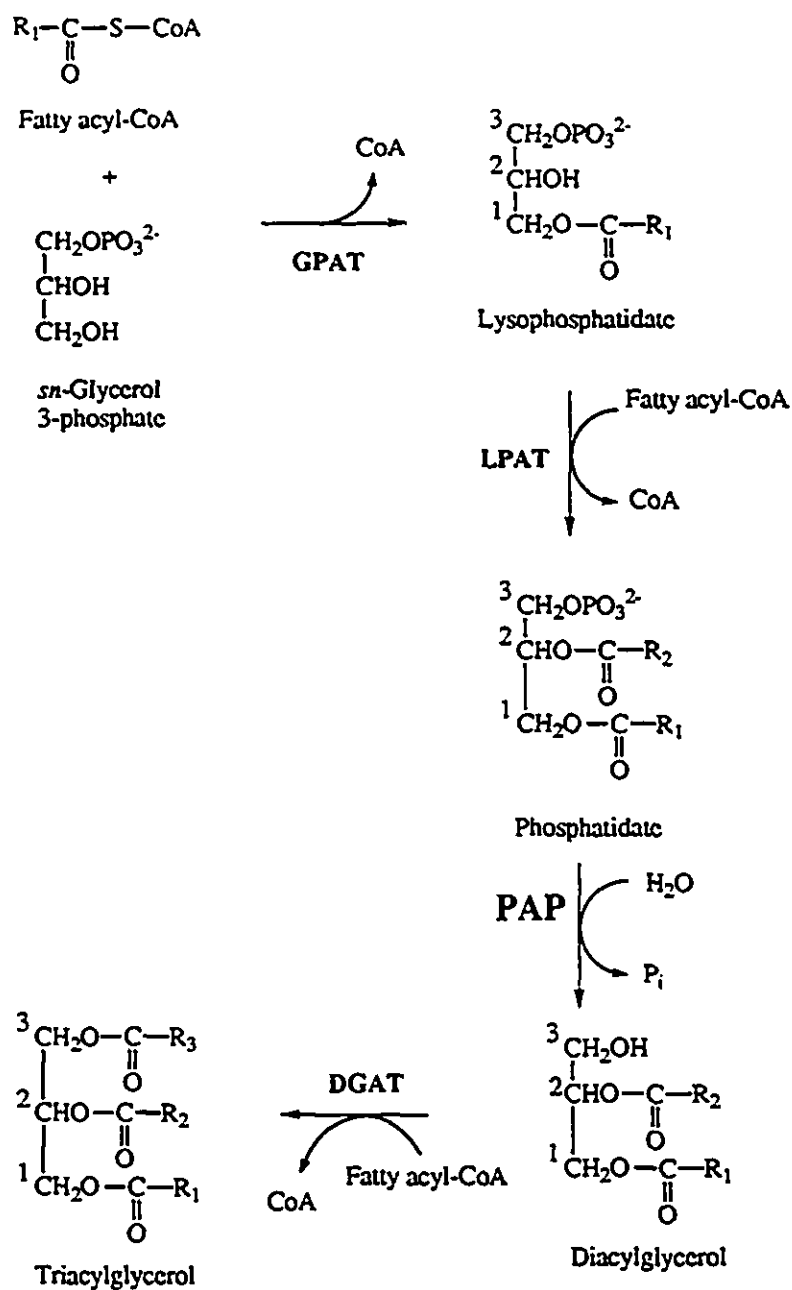


Figure 1. Reactions in the Kennedy pathway leading to the formation of TG. Additional reactions involving the phospholipid fraction are not depicted. (R_1 , R_2 , R_3 = hydrocarbon chains derived from the acyl moieties of fatty acyl-CoA.)

glycerol, which is catalyzed by the enzyme glycerol kinase (Gurr, 1980). In some animal systems a slight variation of the Kennedy pathway involves the direct acylation of DHAP at the *sn*-1 position (Gurr and Harwood, 1991), to yield acyldihydroxyacetone phosphate, which is then converted to lysophosphatidate (LP) and enters the Kennedy pathway at the point of lysophosphatidate acyltransferase (LPAT, EC 2.3.1.51). There are no indications that plant glycerolipids are synthesized via the direct acylation of DHAP (Frentzen, 1993).

In plants, *de novo* biosynthesis of fatty acids occurs exclusively within the plastids (Sparace and Kleppinger-Sparace, 1993). Starting with acetate, the process involves several enzymes and a sequence of repeating reactions which results in the formation of fatty acids up to the level of 16:0, 18:0, and 18:1 (Roughan and Slack, 1982; Sparace *et al.*, 1992). These fatty acids are generated as thioesters linked to acyl carrier protein (ACP), and are either used directly for plastidular glycerolipid biosynthesis or exported as coenzyme A (CoA) thioesters into the cytoplasm (Browse and Somerville, 1991). Once exported, the activated fatty acids can be utilized directly or modified for lipid biosynthesis at extraplastidular sites (Frentzen, 1993). An active acyl-ACP hydrolase, with a broad substrate specificity, is present in the plastid and participates in liberating free fatty acid for movement across the membranes of the plastid envelope. The ligation of CoA to the free fatty acid is catalyzed by the action of an acyl-CoA synthetase which is located in or on the outer membrane of the plastid (Stymne and Stobart, 1987). Whether acyl-CoA thioesters diffuse into the cytoplasm in a free form or bound to specific fatty acid binding proteins requires further clarification (Frentzen, 1993; Kader, 1993). In plants, factors which affect the possible availability of G3P for glycerolipid synthesis have received little attention. On the other hand, Sparace *et al.* (1992) have shown that glycerolipid biosynthesis and fatty acid formation are tightly coordinated, since close similarities exist in the cofactor requirements for the two processes.

Microsomal membranes, and to some extent, oil bodies, are capable of catalyzing the formation of TG (Browse and Somerville, 1991), suggesting that Kennedy pathway enzymes are primarily located on the ER. Strong evidence for this was provided by Stobart *et al.* (1986), who demonstrated that microscopically pure microsomal preparations from safflower seeds were capable of TG synthesis at rates comparable to the maximum *in vivo* (Slack *et al.*, 1985) rate of TG deposition. The first enzyme of the Kennedy pathway, glycerophosphate acyltransferase (GPAT, EC 2.3.1.15), catalyzes the acylation of *sn*-glycerol-3-phosphate at the *sn*-1 position to form LP. The second acyltransferase, LPAT, is responsible for the *sn*-2 acylation of LP to yield PA. It is the acyl specificity and selectivity properties of these two enzymes that govern the non-random distribution of fatty acid species at the *sn*-1 and *sn*-2 positions of PA and DG, and the other glycerolipids which are subsequently derived from them (Murphy, 1993, 1994). PAP catalyzes the cleavage of PA to release P_i and the key intermediate in TG biosynthesis, DG. Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) is the enzyme responsible for the ultimate step in the biosynthesis of TG, and catalyzes the acylation of the *sn*-3 position previously occupied by the P_i group. DGAT is the only enzyme which can be considered solely dedicated to the formation of TG (Stobart and Stymne, 1990) and, therefore, is partially responsible for regulating the quality of reserve lipid.

TG Biosynthesis in Relation to Membrane Lipid Metabolism in Developing Oilseeds

The glycerolipids produced in biological systems serve two primary functions. First, as discussed in the previous section, they can act as an oil or fat store of respiratory fuel, which is always in the form of TG (Stymne and Stobart, 1987; Saggerson, 1988; Gurr and Harwood, 1991). Second, the phospholipids (Mudd, 1980; Moore, 1982), galactolipids (Joyard and Douce, 1987), and sulfolipid (Mudd and

Kleppinger-Sparace, 1987) formed are necessary components of biological membranes. This is particularly important since biological membranes are a cytological feature of all living things (Quinn and Williams, 1990). The membranes not only define the cell, but they also serve as permeability barriers and provide suitable environments for the many enzymes which carry out the reactions necessary for cell maintenance and growth (Raison, 1980; Beevers, 1991).

It is the production of amphipathic lipids, and the way that they become ordered in an aqueous environment, that is responsible for the compartmentation observed in the cell. In general, membrane lipids are confined to a bilayer which is assembled by a spontaneous process similar to that observed in artificial systems (Gershfeld *et al.*, 1986). According to the fluid mosaic model of Singer and Nicolson (1972), this bilayer configuration serves as a matrix for the insertion or attachment of membrane proteins. The membranes are considered to be fluid since the components from which they are made can, supposedly, readily diffuse in the lateral plane of the membrane, even though they are restricted in their ability to move from one side of the membrane to the other. This model has been generally accepted and the concept of lateral membrane fluidity is now central to current views of membrane organization. Additionally, there is a working hypothesis which suggests that the amount of membrane lipid formed is regulated by the need to accommodate integral membrane proteins (Somerville, 1991). In other words, the control of membrane lipid formation may be closely linked to the regulation of protein synthesis.

The biosynthesis of membrane lipids shares the same steps with the Kennedy pathway up to, and including, the point where DG is formed (Gardiner *et al.*, 1984; Frentzen, 1993). These activities are localized in several compartments of the cell and are often collectively referred to as the Kornberg-Pricer pathway (Joyard and Douce, 1987; Joyard *et al.*, 1994). This brings into focus the importance of the two-pathway system of plant lipid biosynthesis (Roughan and Slack, 1982; Browse and Somerville,

1991). Briefly, glycerolipids synthesized in the plastid are generally referred to as "prokaryotic" lipids, while those synthesized outside the plastid, primarily in the ER, are referred to as "eukaryotic" lipids. This distinction is made based on the fatty acids esterified to the *sn*-2 position of the glycerol moiety and, therefore, on the dissimilar fatty acid specificities of LPAT from the different subcellular compartments. Plastidular LPAT is specific for 16-carbon fatty acids, while the corresponding enzyme located in the ER is specific for acyl groups containing 18 carbons. Also, in the plastids, fatty acids utilized by the acyltransferases are in the form of acyl-ACP thioesters rather than the acyl-CoA thioesters consumed by the Kennedy pathway (Harwood, 1989).

In all higher plants, a portion of the DG moiety of phosphatidylcholine (PC), which is almost exclusively synthesized in the ER, is routed to the plastids. In many families of angiosperms, designated as "18:3" plants, phosphatidylglycerol (PG) is the only major product of the prokaryotic pathway, and the remaining plastidular lipids are synthesized from DG obtained entirely from the eukaryotic pathway. On the other hand, in some primitive angiosperm families such as the Brassicaceae (Frentzen, 1993), both pathways contribute to the synthesis of monogalactosyldiacylglycerol (MGDG), and to a lesser extent, digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG). These species are designated as "16:3" plants, and can be readily distinguished from 18:3 plants since the MGDG formed via the prokaryotic pathway contains a high level of 16:3 at the *sn*-2 position, whereas in 18:3 plants this plastidular glycerolipid has predominantly α -linolenate attached to the *sn*-2 carbon (Mudd and Kleppinger-Sparace, 1987). The level of PAP activity in the plastid envelope membranes is probably responsible for the distinction between 16:3 and 18:3 plants, since plastids from 18:3 plants generally have very low PAP activity and are unable to synthesize DG and, as a consequence, glycerolipids with 16-carbon fatty acids at the *sn*-2 position of the glycerol backbone (Joyard *et al.*, 1994). A missing

link in 18:3 plants, however, is the conversion of PC into DG, since envelope membranes seem to lack the phospholipase C activity necessary to produce the required DG (Joyard *et al.*, 1993). Apparently, the plastids from all plants contain PC which is solely eukaryotic and PG which is strictly prokaryotic (Frentzen, 1993).

The mitochondria, like plastids, are capable of *de novo* biosynthesis of glycerolipids. These organelles are also similar to plastids in that they are semiautonomous in their lipid biosynthetic ability. Mitochondria produce only a part of their own membrane lipids, mainly PG and diphosphatidylglycerol (DPG) or cardiolipin, while the remainder have to be derived from glycerolipids imported from the ER (Moore, 1982). Unlike plastids, the PG of mitochondria can possess both eukaryotic and prokaryotic fatty acid patterns (Frentzen, 1993). It has been suggested that the transfer of PC from the ER to both the plastids (Kader, 1993) and the mitochondria (Mazliak and Kader, 1980) may be mediated by some form of cytosolic lipid transfer protein (LTP).

Several generalizations can be made about lipid biosynthesis in plant tissues. First, in both leaf mesophyll cells and the cells of developing seeds, 16:0-ACP and 18:1-ACP are the major products of plastid fatty acid synthesis and 18:0-ACP desaturase activity (Gurr and Harwood, 1991). Second, the thioesterase activity involved in acyl-ACP hydrolysis is presumably selective for 16:0 and 18:1 (Ohlrogge *et al.*, 1978; Browse and Somerville, 1991), and the acyl-CoA thioesters produced from these free fatty acids are the primary substrates for reactions in other cellular compartments (Stymne and Stobart, 1987). Although some fats (oils which are solid at room temperature) such as cocoa butter contain a high proportion of 18:0 (Griffiths and Harwood, 1991), 18:0-ACP is considered to be intermediary and stearate is usually only a minor component of TG and other plant lipids (Browse and Somerville, 1991). Finally, the specificity and selectivity characteristics of microsomal LPAT are such that 16:0 and, if present, 18:0 are essentially excluded from the *sn*-2 position of PA and,

therefore, TG (Griffiths *et al.*, 1985; Stymne and Stobart, 1987). On the other hand, microsomal GPAT will preferentially esterify these saturated fatty acids to the *sn*-1 carbon of the glycerol backbone (Gurr, 1980; Griffiths *et al.*, 1985).

In the developing seed, however, the acyl-CoA pool is a much more complex collection of fatty acids than in the leaf (Browse and Somerville, 1991). The molecular medium for the desaturation of 18:1 to 18:2 and, in some cases, 18:3, has been determined to be PC, and the production of TG enriched in polyunsaturated fatty acids has been attributed to an acyl-exchange mechanism for releasing the desaturated products from the phospholipid back to the acyl-CoA pool (Stymne *et al.*, 1983; Stymne and Stobart, 1987). The enzyme responsible, lysophosphatidylcholine acyltransferase (LPCAT, EC 2.3.1.23), catalyzes the acylation of the *sn*-2 position of lysophosphatidylcholine (LPC) to form PC, and is also able to catalyze the exchange of fatty acids between 18:1-CoA and the polyunsaturated 18-carbon fatty acids esterified to PC (Stobart and Stymne, 1990). Acyl exchange has been demonstrated between the *sn*-2 position of PC and the cytosolic acyl-CoA pool in safflower (Stymne *et al.*, 1983) and other species (Stymne and Stobart, 1987; Frentzen, 1993). The result of oleate desaturation on PC combined with acyl exchange is that the acyl-CoA pool becomes enriched in linoleate and linolenates, which are subsequently utilized by the Kennedy pathway enzymes GPAT, LPAT, and in some cases, DGAT (Stymne and Stobart, 1987; Frentzen, 1993).

The production of TG enriched with polyunsaturated fatty acids has also been attributed to the flux of DG through the PC pool (Roughan and Slack, 1982; Stymne and Stobart, 1987; Stobart and Stymne, 1990). According to Harwood (1989), DG, resulting from the hydrolysis of PA by PAP in the Kennedy pathway, is converted to PC by the freely reversible reaction catalyzed by cholinephosphotransferase (EC 2.7.8.2). The PC is capable of being rapidly desaturated at the *sn*-2 position, as described above, but in this case the DG skeleton of PC is returned to the DG pool and

converted into TG. Using microsomal preparations of cotyledons from developing safflower seeds, Stobart and Stymne (1985a) were able to demonstrate the rapid exchange of DG with PC, while Griffiths *et al.* (1985) showed that DG equilibrated with the PC pool when the glycerol backbone, and associated acyl groups, flowed from PA to TG. Thus, in some oilseeds, the DG pool used for TG biosynthesis is fed as a consequence of the activities of both PAP and cholinephosphotransferase.

It is the coordination of the Kennedy pathway enzymes with cholinephosphotransferase, LPCAT, and the oleate and linoleate desaturases which is responsible for the synthesis of TG having linoleate and linolenate at the *sn*-2 position of the glycerol backbone. It is not surprising that PC in the ER membranes is the complex lipid substrate for the desaturation of 18-carbon fatty acids used in TG biosynthesis since PC, a ubiquitous component of higher plant membranes, is synthesized predominantly on the ER, which is also the intracellular location of the Kennedy pathway. Nonetheless, a key problem in understanding storage lipid metabolism is trying to determine the relative contributions of cholinephosphotransferase activity and PAP activity to the DG pool used for TG formation (Browse and Somerville, 1991). What causes difficulty in deciding which of the two alternative routes is more important in the regulation of TG quality is the very fact that the reactions involved in both PC and TG synthesis do occur within the same subcellular compartment. This difficulty is further emphasized by the fact that the seed oils of many plants contain fatty acids synthesized *in situ* which are largely or completely excluded from the membrane lipids (Fehling and Mukherjee, 1990; Fehling *et al.*, 1990; Frentzen, 1993). Additionally, using homogenates obtained from the developing seeds of safflower, *Crambe abyssinnica*, and *Cuphea wrightii*, Battey and Ohlrogge (1989) demonstrated that in these species exogenously supplied medium and long chain fatty acids were targeted to TG and not to phospholipids.

There are two particularly well known cases where unusual fatty acids are targeted to the reserve lipid, and each deserves a brief discussion. First, several varieties of *Brassica napus* and *B. rapa* (syn. *campestris*) produce seed oil characterized by a large amount of erucic acid. Seed oils high in 22:1 are desirable as industrial feedstocks for the production of high temperature lubricants, nylon, and plasticizers (Taylor *et al.*, 1991), and there is considerable interest directed at developing genetically manipulated *B. napus* which produces trierucin (trierucoylglycerol). In high erucic acid rapeseed lines, this long chain monounsaturated fatty acid is associated with the *sn*-1 and *sn*-3 positions of the TG molecules (Stymne and Stobart, 1987; Taylor *et al.*, 1992c), but is essentially excluded from membrane constituents where long chain fatty acids may adversely affect membrane structure and function (Frentzen, 1993). Using *B. napus* MD embryos of the high erucic acid cultivar, Reston, Taylor *et al.* (1991) demonstrated that ^{14}C -erucoyl moieties from ^{14}C -22:1-CoA were rapidly incorporated into TG but virtually excluded from complex polar lipids. Erucic acid is synthesized by the elongation of oleoyl-CoA (Downey and Craig, 1964; Stumpf and Pollard, 1983; Browse and Somerville, 1991) and the process does not involve, to any extent, a PC intermediate (Roughan and Slack, 1982). Elongation occurs via eicosenoic acid (20:1 Δ -11) and the double bond introduced at the 18-carbon (oleate) level is retained (Harwood, 1989). Low erucic acid cultivars appear to simply lack elongase activity (Stymne and Stobart, 1987) which, when present, is thought to be associated with the ER membranes (Harwood, 1989). In *B. napus*, the levels of 20:1 and 22:1 can be independently varied by selection (Browse and Somerville, 1991), but seed oils containing significant amounts of erucic acid at the *sn*-2 position have not been found in this species (Taylor *et al.*, 1992b). Therefore, without the introduction of foreign genes encoding LPAT specific for erucoyl-CoA, the theoretical breeding limit for 22:1 in *B. napus* seed oils is 66 mol% (Taylor *et al.*, 1992b). Introducing the LPAT gene

from *Tropaeolum majus* or *Limnanthes douglasii*, which encodes for an enzyme capable of inserting erucoyl moieties into the *sn*-2 position of TG (Taylor *et al.*, 1992c; Frentzen, 1993), may lead to the elusive trierucin *B. napus* cultivar. Although Taylor *et al.* (1992c) have shown that native DGAT from *B. napus* will readily accept *sn*-1,2-dierucoyl-DG, the success of this approach is based on the assumption that native PAP will readily hydrolyze *sn*-1,2-dierucoyl-PA.

The second case where unusual fatty acids are selectively targeted to storage TG involves the ricinoleic acid (12-hydroxy-18:1 Δ -9) of castor bean (*Ricinus communis*). Hydroxylated fatty acids are particularly useful because they allow many chemical conversions (Frentzen, 1993), and economically desirable castor bean oils contain ricinoleic acid in excess of 90% of the total acyl composition (Stymne and Stobart, 1987). The relative abundance of ricinoleate in this oil is particularly interesting since one of the major aims of some plant breeding programs is to produce strains of common oil crop species, such as *B. napus*, which will generate TG containing only one type of fatty acid (Scarth *et al.*, 1992; Taylor *et al.*, 1992b). The hydroxylation step of ricinoleic acid biosynthesis requires molecular O₂ and NADH (Stymne and Stobart, 1987), and appears to take place in the ER using oleoyl groups esterified to the *sn*-2 position of PC (Banas *et al.*, 1992; Frentzen, 1993) in a manner that is similar to the formation of polyunsaturated fatty acids. That is, PC is the central intermediate in ricinoleate synthesis where, once again, cholinephosphotransferase is involved in DG-PC interconversion. DG containing ricinoleic acid does not accumulate in the DG pool, however, because it is rapidly converted into TG as a result of the ricinoleoyl-containing DG preference of the DGAT. Unlike the polyunsaturated fatty acid system, ricinoleoyl groups are not released from PC as CoA thioesters via acyl exchange. Instead, they are liberated as free fatty acids as a result of a phospholipase A₂ activity which rapidly digests PC species with hydroxylated, but not unsaturated, acyl groups (Frentzen, 1993). The free fatty acids are subsequently

converted to acyl-CoA thioesters which are then used by the Kennedy pathway acyltransferase enzymes. The thermodynamically favorable degradation of PC to LPC and ricinoleic acids effectively removes PC with hydroxylated fatty acids from the membranes. The specific degradation of PC with hydroxylated acyl groups is not confined to castor bean seeds, but also occurs using enzyme systems from other plant species such as *B. napus*, which do not accumulate ricinoleic acid (Frentzen, 1993). Using microsomal preparations obtained from MD embryos of *B. napus* cv Topas, Banas *et al.* (1992) showed that PC containing ricinoleate was catabolized much more readily than PC containing either oleate or linoleate. These investigators also demonstrated that DG moieties containing ricinoleic acid were preferentially released from PC, and suggested that this, along with other observations, may be attributable to phospholipase D and microsomal PAP with high specificities for substrates containing ricinoleoyl groups. The role of PAP in directing DG carbon skeletons during phospholipid turnover and TG bioassembly is in need of much further investigation.

Available evidence suggests that the unusual fatty acids described above are components of the acyl-CoA pool and are one way or another restricted to TG synthesis. As mentioned earlier, the biosynthesis of the DG moiety of the membrane phospholipid, PC, also occurs via the Kennedy pathway. Additionally, it is known that the reactions of TG formation occur alongside those of phospholipid synthesis in the ER (Browse and Somerville, 1991). Therefore, it is most likely that certain enzymic properties involved in TG and PC formation, rather than any type of spatial separation, are what control the specific targeting of acyl groups. In other words, the unusual fatty acids are probably confined to TG by the specificity characteristics of the Kennedy pathway acyltransferases, especially DGAT, and by the specificities of the enzymes involved in phospholipid synthesis. For example, if cholinephosphotransferase specifically excluded DG molecular species containing unusual fatty acids, then these would accumulate in the DG pool and could be used for TG synthesis by DGAT with

broad substrate specificity. Alternatively, it has been suggested that there may be a subtle difference in the intracellular location of phospholipid and TG synthesis which has not yet been recognized (Thompson, 1993). In this case the two processes would utilize distinct acyltransferase isoforms, each with its own substrate specificity. Ultimately, however, the source of all unmodified DG molecules in the cell is the PAP catalyzed hydrolysis of PA via either the so-called Kennedy or Kornberg-Pricer pathways.

In general, the fatty acid composition of plant storage TG is species and, in some cases, variety specific. Although the acyl composition of plant lipids is genetically controlled, the temperature during seed maturation has a modifying influence on the proportions of the different unsaturated 18-carbon fatty acids (Roughan and Slack, 1982). An elevation in temperature causes an increase in 18:1, while a decrease in temperature produces an increase in 18:2 and 18:3 (Raison, 1980). Palmitic acid, however, is usually the only 16-carbon fatty acid found in plant TG. This is because the desaturation of 16:0 occurs only at position *sn*-2 of the prokaryotic glycerolipids, PG and MGDG (Roughan and Slack, 1982). In plastids, the rate of TG formation is normally very low, although under certain physiological conditions it can increase significantly. When plants are stressed, DG released by the degradation of plastidular membrane lipids is converted into TG by a form of DGAT located in the envelope membranes (Frentzen, 1993). In oil-forming tissues, however, there are no indications that plastidular DGAT activity is of any importance to the huge accumulation of TG in the cell.

In species where it is present in significant amounts, plastidular PAP catalyzes the formation of prokaryotic DG which, in turn, is used for the synthesis of MGDG, DGDG (Joyard *et al.*, 1993), and SQDG (Mudd and Kleppinger-Sparace, 1987), but not PG (Kinney, 1993). PG is formed directly from PA by a series of enzymic activities starting with cytidine diphosphate-diacylglycerol synthetase (CDP-DG

synthetase, EC 2.7.7.41). CDP-DG synthesized from PA is converted into phosphatidylglycerol-3-phosphate (PGP), and then PGP is dephosphorylated to form PG. Therefore, CDP-DG synthetase and PAP compete for PA, and it is the level of PAP in the organelle, relative to CDP-DG synthetase, which determines the difference between 16:3 and 18:3 plants (Kinney, 1993).

One final, but very important, aspect of TG biosynthesis involves the subcellular compartmentation of the storage lipid once it has been formed. The most conspicuous change in the cotyledonary cells of a developing oil seed is the appearance of spherical bodies containing lipid (Roughan and Slack, 1982). These oil bodies do not coalesce (Stymne and Stobart, 1987), and their surfaces appear to be very stable (Cummins *et al.*, 1993). The most popular school of thought proposes that oil bodies originate as membrane-bound vesicles formed from the ER by the accumulation of nonpolar TG in the hydrophobic region of the membrane (Frey-Wyssling *et al.*, 1963; Roughan and Slack, 1982; Stymne and Stobart, 1987). Supposedly, newly formed oil accumulates between the phospholipid bilayer of the ER and, when the oil-filled vesicle reaches a certain critical size, it separates from the ER and becomes an independent particle with a half-unit membrane (Gurr, 1980; Murphy, 1993). This model is consistent with results which implicate both the ER and oil bodies as the sites for TG biosynthesis (Stobart *et al.*, 1986; Browse and Somerville, 1991). Additionally, oil bodies do in fact appear to be enclosed by a half-unit membrane (Gurr, 1980; Frentzen, 1993). Although this hypothesis reflects the mainstream view concerning the ontogeny of oil bodies in seeds, there is some ultrastructural evidence, from a number of species, that suggests that oil bodies may actually arise directly from a granular area of the cytoplasm which consists of a matrix of lipid and protein (Murphy, 1993; Murphy *et al.*, 1993). At any rate, the increase in TG concentration within the cell is paralleled by an increase in the number, rather than the size, of oil bodies (Gurr, 1980; Roughan and Slack, 1982). These spherical entities are 0.2 to 2.5 μm in diameter, and consist

of a core of reserve lipid stabilized by a 2 to 4 nm wide annulus of phospholipid and protein (Frentzen, 1993).

From experiments using the seeds from a wide range of plant species, it is now well established that the major oil body protein, termed oleosin, represents a unique class of polypeptide which is not associated with any other subcellular compartment (Murphy, 1993, 1994). Oil body fractions prepared from mature dry seeds of *B. napus* cv Topas contain over 10% (w/w) oleosin (Cummins *et al.*, 1993), and it has been suggested that this protein is involved in the ontogeny of lipid bodies during seed development (Murphy, 1993, 1994). Also, it is believed that oleosins, by preventing the coalescence of oil droplets, play a role in the stabilization of oil bodies during seed desiccation and, thus, maintain optimal oil body surface area to volume ratios for the rapid mobilization of storage lipid during germination (Cummins *et al.*, 1993; Frentzen, 1993; Murphy, 1994). Using the cotyledons of developing safflower seeds, Ichihara *et al.* (1989) have shown that PAP is associated with several different subcellular fractions resulting from the differential centrifugation of tissue homogenate, including the lipid layer fraction, which probably contains oil bodies. Perhaps oil bodies take the membrane-bound TG and phospholipid bioassembly enzymes with them when they separate from the ER, and then continue to form more oil bodies independently of the ER.

Phosphatidate Phosphatase from Various Organisms

The key intermediate common to the *de novo* synthesis of all glycerolipids in prokaryotes and eukaryotes is PA (Brindley, 1988; Harwood and Price-Jones, 1988). PA is formed as a result of the sequential acylation of G3P by the two Kennedy pathway acyltransferases, GPAT and LPAT. PA may then be converted to DG by the action of PAP, or it may be converted to CDP-DG by the competing reaction catalyzed by CDP-DG synthetase (Kinney, 1993). In oil or fat producing cells, the DG produced

by PAP is the direct precursor of TG (Harwood and Price-Jones, 1988; Saggerson, 1988). Additionally, the DG molecule can provide the carbon skeleton used for the production of plastidular glycerolipids such as the galactolipids, MGDG and DGDG (Harwood, 1989), as well as the sulfolipid, SQDG (Mudd and Kleppinger-Sparace, 1987). PAP also provides the DG skeleton necessary for the synthesis of the predominant phospholipids, phosphatidylethanolamine (PE) and PC (Moore, 1982, 1990). On the other hand, CDP-DG is the precursor for the phospholipids, phosphatidylinositol (PI), phosphatidylserine (PS), and PG (Moore, 1990; Kinney, 1993). Cardiolipin (DPG), which is restricted to the inner mitochondrial membrane, is formed from PG, and is therefore indirectly the result of CDP-DG synthetase activity (Harwood, 1989).

In animals, PAP not only functions in its traditional role of providing DG for the production of TG and nitrogen-containing phospholipids, which are used for energy storage and cellular membranes, respectively, but PAP from vertebrate lungs also functions in the production of pulmonary surfactant (Casola *et al.*, 1982; Walton and Possmayer, 1986; Possmayer, 1988). The major component of pulmonary surfactant is the phospholipid, dipalmitoyl-PC (Possmayer, 1988), which is synthesized via the Kennedy pathway in type II epithelial cells of the alveoli (Casola *et al.*, 1982). Pulmonary surfactant acts to reduce the surface tension of the air-liquid interface of the alveoli, and stabilizes the terminal airways of the lung by preventing alveolar collapse. Copious amounts of dipalmitoyl-PC have been found in the lungs of all the mammalian species studied, which include mice, rats, rabbits, dogs, cattle, and monkeys, as well as humans (Casola *et al.*, 1982; Walton and Possmayer, 1986; Possmayer, 1988). Also, significant amounts of dipalmitoyl-PC have been observed in the lungs of such diverse organisms as chickens, turtles, and frogs (Possmayer, 1988). Magnesium-dependent and Mg^{2+} -independent types of PAP have been identified in the lung, with a microsomal and soluble form of each one (Possmayer, 1988). Using a permanent cell

line of human lung, however, Walton and Possmayer (1986) showed that it is probably the Mg^{2+} -dependent form which plays a regulatory role in the formation of dipalmitoyl-PC, since it was highly responsive to exogenous free fatty acids. That is, in A549 cells, oleate promoted the translocation of the Mg^{2+} -dependent PAP from a predominantly soluble distribution to a location predominantly on cellular membranes. With this translocation was a concurrent increase in the production of DG, suggesting that subcellular translocation of Mg^{2+} -dependent PAP could function to facilitate the hydrolysis of increased amounts of PA.

In addition to its pivotal role in glycerolipid metabolism, the PAP catalyzed conversion of PA to DG is also important in signal transduction (Lavie *et al.*, 1990; Jamal *et al.*, 1991; Martin *et al.*, 1993). In both plant (Gross and Boss, 1991; Thompson, 1994) and animal (Siddiqui and Exton, 1992) tissues, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP₂) are formed from PI in small amounts and localized in the plasma membrane where they are involved in the early response of cells to many agonists (Harwood, 1989). PIP and PIP₂ are precursors of the second messengers, inositol-1,4,5-triphosphate (IP₃) and DG, which are generated as a result of the receptor-mediated stimulation of inositol lipid breakdown by a specific phospholipase C associated with the plasma membrane (Wissing *et al.*, 1992; Thompson, 1994). In plant cells, it has recently been shown that, as in animals, IP₃ stimulates calcium efflux both *in vitro* and *in vivo*, while DG plays an important role as a second messenger in cell signal transduction by activating protein kinase C (Harwood, 1989; Thompson, 1994). Recent studies also indicate that an important alternative route for the agonist-induced production of DG is through the breakdown of PC (Gomez-Muñoz *et al.*, 1992; Jamal *et al.*, 1992; Fleming and Yeaman, 1993; Perry *et al.*, 1993). The generation of DG can occur either directly as a result of phospholipase C activity, or by the sequential action of phospholipase D followed by PAP.

The mechanism involving the phospholipase D degradation of PC, coupled to the hydrolysis of PA by PAP, is quantitatively the most important route for signal transducing DG liberation (Exton, 1990), and has been reported in rat liver (Gomez-Muñoz *et al.*, 1992; Siddiqui and Exton, 1992), rat fibroblasts (Martin *et al.*, 1993), Chinese hamster ovary cells (Perry *et al.*, 1993), NG108-15 cells (Lavie *et al.*, 1990), porcine thymus (Kanoh *et al.*, 1992), human neutrophils (Billah *et al.*, 1989; Perry *et al.*, 1993), and interleukin-1-stimulated human mesangial cells (Bursten *et al.*, 1991). Additionally, there is evidence that PA itself is a potent second messenger involved in the modulation of protein kinase C as well as several other processes (Gomez-Muñoz *et al.*, 1992; Kanoh *et al.*, 1992). Therefore, it is most likely that PAP plays a role in destroying this signal while, at the same time, creating another through the formation of DG (Martin *et al.*, 1993; Perry *et al.*, 1993). The PAP activity involved in signal transduction, however, is clearly different from the microsomal enzyme involved mainly in glycerolipid metabolism (Gomez-Muñoz *et al.*, 1992; Jamal *et al.*, 1992; Martin *et al.*, 1993). The enzyme involved in cell signalling, which is often referred to as PAP-2 (Jamal *et al.*, 1992; Martin *et al.*, 1993), is tightly bound to the plasma membrane (Perry *et al.*, 1993), Mg^{2+} -independent (Jamal *et al.*, 1991), and insensitive to the thioreactive reagent N-ethylmaleimide (Kanoh *et al.*, 1992). On the other hand, the microsomal enzyme, referred to as PAP-1 (Gomez-Muñoz *et al.*, 1992; Jamal *et al.*, 1992), translocates from the cytosol to the ER to become functionally active (Gomez-Muñoz *et al.*, 1992), has an absolute Mg^{2+} requirement (Jamal *et al.*, 1992), and is selectively inactivated by N-ethylmaleimide (Martin *et al.*, 1993). Even though the role of PAP-1 in glycerolipid metabolism has been well established, it has been suggested that the translocation of this enzyme from the cytosol to the plasma membrane may also participate in signal transduction (Gomez-Muñoz *et al.*, 1992). In the literature dealing with plants, however, PAP-1 and PAP-2 have not yet been distinguished.

PAP-2, from porcine thymus membranes, has recently been purified to apparent homogeneity (Kano *et al.*, 1992). The enzyme, which was solubilized using n-octyl β -D-glucopyranoside and Triton X-100, was purified over 2000-fold with an 8% recovery. The native M_r of the enzyme was estimated to be about 218,000 by Superose 12 gel filtration chromatography in the presence of 1% (w/v) Triton X-100. Under both reducing and nonreducing conditions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the final enzyme preparation resulted in a single band with a M_r of 83,000, suggesting a possible oligomeric structure for the enzyme. This cell-signalling form of PAP was specific for PA, and was inhibited by the reaction product, DG. The enzyme did not hydrolyze LPA or any of the following water soluble compounds: *p*-nitrophenyl phosphate (*p*NPP), α -glycerophosphate, or dicyetyl phosphate. Additionally, when the pH of the assay mixture was varied from 5.5 to 9.0, the enzyme exhibited a broad activity peak for the hydrolysis of dioleoyl-PA, with maximal activity occurring at pH 7-7.4.

PAP-2 has also been highly purified from a crude membrane fraction of rat liver (Fleming and Yeaman, 1993). The rat liver enzyme was solubilized using 1% (w/v) n-octyl β -D-glucopyranoside, and then purified over 600-fold using a series of chromatographic steps employing equilibration and elution buffers containing 10% glycerol and 1% Triton X-100. The final preparation was obtained by eluting a heparin-Sepharose column with a 0.05-1 M NaCl gradient, which resulted in two major peaks of activity exhibiting vastly different stabilities at 4°C. Additionally, an N-ethylmaleimide-insensitive form of PAP was identified as a membrane-bound, heat-stable, and vanadate-sensitive impurity in partially purified rabbit kidney Na^+/K^+ -ATPase preparations (Swarts *et al.*, 1992).

Aside from the N-ethylmaleimide-insensitive form of the enzyme from porcine thymus, PAP has not been purified to homogeneity from any other animal source. PAP activity has, however, been extensively characterized using several different

tissues of mammalian origin (Brindley, 1988; Saggerson, 1988; Possmayer, 1988), including human adipose tissue (Belfiore *et al.*, 1983). The Mg^{2+} -dependent and N-ethylmaleimide-sensitive form of PAP, which is mainly involved in glycerolipid metabolism, has been particularly well studied in rat liver (Stewart and Briggs, 1981; Cascales *et al.*, 1984; Tijburg *et al.*, 1989; Asiedu *et al.*, 1992) and rat adipose tissue (Moller and Hough, 1982; Taylor and Saggerson, 1986; Wells *et al.*, 1986; Jamdar *et al.*, 1987) which are, as in most animals, key organs involved in TG biosynthesis and storage, respectively.

The level of Mg^{2+} -dependent PAP activity in rat liver appears to be under both long-term and short-term regulatory control (Tijburg *et al.*, 1989; Martin *et al.*, 1991). In long-term regulation, synthesis of the rat liver enzyme was stimulated by glucagon, glucocorticoids, cyclic AMP, and growth hormone (Butterwith *et al.*, 1984a; Pittner *et al.*, 1985; Martin *et al.*, 1987), and this action was antagonized by insulin (Pittner *et al.*, 1985). The level of this form of PAP also seemed to be somehow stabilized or maintained by glucagon, while insulin decreased the half-life of this activity (Brindley, 1988; Martin *et al.*, 1991). Additionally, Humble and Berglund (1991) have demonstrated that the level of PAP activity can be influenced by a number of different phospholipids, when they are included with PA in substrate emulsions presented to the cytosolic form of the rat liver enzyme, suggesting that changes in the lipid environment may possibly play a role in modulating PAP activity *in vivo*. In relation to animal nutrition, Stewart and Briggs (1981) showed that the activities of both the soluble and microsomal forms of Mg^{2+} -dependent PAP from rat liver were greater in essential-fatty-acid-deficient rats than in rats which were fed diets containing the nutritionally necessary fatty acid, linoleic acid.

In short-term regulation, the metabolic expression of PAP activity is thought to be regulated by the ability of the cytosolic form of the enzyme to translocate to the cellular compartment where its substrate is being generated, in other words, to the

membranes of the ER (Brindley, 1984; Butterwith *et al.*, 1985; Hopewell *et al.*, 1985). In rat liver, this intracellular movement seemed to be controlled mainly by the accumulation of fatty acids (Cascales *et al.*, 1984) and acyl-CoA esters (Martin-Sanz *et al.*, 1984) on the ER membranes. According to Hopewell *et al.* (1985) and others (Brindley 1984, 1988; Tijburg *et al.*, 1989), the binding of long-chain fatty acids and their CoA esters to the ER, as well as the accumulation of PA in this compartment, acts as a signal for more PAP to associate with the membranes and thereby enhances the synthesis of glycerolipids, especially TG. It has been suggested that the translocation of PAP probably depends on the increased negative charge conferred to the ER membranes by these metabolites (Hopewell *et al.*, 1985), and that these compounds act as feedforward regulators which activate PAP activity in order to match the rate of TG synthesis to the supply of fatty acids (Brindley, 1984; Martin *et al.*, 1987). Spermine also facilitated the association of PAP with the membranes of the ER (Martin-Sanz *et al.*, 1985), and its effect was synergistic with fatty acids in this respect (Simpson *et al.*, 1991). It appears that the mode of action for spermine may be in that it promotes the association of hydrophobic proteins with lipids and membranes (Hopewell *et al.*, 1985; Jamdar *et al.*, 1987), possibly by forming complexes with acyl-CoA *in vivo* (Martin-Sanz *et al.*, 1985) and also by causing the aggregation of microsomal membranes *in vitro* (Hopewell *et al.*, 1985). Vasopressin caused an increase in enzyme activity, and TG biosynthesis, within 5 min (Cascales *et al.*, 1984; Brindley, 1988). ADP, GDP, and CDP also activated the enzyme, while ATP inhibited rat liver PAP activity (Berglund *et al.*, 1989). Short-term treatment with the amphipathic cationic drug, chlorpromazine, decreased the rate of TG and PC biosynthesis in isolated rat hepatocytes, and decreased the rate of DG formation in a cell-free system of rat liver, by displacing PAP from the microsomal membranes and also by preventing the soluble form of PAP from interacting with these membranes (Hopewell *et al.*, 1985; Martin *et al.*, 1986). Hopewell *et al.* (1985) have indicated that chlorpromazine could oppose

the intracellular translocation of PAP by donating a positive charge to the membranes. Additionally, there is some evidence indicating that the regulation of PAP activity may be coordinated with that of CTP-phosphocholine cytidyltransferase (EC 2.7.7.15), which is another translocatable enzyme involved in the glycerolipid metabolism of rat hepatocytes (Yao *et al.*, 1990; Houweling *et al.*, 1994).

Freeman and Mangiapane (1989) have demonstrated that cytosolic rat liver PAP also translocates to the mitochondria. This movement of PAP to the mitochondria, which occurs in the presence of oleate or palmitate, happens in a feedforward manner similar to the short-term regulatory mechanism involved in the translocation of PAP to the ER. The physiological significance of this was confirmed by Mangiapane (1991), who showed that mitochondrial PA can be converted to TG in rat hepatocytes.

Although there is evidence indicating that the Mg^{2+} -dependent PAP activity from rat liver is affected by the presence of glucagon and cyclic AMP analogues (Butterwith *et al.*, 1984b; Pittner *et al.*, 1985; Tijburg *et al.*, 1989), it has not yet been established whether the activity of this enzyme is controlled by reversible cyclic AMP-dependent protein phosphorylation. Direct evidence for the existence of a phosphorylated protein has not yet been provided because of the extreme difficulty in purifying the enzyme, and in preparing antibodies against the Mg^{2+} -dependent form of PAP (Gomez-Muñoz *et al.*, 1992). Additionally, Butterwith *et al.* (1984b) were not able to confirm the observations of Berglund *et al.* (1982) that the catalytic subunit of cyclic AMP-dependent protein kinase decreased the *in vitro* enzyme activity of rat liver PAP. The stimulation of PAP activity by the apparent dephosphorylation of the enzyme, which was also demonstrated by Berglund *et al.* (1982), is only another tenuous line of evidence for this type of regulatory control. In fact, it appears that the alkaline phosphatase employed by Berglund *et al.* (1982), to dephosphorylate PAP, actually contributed to the hydrolysis of PA, giving the appearance that dephosphorylation of PAP results in enzyme activation. On the other hand, Butterwith

et al. (1984b) carefully repeated the experiments, using the appropriate controls, and showed that incubating the particle-free supernatant of rat liver with alkaline phosphatase actually decreased the activity of PAP by 21-29%.

Further progress towards elucidating the mechanisms that control the PAP activity involved in glycerolipid metabolism, including its distribution in different cellular compartments, depends on the ability to purify and further characterize the Mg^{2+} -dependent enzyme. Butterwith *et al.* (1984a) have partially purified Mg^{2+} -dependent PAP from the cytosolic fraction of rat liver by using the nonionic detergent, Tween 20, as a stabilizer. The enzyme was purified about 400-fold and, based on the inhibitor studies that were conducted, it contained functional thiol groups and arginine residues. Bio-Gel A-5m gel filtration chromatography of both the cytosolic and solubilized microsomal fractions, carried out in the presence of 0.075% Tween 20, resulted in major chromatographic peaks which indicated an apparent M_r of approximately 500,000 for both forms of Mg^{2+} -dependent PAP from rat liver (Ide and Nakazawa, 1985). The majority of the microsomal enzyme activity, which was solubilized either by sonication or with 2% Tween 20, eluted with the void volume in the absence of detergent. Similarly, about half the cytosolic activity eluted in the void volume when detergent was lacking in the elution buffer, indicating a potential relationship between the cytosolic and microsomal activities. Additionally, Lamb *et al.* (1987) have addressed the observation that phospholipase C from rat liver can also catalyze the hydrolysis of PA into DG and P_i . These researchers have shown that, even though both enzymes have the common ability to hydrolyze PA, rat liver PAP activity and phospholipase C PA-hydrolase activity are clearly distinct. The two activities can be distinguished from each other based on the effects of a number of different activators and inhibitors, and by the differential effects of certain divalent cations, such as Mg^{2+} and Ca^{2+} . To further aid in the characterization of PAP, Martin *et al.* (1987) have described a rapid assay method which can be used, not only

to measure the activities of the cytosolic and microsomal forms of the rat liver enzyme, but also to determine their Mg^{2+} and Ca^{2+} requirements.

The Mg^{2+} -dependent form of PAP from rat adipose tissue has many characteristics in common with the enzyme from rat liver (Saggerson, 1988). The adipose enzyme, like the enzyme from liver, exists in both particulate and soluble form, with the particulate enzyme being associated primarily with the microsomal membranes where, again, it plays a pivotal role in TG biosynthesis (Jamdar and Fallon, 1973b; Wells *et al.*, 1986). As well, the adipose enzyme was inhibited by N-ethylmaleimide, indicating that sulfhydryl groups were necessary for the activity of this enzyme (Wells *et al.*, 1986). Like hepatic PAP, the activity and subcellular distribution of the adipose enzyme appeared to be regulated by a number of different physiologically relevant compounds. For example, the polyamines, spermine, spermidine, and putrescine, all activated the Mg^{2+} -dependent PAP in both the microsomal and cytosolic fractions of rat adipocytes (Jamdar *et al.*, 1987; Saggerson, 1988). It was also shown that spermine interacted directly with the soluble enzyme and translocated this activity to the microsomes (Moller and Hough, 1982; Jamdar *et al.*, 1987). Additionally, Taylor and Saggerson (1986) have demonstrated that the relative proportion of PAP activity associated with the microsomal fraction can be increased by incubating rat adipocytes with palmitic acid, or the hormone, noradrenaline. They found the effect of noradrenaline to be extremely rapid, with maximum change relative to the control occurring within 10 min. These researchers also found that insulin decreased the proportion of particulate PAP and abolished the effect of noradrenaline, but it did not reverse the effect of palmitate. Unlike the hepatic enzyme, the Mg^{2+} -dependent PAP activity of rat adipose tissue was not affected by the addition of ATP or ADP, suggesting that the enzymes from adipose tissue and liver may be regulated by different mechanisms, and may thus represent two different isoenzymes (Al-Shurbaji and Berglund, 1989). Using rat adipocytes, Moller and Hough (1982)

have reported that isosmotic replacement of sucrose by KCl in the homogenizing buffer resulted in an increase in microsomal PAP activity at the expense of cytosolic activity. Studies by Jamdar and Fallon (1973a) suggested that the effect of Mg^{2+} and several other divalent cations on PAP activity may be important in the regulation of neutral lipid formation in rat adipose tissue. Also, the subcellular distribution of Mg^{2+} -dependent PAP was found to be similar when brown and white adipocytes from normal animals were compared but, with respect to PAP activity, the two cell types responded very differently to diabetes and hypothyroidism (Taylor and Saggerson, 1986; Baht and Saggerson, 1988).

In animals, there is abundant evidence indicating that the PAP reaction associated with the ER is generally the rate-limiting step in TG biosynthesis (Brindley, 1984, 1988). Also, it is becoming increasingly more apparent that the enzyme from mammals is regulated by a mechanism involving the translocation of Mg^{2+} -dependent PAP between the cytosol and the membranes of the ER (Brindley, 1988; Possmayer, 1988; Saggerson, 1988). In this way, the functionally inactive cytosolic form of PAP becomes activated by associating with the cellular compartment involved in the formation of its substrate. Besides the extensive studies on Mg^{2+} -dependent PAP from rat liver and adipose tissue, the animal enzyme has also been examined in a number of tissues from many other species. For example, the enzyme has been studied in rod outer segments (Pasquaré and Giusto, 1986) and other subcellular fractions of bovine retina (Pasquaré and Giusto, 1993), as well as in pig brain (Hajra and Agranoff, 1969). PAP may play an important role in myocardial TG homeostasis, and the enzyme from rat heart shares many characteristics with the enzymes from rat liver and adipose tissue, including its stimulation by Mg^{2+} and oleate, and its regulation by subcellular translocation to the microsomal membranes in response to oleate, KCl, and glucagon (Schoonderwoerd *et al.*, 1990). Ng and Tam (1986) showed that acid phosphatase, alkaline phosphatase, and PAP are distinct enzymes in developing mouse

brain, and that they are differentially regulated during fetal development. Many of the characteristics associated with the enzymes from animals seem to be highly conserved across species boundaries. In an extreme case, for example, Mg^{2+} -sensitive PAP was observed in the microsomal and cytosolic fractions obtained from the fourth instar larvae of the insect, *Culex pipiens fatigans* (Palakodery *et al.*, 1986). The activities associated with both fractions were sensitive to a number of sulfhydryl reagents, inhibited by Ca^{2+} , and were not inhibited by 0.1% (w/v) Tween 20.

TG is present in the yeast, *Saccharomyces cerevisiae*, and PAP plays an important role in the biosynthesis of both TG and phospholipids in this unicellular, eukaryotic organism (Hosaka and Yamashita, 1984b; Carman and Henry, 1989). In *S. cerevisiae*, the regulatory mechanisms and basic biosynthetic pathways involved in the formation of TG and other glycerolipids are, with only a few exceptions, very similar to those which occur in higher eukaryotes (Hosaka and Yamashita, 1984b; Harwood and Price-Jones, 1988; Carman and Henry, 1989). Additionally, as in the many other organisms which have been studied, several forms of Mg^{2+} -dependent PAP have been identified in this organism (Hosaka and Yamashita, 1984a; Carman and Lin, 1991; Carman and Quinlan, 1992). Hosaka and Yamashita (1984a) first described the enzyme in *S. cerevisiae* when they showed that PAP activity was associated with both membrane and cytosolic fractions. At the same time, they partially purified and characterized the soluble form of PAP from this microorganism. The soluble enzyme was purified 600-fold using $(NH_4)_2SO_4$ and polyethylene glycol (PEG) 6000 fractionation, followed by column chromatography on DEAE-Sepharose, Sephadex G-100, and blue-Sepharose. The cytosolic form of PAP from *S. cerevisiae* showed a strict specificity for PA, had an absolute Mg^{2+} requirement, and exhibited optimal activity between pH 7.0 and 8.0. Additionally, the M_r of the enzyme was estimated to be about 75,000 by analytical gel filtration on a column of Sephadex G-100.

More recently, both a microsomal PAP and a form of the enzyme present in both microsomes and mitochondria have been identified in *S. cerevisiae* (Lin, 1991; Morlock *et al.*, 1991). A 91-kDa form of the enzyme was purified to homogeneity, based on analysis by SDS-PAGE, from the total membrane fraction of this yeast (Lin and Carman, 1989; Carman and Lin, 1991). The enzyme was solubilized using 1% sodium cholate in the presence of 20% glycerol, and the purification procedure included column chromatography with DEAE-cellulose, Affi-Gel blue, hydroxyapatite, Mono Q, and Superose 12. Immunoblot analysis, using antibodies raised against this membrane-associated 91-kDa enzyme, showed that it was actually the proteolytic product of a 104-kDa form of microsomal PAP (Lin, 1991; Morlock *et al.*, 1991). Subsequently, using the same procedure employed to purify the 91-kDa protein, the 104-kDa form of the enzyme was purified from microsomes obtained from a strain of protease-deficient cells. The enzymological properties of the 104-kDa PAP were indistinguishable from the 91-kDa enzyme. Immunoblot analysis using antibodies specific for the 91-kDa enzyme also revealed the existence of a 45-kDa form of PAP, which was then purified to homogeneity from the mitochondrial fraction of *S. cerevisiae* (Morlock *et al.*, 1991; Carman and Quinlan, 1992). Again, this was done by employing the same procedure initially used to purify the 91-kDa protein from the total membrane fraction. The 45-kDa form of the enzyme was shown to be associated with both the microsomal and mitochondrial fractions.

The strictly microsomal, 91-kDa enzyme was monomeric, Mg^{2+} -dependent, stimulated by Triton X-100, and exhibited optimal activity at pH 7 (Lin and Carman, 1989). Also, this 91-kDa PAP was sensitive to Hg^{2+} ions and other thioreactive compounds, such as N-ethylmaleimide and *p*-chloromercuriphenylsulfonic acid (Carman and Lin, 1991). Using a preparation of pure enzyme, the apparent K_m for dipalmitoyl-PA and V_{max} were determined to be 50 μM and 30 $\mu mol/min/mg$, respectively (Lin and Carman, 1989). The molecular activity or turnover number for

the enzyme was $2.7 \times 10^3 \text{ min}^{-1}$ at pH 7 and 30°C. The activation energy for this form of PAP was found to be 11.9 kcal/mol, and the enzyme appeared to be labile above 30°C (Lin and Carman, 1989). Additionally, detailed kinetic analysis of purified 91-kDa PAP, using Triton X-100/PA mixed micelles, showed that enzyme activity was dependent on the bulk and surface concentrations of PA (Lin and Carman, 1990). This is consistent with a "surface dilution" kinetic scheme where PAP binds to the mixed micelle surface before substrate binding and catalysis occur. Based on the kinetic constants, V_{\max} and xK_m^B (interfacial Michaelis constant), dicaproyl-PA was a better substrate than either dioleoyl-PA or dipalmitoyl-PA which, in relation to each other, were equally good substrates. Interestingly, this 91-kDa form of PAP from *S. cerevisiae* was inhibited by the phospholipid intermediate, CDP-DG, and by the neutral lipids, DG and TG (Lin and Carman, 1989).

The 45-kDa form of PAP exhibited optimal activity at pH 6-7 and, like the 91-kDa enzyme, was also Mg^{2+} -dependent and stimulated by Triton X-100 (Morlock *et al.*, 1991; Carman and Quinlan, 1992). Although the 45-kDa enzyme was associated with both the microsomal and mitochondrial fractions, the kinetic properties of this form of PAP were similar to those of the strictly microsomal enzyme when uniform mixed micelles containing Triton X-100 and PA were used. The 45-kDa and 91-kDa forms of the enzyme were also similar to each other with respect to their inhibition by various compounds and their temperature stability. On the other hand, the 45-kDa and 91-kDa forms of PAP differed in their isoelectric points, and in the peptide fragments generated as a result of V8 proteolysis and cyanogen bromide cleavage.

A number of possible regulatory mechanisms for controlling lipid metabolism at the level of PAP have been investigated in *S. cerevisiae*. Wu *et al.* (1993) suggested that PAP activity may be regulated *in vivo* by certain sphingoid bases. They were able to demonstrate that the dependence of PAP activity on PA was cooperative in the

absence and presence of sphingosine, phytosphingosine, and sphinganine, and that these compounds inhibited purified preparations of both the 104-kDa and 45-kDa forms of PAP in a dose-dependent manner. They also found that these sphingoid bases were parabolic competitive inhibitors of PAP activity, indicating that more than one inhibitor molecule contributed to the exclusion of PA from the enzyme, and that both phytosphingosine and sphinganine were inhibitory at near endogenous concentrations.

Quinlan *et al.* (1992) have provided evidence indicating that the 45-kDa enzyme from *S. cerevisiae* is probably regulated by a mechanism involving reversible cyclic AMP-dependent protein phosphorylation. For example, cyclic AMP-dependent protein kinase catalyzed the phosphorylation of pure 45-kDa PAP at a serine residue, which resulted in a 2.4-fold stimulation of PA-hydrolase activity. Further, dephosphorylation of the pure 45-kDa enzyme was catalyzed by alkaline phosphatase, and resulted in a 1.3-fold inhibition of PAP activity. Purified 104-kDa PAP, however, was not phosphorylated by cyclic AMP-dependent protein kinase, strongly suggesting that the two yeast enzymes are differentially regulated. These findings were corroborated by *in vivo* phosphorylation studies using *S. cerevisiae* mutants (*bcy 1* and *bcy 2*) defective in cyclic AMP-dependent protein kinase activity.

At the genetic level, certain factors regulate the expression of PAP in *S. cerevisiae*. These regulatory factors include the availability of inositol in the growth medium and the growth phase of the cells (Morlock *et al.*, 1988, 1991). The expression of CDP-DG synthetase activity is also highly regulated in response to these two factors (Carman and Henry, 1989). So far, it is not clear what the relative contributions of the 45-kDa and 104-kDa forms of PAP are to the overall TG and phospholipid synthesis rates in *S. cerevisiae*, but it has been found that PAP activity increases when wild-type cells are supplemented with inositol (Morlock *et al.*, 1988) and when cells enter the stationary phase of growth (Hosaka and Yamashita, 1984b; Morlock *et al.*, 1988). Although the 104-kDa form of PAP was not affected, the

45-kDa form of PAP was induced in the presence of inositol (Morlock *et al.*, 1991), while the activity of CDP-DG synthetase was repressed (Carman and Henry, 1989). A similar effect was observed when yeast cells entered the stationary phase of growth, where both the 104-kDa and the 45-kDa PAP were induced (Morlock *et al.*, 1991), and CDP-DG synthetase activity was, once again, repressed (Carman and Henry, 1989). Additionally, the increase in PAP activity in response to inositol supplementation correlated with an increase in phospholipid content at the expense of TG (Morlock *et al.*, 1988). On the other hand, the increase in PAP activity in the stationary phase of growth correlated with an increase in TG content at the expense of phospholipid (Hosaka and Yamashita, 1984b). Thus, in *S. cerevisiae*, the flux of acyl chains through DG and CDP-DG appears to be controlled by the relative activities of both forms of PAP, and CDP-DG synthetase.

In light of the above studies in yeast and animals, further investigation of plant PAP activities would most likely illuminate the situation in plant cells, and aid in the elucidation of factors which control the rate of synthesis and the quality of the TG formed in oilseeds. Although they have not been nearly as well studied as the comparable enzymes in either animal tissues or yeast, PAP activities have been detected and partially characterized in the plastids, microsomes, and soluble fractions of a number of plant tissues (Moore, 1982; Block *et al.*, 1983; Alban *et al.*, 1989; Ichihara *et al.*, 1989; Ichihara, 1991). It seems probable, by analogy with *S. cerevisiae* and mammals, that there is also a mitochondrial form of PAP in plants. In fact, all four of the particulate fractions obtained from the developing seeds of safflower showed high specific PA-hydrolase activities when assayed at pH 6.75 and, next to the microsomal fraction, the highest specific activity was associated with the membrane fraction sedimenting from 3,000-20,000 x g (Ichihara *et al.*, 1989). It has been postulated that the animal enzyme is activated by a mechanism involving the translocation of a functionally inactive form of PAP from the cytoplasm to the microsomal membranes

(Brindley, 1984, 1988; Walton and Possmayer, 1986; Saggerson, 1988), and there is some evidence indicating that a similar mechanism may operate in plants (Ichihara *et al.*, 1990). This may explain some of the conflicting reports resulting from studies using plant tissues, with some investigators believing PAP to be a membrane-associated enzyme and some claiming that it is soluble (Moore, 1982; Harwood and Price-Jones, 1988; Harwood, 1989). In developing oilseeds (Harwood, 1989; Stobart and Stymne, 1990), the DG produced by PAP is the direct precursor of TG, while in chloroplasts (Joyard and Douce, 1977, 1987; Joyard *et al.*, 1994) as well as in non-green plastids (Alban *et al.*, 1989; Joyard *et al.*, 1994), DG is a source of acyl chains for galactolipids and sulfolipid. In plant microsomes, PAP also provides DG for the phospholipids, PE and PC (Browse and Somerville, 1991).

The first demonstration of PAP activity in plant subcellular fractions was by Kates (1955). His assay for the enzyme was indirect, however, and further studies using exogenous PA and short incubation periods were necessary. That is, although Kates showed that the 1,800-15,000 x g sediment from spinach leaf homogenate could catalyze the hydrolysis of PA to form P_i , he generally employed PC as the substrate and followed the liberation of both choline and P_i , which was catalyzed by the crude plastidular preparations obtained from a number of plant sources. The formation of P_i was slower than that of choline and was preceded by a lag period, suggesting that PC was converted to PA and subsequently hydrolyzed to DG and P_i . In the same study, Kates showed that the P_i could have arisen from either, or both, of the PC degradation products, PA or G3P, but not from LP.

More recently, plastidular PAP has been studied in the non-green starch-containing plastids from sycamore and cauliflower (Alban *et al.*, 1989) as well as in the chloroplasts of spinach (Joyard and Douce, 1979; Block *et al.*, 1983; Malherbe *et al.*, 1992) and pea (Andrews *et al.*, 1985). As discussed earlier, it is now known that the PA synthesized in plastids is further metabolized into either DG or

CDP-DG, and it is most likely that here lies one of the major regulatory points for controlling the biosynthesis of plastidular glycerolipids and the final proportions of PG, MGDG, DGDG, and SQDG (Joyard *et al.*, 1993, 1994). The formation of DG occurs in the plastids as the result of a membrane-bound PAP (Joyard and Douce, 1977, 1979) which is exclusively located on the inner envelope membrane (Block *et al.*, 1983; Andrews *et al.*, 1985). In contrast to plastids from 16:3 plants, those from 18:3 plants have a rather low PAP activity and cannot deliver DG fast enough to sustain the full rate of glycolipid synthesis (Joyard *et al.*, 1993). This is true for the chloroplasts as well as the non-green plastids from 18:3 plants (Alban *et al.*, 1989). These results explain why 18:3 plants contain only small amounts of galactolipids and SQDG with 16-carbon fatty acids at the *sn*-2 position, but contain PG, which is synthesized from PA via CDP-DG, with such a structure. It is not known, however, whether the reduced level of plastidular PAP activity in 18:3 plants is due to a lower species-specific expression of the gene coding for the enzyme, or to the presence of regulatory molecules which control the activity of PAP in this compartment (Joyard *et al.*, 1993).

According to Joyard and Douce (1979, 1987), the PAP activity associated with plant plastids exhibits a number of biochemical properties which are clearly different from those described for the PAP activities involved in glycerolipid metabolism in the various cell fractions obtained from animals and yeast. For example, the plastidular enzyme is tightly membrane-bound and localized only on the inner envelope membrane, whereas in yeast or animal cells PAP activity is recovered as both cytosolic and microsomal. Although, there is a separate PAP associated with the mitochondria in *S. cerevisiae*. Excluding the cell-signalling form of PAP associated with the plasma membrane, all the PAP activities from yeast (Lin and Carman, 1989; Lin, 1991) and animals (Butterwith *et al.*, 1984a; Taylor and Saggerson, 1986; Schoonderwoerd *et al.*, 1990; Martin *et al.*, 1991) were Mg^{2+} -dependent, regardless of the cell fraction

serving as the source of enzyme activity. Additionally, the PAP activities from yeast (Hosaka and Yamashita, 1984a; Carman and Lin, 1991; Carman and Quinlan, 1992) and animals (Jamdar and Fallon, 1973b; Saggerson, 1988) usually exhibited pH optima which were within one pH unit of neutrality. In marked contrast, the plastidular enzyme appeared not to need divalent cations, which, in fact, seemed to inhibit the enzyme, and plastidular PAP exhibited maximal activity at pH 9.0. This alkaline pH optimum makes physiological sense since Sauer and Heise (1983) have shown that light-activated fatty acid synthesis in spinach chloroplasts was dependent on stromal pH, and that the optimum pH for fatty acid synthesis was close to 8.5. Therefore, it appears that plastidular glycerolipid metabolism may be linked to the biosynthesis of fatty acids, at least, partly, through the effects of pH in this subcellular compartment. Furthermore, the extraplastidular PAP activities involved in plant glycerolipid metabolism, which have been described in subcellular fractions from a number of different tissues, are also very different from the PAP activity associated with the inner membrane of the plastid envelope, and strongly resemble their counterparts from animals and yeast (Harwood and Price-Jones, 1988).

Feedback inhibition by DG probably plays a pivotal role in the regulation of plastidular PAP and, as a consequence, may play a major role in controlling the rate of galactolipid and sulfolipid biosynthesis (Joyard *et al.*, 1994). Using isolated intact chloroplasts and envelope membranes from thermolysin-treated chloroplasts, Malherbe *et al.* (1992) have provided strong evidence that DG is a powerful competitive inhibitor of the plastidular enzyme in spinach. Using *sn*-1,2-diolein, they showed that the apparent K_i value was 70 μM , whereas the apparent K_m for *sn*-1,2-dioleoyl-PA was as high as 600 μM . Additionally, when the incubation was done in the presence of different molecular species of *sn*-1,2-DG, at the same concentration as *sn*-1,2-diolein, PAP inhibition was in the same range. In contrast, *sn*-1,3-dipalmitin had almost no inhibitory effect. Furthermore, it appears that the *in vivo* steady state activity of

plastidular PAP is sensitive to the *sn*-1,2-DG/PA molar ratio, since increasing this ratio resulted in a logarithmic increase in PAP inhibition. Also, because the apparent K_i value for DG was much lower than the apparent K_m for PA, it has been suggested that the rate of DG formation is closely related to the rate of its utilization by the plastidular enzymes involved in galactolipid and sulfolipid synthesis (Joyard *et al.*, 1994). Thus, feedback inhibition of PAP by DG would most likely lead to an accumulation of PA and would favor PG synthesis in the plastid envelope (Malherbe *et al.*, 1992). There are only a few reports of PAP inhibition by DG in other eukaryotes. For instance, the soluble PAP from rat adipocytes (Saggerson, 1988) and the 91-kDa, membrane-associated enzyme from *S. cerevisiae* (Lin and Carman, 1989; Lin, 1991) were shown to be inhibited by DG. In these cases, it was understood to be a regulatory mechanism involving feedback inhibition of TG biosynthesis.

As mentioned earlier, extraplastidular PAP activities have been described in a number of plant tissues. A few of these investigations involve tissue obtained from non-oilseed sources. The cotyledons of fully imbibed or germinating mung beans (*Vigna radiata*), which contain at least two distinct PAP activities (Herman and Chrispeels, 1980), are one example. In this case, an enzyme exhibiting a pH optimum of 7.5 was located on the ER, while a second PAP with a pH optimum of 5.0 was found associated with the protein bodies. The PAP activity exhibiting the acidic pH optimum was very easily released into the soluble fraction during subcellular fractionation, and could be partially separated from the peak of general acid phosphatase activity by DEAE-cellulose and Sephadex G-200 column chromatography. The identification of two separate phosphatase activities with acidic pH optima, one representing PAP, and the other, a presumably nonspecific acid phosphatase, was facilitated by the observation that the partially purified PAP did not readily hydrolyze *p*NPP, and that partially purified nonspecific acid phosphatase showed little activity towards PA. The apparent molecular weight of the acidic PAP was estimated to be

about 37,000 by gel filtration chromatography on Sephadex G-200, and the enzyme was inhibited 57% and 43% by 10 mM KF and 10 mM sodium phosphate, respectively.

In another case, Königs and Heinz (1974) investigated the subcellular distribution of PAP in broadbean (*Vicia faba*) leaves and partially characterized the enzyme. When the crude subcellular fractions obtained by the differential centrifugation of tissue homogenate were assayed at pH 5.6, PAP activity was almost exclusively associated with the cytosolic (40,000 x g supernatant) fraction and the 600-15,000 x g pellet. Oddly, very little PA-hydrolase activity was associated with the plastidular (200-600 x g sediment) or microsomal (15,000-40,000 x g sediment) fractions. Although the 600-15,000 x g sediment and 40,000 x g supernatant exhibited similar specific activities, about 80% of the total activity of the homogenate was found in the cytosolic fraction. The cytosolic and particulate enzymes exhibited pH optima of 5.1 and 5.6, respectively, and both forms of PAP activity were inhibited by Ca^{2+} ions. The investigators were unable to determine whether the PAP activity associated with the 600-15,000 x g pellet was located in the mitochondria or the microbodies because no phosphatase activity could be recovered once these organelles were separated by sucrose density centrifugation. The lack of phosphatase activity after subjecting the 600-15,000 x g sediment to sucrose density fractionation was attributable to the inhibitory effects of high sucrose concentration on the membrane-bound enzyme. Finally, the particulate enzyme could not be solubilized by sonication alone, but it was solubilized by sonicating the 600-15,000 x g sediment in the presence 0.2% Triton X-100.

Besides its important role in the biosynthesis of glycerolipids, which includes the formation of TG, phospholipids, galactolipids, and sulfolipid, PAP is also involved the degradation of phospholipids and in general phospholipid turnover. For example, Paliyath and Thompson (1987) have shown that, in the cotyledons of germinating seeds

from the common bean (*Phaseolus vulgaris*), microsomal PAP activity is involved in the catalysis of membrane phospholipids. This activity was stimulated by Ca^{2+} and was calmodulin-dependent. A similar mechanism also seems to be operative in the microsomal membranes from senescing carnation flowers (Paliyath *et al.*, 1987; Brown *et al.*, 1990). This may be significant because flower senescence and the development and filling of seeds occur simultaneously in many plants, especially in annuals such as *Brassica napus*. Perhaps TG deposition in oilseeds and the degradation of phospholipids in senescing tissues and organs are somehow related by some common hormonally controlled mechanism. Additionally, the decrease in microsomal PAP activity, relative to the other enzymes involved in phospholipid degradation, has been implicated in chilling injury in tomato fruit (Todd *et al.*, 1992). That is, it appears that the chilling injury occurring during low temperature storage of tomato fruit may be the result of the accumulation of PA and other lipid degradation intermediates in the membranes, and that the differential effects of reduced temperature on the activity of PAP and other lipid-degrading enzymes results in this detrimental accumulation of phospholipid catabolites.

PAP Activity in Oilseeds

The oleaceous seeds (Stymne and Stobart, 1987; Stobart and Stymne, 1990) and fruits (Gurr, 1980; Sanchez *et al.*, 1992) of many plant species are significant sources of economically important oils. In the temperate regions, the oils derived from seed storage lipids are by far the most important source of vegetable oils (Gurr, 1980; Downey, 1983). Seed oil is synthesized via the Kennedy pathway, and PAP catalyzes the penultimate step in the formation of TG by removing P_i from PA to generate *sn*-1,2-DG (Stobart and Stymne, 1990). In developing oilseeds, the DG formed by the PAP-catalyzed hydrolysis of PA is not only a precursor of TG but is also a substrate used for the synthesis of membranes (Harwood and Price-Jones, 1988). Furthermore,

it should be noted that although PA is technically the simplest phospholipid, it is not a characteristic membrane lipid. In fact, the isolation of PA from plants was shown to be largely an artifact formed by enzymatic degradation during lipid extraction (Kates, 1970). Even the PA produced in non-oil-storing plant tissues by the action of LPAT in the Kornberg-Pricer pathway (Joyard and Douce, 1987), or by the phospholipase D degradation of PC during membrane catabolism (Brown *et al.*, 1990), is often coupled to the hydrolytic reaction catalyzed by PAP, so that PA does not accumulate.

Typically, seeds accumulate storage lipid in a discontinuous manner (Harwood, 1989). In the initial period after flowering there is little or no accumulation of oil, and analysis of seeds in this stage of development shows a preponderance of membrane lipids, including those associated with the plastids. In the second stage of development, there is a period of rapid oil accumulation during which most of the storage TG is produced. In the final developmental stage, seed dehydration takes place and there is almost no further synthesis of any form of lipid. Obviously, the study of PAP, and other enzymes involved in TG formation, require seeds in the second stage of development. In oilseed rape, and in certain other oilseeds, it has been found that the embryo has to attain a certain level of development before storage reserve deposition can begin, that is, cell division and organ formation must be complete (Norton and Harris, 1983).

With respect to oilseed species, PAP activity has been investigated in castor bean, groundnut (*Arachis hypogaea*), and safflower (Sukumar and Sastry, 1987; Harwood and Price-Jones, 1988). The first study specifically involving PAP activity in an oilseed was carried out by Moore *et al.* (1973), and employed subcellular fractions obtained from the endosperm of germinating seeds of castor bean. Initially, PAP activity was found to be located mainly in the microsomes of this tissue, with some activity in the soluble fraction. That is, when sucrose gradient fractions were assayed at pH 7.4, it appeared that the major site of PAP was in the ER. Later, when

subcellular fractions were assayed under optimal conditions by Moore and Sexton (1978), it was found that the cytosolic fraction contained most of the PA-hydrolase activity, and that there were two peaks of soluble enzyme activity in response to pH. One peak was at about pH 6.0 and the other at pH 4.25. In light of the report by Blank and Snyder (1970) that wheat germ contains a nonspecific acid phosphatase capable of dephosphorylating PA, and with the more recent discovery that oilseeds such as sunflower also contain acid phosphatases with broad substrate specificities (Park and Van Etten, 1986), care must always be taken to ensure that the PA-hydrolase activity being measured is not due to the activity of a nonspecific phosphatase. It is interesting that a preparation of wheat germ acid phosphatase was first described by Singer (1948) as wheat germ lipase. The ER-associated enzyme described by Moore *et al.* (1973) was shown not to hydrolyze glucose-6-phosphate or *o*-carboxyphenyl phosphate, suggesting that the PA-hydrolase activity in this fraction was not due to a nonspecific phosphatase, although, *p*NPP was not tested as a possible substrate. At any rate, the PA-hydrolase activity associated with the endosperm from germinating castor beans is most likely not involved in TG biosynthesis, but instead probably plays a role in membrane formation and other processes involved in germination and seedling growth. Therefore, in order to study the PAP activity involved in TG biosynthesis via the Kennedy pathway, it is much more appropriate to use the tissues obtained from the developing seeds of oilseed species.

Using the developing seeds from groundnut, Sukumar and Sastry (1987) showed that PAP was associated with both the microsomal and mitochondrial fractions, and that PAP activity depended upon the inclusion of $MgCl_2$ in the homogenizing buffer. Interestingly, no PA-hydrolase activity was found in the cytosolic fraction, and only a negligible amount (<1%) was associated with the fat fraction containing the oil bodies. The PAP activity associated with the post-nuclear supernatant exhibited a broad pH optimum between pH 6 and 7, and was inhibited 87% by 10 mM NaF. Both the

microsomal and mitochondrial PAP activities showed a significant increase in specific activity between 20 and 30 days after fertilization, with maximal specific activity occurring 30 days after fertilization. By 40 days after fertilization the enzyme activities had decreased to the levels observed at 20 days after fertilization. Furthermore, not only did the mitochondrial and microsomal enzymes show the same activity profiles, but the activity profiles of DGAT and both forms of PAP closely paralleled the lipid accumulation profiles, suggesting that there is a coordinate induction of the enzymes involved in TG biosynthesis during seed maturation. In addition to the study of PAP and its role in TG formation in the developing seeds of groundnut, the effect of herbicides on PAP and other enzymes involved in glycerolipid metabolism has been investigated in the leaves of this oilseed species. Rajasekharan and Sastry (1989, 1990) have shown that, although other enzyme activities involved in lipid metabolism were inhibited, both the plastidular and microsomal PAP activities from leaf disks of groundnut were insensitive to phenoxy, thiocarbamate, urea, and uracil herbicides.

The dephosphorylation of PA during TG formation by microsomal fractions obtained from the cotyledons of developing safflower seeds was demonstrated by Stymne *et al.* (1983). The reaction was demonstrated in each of three cultivars differing in seed oil fatty acid composition. These cultivars included a high oleate (75% oleate, 16% linoleate) variety, a very high linoleate (6% oleate, 88% linoleate) variety, and a cultivar known as Gila (16% oleate, 75% linoleate). At the same time, an examination of the ability of different molecular species of acyl-CoA to be incorporated into phospholipids and acylglycerols in Gila and high oleate varieties gave some information about the substrate specificities of microsomal PAP (Stymne *et al.*, 1983). The results from this study strongly suggested that microsomal PAP did not show a marked substrate specificity. Ichihara (1991) showed that the microsomal PAP in maturing seeds of safflower was specific and selective for unsaturated forms of PA, and when the different forms of PA were ranked, the order of selectivity was similar to

that of the specificity. Additionally, in agreement with Stymne *et al.* (1983), Ichihara (1991) concluded that the broad selectivity for unsaturated PA species (*sn*-1,2-diunsaturated-PA and *sn*-1-saturated, 2-unsaturated-PA) indicated that the PAP reaction probably has little effect on the fatty acid composition of TG in the oil obtained from safflower. Furthermore, compared with the safflower microsomal enzyme, the chloroplast PAP of spinach leaves showed a broader specificity (Ichihara, 1991), which agreed with the selectivity profile indicated by labelling patterns of PA and DG synthesized from ^{14}C -acetate previously demonstrated in spinach chloroplasts (Gardiner *et al.*, 1984).

Griffiths *et al.* (1985) showed that microsomal PAP from the developing seeds of safflower has an absolute Mg^{2+} requirement. This was confirmed by Ichihara *et al.* (1989), who went on to show that other divalent cations such as Ca^{2+} and Mn^{2+} could not be substituted for Mg^{2+} . Additionally, Ichihara *et al.* (1989) showed that the microsomal enzyme exhibited a pH optimum of 6.75 and was inhibited by the polyamines, spermidine and spermine, but not by putrescine, which is the diamine precursor of spermidine and spermine. It has been suggested that polyamines, which are ubiquitous in plant tissues, may affect the synthesis of TG in maturing oilseeds by modulating the enzyme activity of each step in the Kennedy pathway (Ichihara *et al.*, 1989). The soluble form of the enzyme from developing safflower seeds has not been characterized, but has been suggested to be a functionally inactive cytosolic reservoir of the microsomal form of the enzyme (Ichihara *et al.*, 1990). That is, as in the animal system (Brindley, 1984), it appears that in maturing safflower seeds PAP translocates between the cytosol and the ER membrane in response to the fatty acid level in the cytosol (Ichihara *et al.*, 1990).

Ichihara *et al.* (1990) have reported that the combined and relative activities of the microsomal and cytosolic forms of PAP varied with seed maturation. Combined PAP activity in the cell was highest during the period of rapid oil accumulation (13-15

days post-anthesis), when the majority of the PAP was membrane-bound. At the initial and last stages of seed development, when TG synthesis was at an insignificant level, the majority of the activity was soluble. Additionally, the potassium salts of palmitic acid, stearic acid, and oleic acid, which are the fatty acid products formed in the plastids, caused the translocation of PAP from the cytosol to the microsomes. Laurate and linoleate, which are not products of plastidular fatty acid biosynthesis, showed no effect. The intracellular translocation induced by oleate was reversed by its removal. Oddly, the metabolically significant thioester, oleoyl-CoA, did not convert the soluble form of the enzyme into the membrane-bound form. In the presence of 1 mM oleate, the cytosolic form of PAP from safflower seeds could be induced to translocate to the microsomes prepared from the seeds of sunflower, but not to the microsomes prepared from the seeds of oilseed rape or soybean. This may possibly be explained by the phylogenetic relationship of the oilseed species studied. Sunflower is in the same family as safflower, Asteraceae, whereas oilseed rape and soybean are members of the Brassicaceae and Fabaceae, respectively. It has been suggested that a species-specific mechanism may exist for binding the cytosolic PAP to the ER membrane (Ichihara *et al.*, 1990).

The reaction catalyzed by PAP is considered to be the rate-limiting step in TG biosynthesis in animals (Brindley, 1988; Saggerson, 1988), and both PAP and DGAT have been implicated in having a rate-limiting role in the formation of TG in oilseeds (Ichihara *et al.*, 1988, 1989, 1990; Perry and Harwood, 1993a, 1993b). On the one hand, it appears that PAP activity in microsomal preparations of most oilseed species tends to be limiting to some extent (Stymne and Stobart, 1987; Stobart and Stymne, 1990). For example, based on analysis by thin layer chromatography (TLC), PA was an obvious product of the polar lipid fraction when microsomes obtained from developing safflower cotyledons were incubated with the Kennedy pathway precursors, fatty acyl-CoA and G3P (Stobart and Stymne, 1985b). On the other hand, Ichihara *et*

al. (1989) have indicated that the amount of PAP activity in developing safflower seeds was enough to account for the amount of TG being formed *in vivo*, and that DGAT may catalyze the rate-limiting step in TG biosynthesis. These researchers did not, however, account for the fact that the DG formed by PAP is also used to synthesize the major phospholipids, PC and PE (Browse and Somerville, 1991), or that the PC formed in the microsomal membranes is a major source of DG moieties for plastidular galactolipids and SQDG in 18:3 plants (Joyard and Douce, 1987). In addition, it should be noted that microsomal PC also provides some of the DG skeletons for the glycerolipids formed in the mitochondria (Moore, 1982), and that oil bodies themselves consist of a half-unit membrane encompassing small droplets of TG (Frentzen, 1993). Therefore, in order to determine if PAP catalyzes the rate limiting step in plants one must take into account not only the formation of TG, but also the formation of the major phospholipids and their contribution to plastidular glycolipid formation, as well as their possible contribution to the formation of glycerolipids in the mitochondria. Although, using isotopic feeding studies, Perry and Harwood (1993b) have shown that radiolabelled DG is the only Kennedy pathway intermediate which accumulates during the period of rapid TG accumulation in the zygotic embryos of *Brassica napus* cv Shiralee, suggesting again that only DGAT is likely to exert significant flux control. It would seem odd, however, that if the reaction catalyzed by PAP was not the rate-limiting step in the Kennedy pathway, that a form of regulatory control involving intracellular translocation of the enzyme would be evident. That is, if the PAP-catalyzed reaction is not the "bottle-neck" in the formation of TG in oilseeds, then the question is raised: What is being regulated by the apparent translocation of the enzyme observed by Ichihara *et al.* (1990) if it is not the formation of DG? If Ichihara *et al.*'s observations are valid, then, as in the animal system (Brindley, 1984, 1988), PAP in plants may play both a rate-limiting and regulatory role in TG biosynthesis.

At this point, it is premature to draw general conclusions, and further work will be required to elucidate the mechanisms which control oil quality and rate of TG formation in oil-storing seeds. In no case has the cytosolic enzyme been adequately characterized to enable conclusions to be drawn about its physiological significance. Additionally, no form of PAP has been significantly purified from any plant source and, to date, there are no indications in the literature of the solubilization of the microsomal plant enzyme. Since the ER is considered to be the primary site of TG biosynthesis in developing seeds, a necessary step on the path to the eventual purification of the enzyme and its extensive characterization is the ability to solubilize microsomal PAP in an active form. This, in turn, would lead to a better understanding of plant lipid metabolism, and may eventually allow the manipulation of the biosynthetic rate and quality of TG formed in oilseeds through biotechnological approaches.

MATERIALS AND METHODS

Plant Material

Oilseed rape (*Brassica napus* L. cv Westar) was field grown at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta. *B. napus* L. cv Topas was grown in a temperature-controlled growth cabinet as described by Pomeroy *et al.* (1991). In both cases, flowers were tagged at anthesis and the developing siliques were collected 3-4 weeks post-anthesis. Maturing seeds were removed from the harvested siliques, flash frozen in liquid N₂, and stored at -20°C for enzymic analysis.

Microspores of oilseed rape (*B. napus* L. cvs Reston and Topas) were isolated and induced to form MD embryos (Pomeroy *et al.*, 1991), which were kindly supplied on a regular basis by Dr. M. Keith Pomeroy, Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa. MD embryos at the early to mid-cotyledonary stage of development were rinsed with deionized distilled water over a nylon sieve (60 µm), blotted with filter paper, and weighed. MD embryos were either used immediately or were flash frozen in liquid N₂ and stored at -20°C prior to tissue homogenization and subsequent fractionation.

An embryogenic MD cell-suspension culture of 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. The culture was maintained according to Orr *et al.* (1986). These cells were harvested by rinsing and storing as described for MD embryos.

Safflower (*Carthamus tinctorius* L. experimental line Lesaf 241L-138) was grown to maturity in a temperature-controlled growth chamber under the conditions described by Weselake *et al.* (1993). Developing seeds were harvested 14 days post-anthesis, flash frozen in liquid N₂, and stored at -20°C until enzyme extracts were prepared.

Chemicals

The L- α -PA derived from egg yolk lecithin, egg yolk L- α -PC (Type XI-E), soybean L- α -PC (Type III-S), BSA (bovine serum albumin), CTAB (cetyltrimethylammonium bromide), CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate), CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate), sodium DOC (deoxycholate), n-dodecyl β -D-maltoside, SDS (sodium dodecyl sulfate), MEGA-8 (octanoyl-N-methylglucamide), n-octyl β -D-glucopyranoside, Triton X-100, Tween 20, Tris, Hepes, L-ascorbic acid, DTT (dithiothreitol), sucrose, triolein, and oleic acid were from the Sigma Chemical Co. (St. Louis, Missouri). *sn*-1,2-Diolein (15% *sn*-1,3-diolein) was from NuChek Prep Inc. (Elysian, Minnesota). *p*-Nitrophenyl phosphate (*p*NPP) was from Boehringer Mannheim (Mannheim, Germany). HPLC-grade organic solvents were purchased from BDH Chemicals Inc. (Toronto, Ontario). Silica Gel 60 G aluminum-backed TLC plates were obtained from E. Merck (Darmstadt, Germany). A Superose 6 (HR 10/30) gel filtration column (1.0 cm x 30.0 cm) was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Dye reagent concentrate for protein assays was from Bio-Rad Laboratories (Richmond, California). Other chemicals were from Fisher Scientific.

Preparation and Fractionation of Tissue Homogenate

All procedures were conducted at 0-4°C unless indicated otherwise. Tissues obtained from oilseed rape were ground in homogenizing buffer consisting of 60 mM Tris/60 mM maleic acid-NaOH (pH 6.75) containing 2.5 mM MgCl₂, 0.12 mM EDTA, and 0.5 M sucrose. *B. napus* tissues were usually homogenized in 4 volumes of buffer per gram of plant material. Developing seeds and Jet Neuf cells were ground using a chilled mortar and pestle with a small amount of fine acid-washed sand (40-100 mesh, BDH Chemicals Inc.). MD embryos were homogenized in the same manner except that sand was often not included. Homogenates were filtered through 2 layers

of nylon mesh (60 μm) and then assayed immediately and processed further, or frozen in liquid N_2 and stored at -20°C .

Crude subcellular fractions were obtained by differential centrifugation of *B. napus* tissue homogenate. Homogenate was centrifuged at 1,500 x *g* (Beckman J2-21M induction drive centrifuge with JA-20 34° fixed angle rotor using 50 mL polycarbonate centrifuge tubes) for 15 min to obtain the first particulate fraction. The second particulate fraction was obtained by centrifuging the 1,500 x *g* supernatant for 20 min at 10,000 x *g* (Beckman J2-21M induction drive centrifuge with JA-20 34° fixed angle rotor using rubber adaptors and 16 mm x 76 mm Beckman Ultra-Clear centrifuge tubes). The microsomal fraction was prepared by centrifugation of the 10,000 x *g* supernatant for 2 h at 100,000 x *g* (Beckman Optima TLX ultracentrifuge with TLA-100.3 30° fixed angle rotor using 13 mm x 51 mm Beckman pollyallomer centrifuge tubes, or Beckman model L3-50 ultracentrifuge with type 65 23.5° fixed angle rotor and 16 mm x 76 mm Beckman Ultra-Clear tubes). Floating fat layers obtained by these centrifugation steps were collected by aspiration, combined, and washed 3 times by suspending the combined fat layer in a volume of homogenizing buffer equal to one-half of the tissue weight and centrifuging at 10,000 x *g* for 20 min. Usually, the microsomal fraction was washed by suspending the 100,000 x *g* pellet in a volume of homogenizing buffer equal to one-half the tissue weight, with the aid of a chilled glass Potter-Elvehjem homogenizer (7 mL, Pyrex), and recentrifuging at 100,000 x *g* for 2 h. Each of the particulate fractions, as well as the combined fat layer, were eventually suspended in a volume of homogenizing buffer equal to one-half the tissue weight. Again, the microsomal pellet was resuspended with the aid of a chilled glass homogenizer. The soluble fraction (100,000 x *g* supernatant) was usually dialyzed against Tris-maleic acid-NaOH assay buffer (pH 6.75, no sucrose) for 16 h to reduce the level of endogenous P_i . Fractions obtained by differential centrifugation were

either assayed immediately or were frozen as small aliquots using liquid N₂ and stored at -20°C.

Developing seeds of safflower were ground in 8 volumes of homogenizing buffer per gram of tissue, in the same manner as *B. napus* seeds, except the safflower grinding medium consisted of 0.2 M Hepes-NaOH (pH 7.4) containing 0.5 M sucrose and 1 mM DTT. Safflower homogenate was immediately centrifuged at 1,500 x g for 15 min. The 1,500 x g supernatant was filtered through fine glass wool, to remove the well-defined lipid layer, and then frozen with liquid N₂ and stored at -20°C. The 1,500 x g supernatant was thawed on ice prior to fractionation. The 10,000 x g pellet and the floating fat layers were discarded. The 10,000-100,000 x g pellet was not washed and the 100,000 x g supernatant was not dialyzed. The microsomal and soluble fractions obtained from safflower were frozen with liquid N₂ and stored at -20°C before being assayed for enzyme activity.

Assay for Phosphatidate Phosphatase Activity

Frozen extracts were thawed on ice. PAP activity was determined by following the formation of P_i using the colorimetric method of Chen *et al.* (1956). The method of substrate preparation, as well as the typical assay conditions, were essentially those outlined by Ichihara *et al.* (1989). Glassware was routinely washed in phosphate-free Extran 300 concentrate (E. Merck).

Stock substrate was prepared by adding an appropriate amount of PC, which was stored in chloroform, to a vial. Chloroform was evaporated under a stream of N₂ gas, and appropriate amounts of sodium PA and BSA were added. PA, PC, and BSA were dissolved and mixed in a small amount of diethyl ether. The ether was evaporated to dryness under a stream of N₂ gas and water was added to the residue. The mixture was sonicated for 1 min at 0°C using a sonicating probe (Sonic Dismembrator, Quigley-Rochester Inc.) set at 60% maximum intensity.

The assay reaction mixture generally consisted of 50 mM Tris/50 mM maleic acid-NaOH (pH 6.75), 2 mM MgCl₂, 0.1 mM EDTA, 1 mM sodium PA, 0.65 mM PC, and 1 mg BSA in a final volume of 1 mL. PC was from egg yolk unless otherwise indicated. Reactions were initiated by the addition of enzyme extract and, unless indicated otherwise, were allowed to proceed for 30 min at 30°C. The reactions were terminated with the addition of 1 mL 20% (w/v) trichloroacetic acid (TCA). Tubes were vortexed and then centrifuged for 10 min at 1,500 x g (IEC model K portable centrifuge). A 1.5 mL aliquot of the supernatant was mixed with 3 mL of color reagent C, which consisted of 2:1:1:1 (v/v/v/v) water/6 N H₂SO₄/2.5% (w/v) ammonium molybdate/10% (w/v) ascorbic acid. The mixture was incubated at 37°C for 1.75 h, allowed to cool to room temperature for 15 min, and then the absorbance at 820 nm was measured using a Pharmacia LKB Novaspec II spectrophotometer. The P_i concentration of the supernatant was calculated using KH₂PO₄ standards. Control incubations were carried out for zero time by adding TCA to the assay medium prior to the addition of enzyme extract. Controls were used to correct for the presence of endogenous P_i by subtracting their absorbance values from those of reactions which were not prematurely quenched. The specific activity of PAP was expressed as nmol P_i liberated from PA/min/mg protein, and in most cases data were reported as the means of triplicate assays.

Assay of *p*-Nitrophenyl Phosphate Hydrolysis Catalyzed by Phosphatase

Frozen extracts were thawed on ice prior to activity determination. Phosphatase activity was measured by colorimetrically monitoring the release of *p*-nitrophenol from the synthetic substrate analog *p*NPP (Bessey *et al.*, 1946; Andersch and Szczypinski, 1947). Assays were carried out based on the procedure described by Crandall (1983), and were conducted at pH values of 6.75 and 5.0. Unless indicated otherwise, the assay medium consisted of 50 mM Tris/50 mM maleic acid-NaOH containing 2 mM

MgCl₂, 0.1 mM EDTA, and 5 mM *p*NPP in a total volume of 5 mL. Reactions were initiated by the addition of enzyme extract and allowed to proceed at 30°C. After incubation, 2.5 mL of 0.5 N KOH was added to each tube to stop the reaction and facilitate color development. The resulting mixture was centrifuged for 5 min at 1,500 x *g* to remove the precipitate that formed. The supernatant was read against a blank at 405 nm. Control incubations were carried out for zero time. The quantity of *p*-nitrophenol produced was calculated from the molar extinction coefficient, $\epsilon = 1.88 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ (Biggs, 1954; Crandall, 1983; Bretauiere and Spillman, 1984). Data were reported as the means of duplicate or triplicate assays, and phosphatase specific activity was expressed as nmol *p*-nitrophenol formed/min/mg protein.

Microdetermination of Phosphorus in *Brassica napus* Seed Homogenate

Ground tissue prepared from developing seeds of *B. napus* L. cv Westar was ashed according to the micromethod described by Isaac (1990). In a small porcelain crucible, 1 mL of 95% (w/v) magnesium nitrate was added to 2 mL of homogenate. The control contained KH₂PO₄ (equivalent to 400 μ g phosphorus) in 2 mL of homogenizing buffer. Crucibles were placed on a steam bath and 20 drops of concentrated HCl were slowly added to the mixture. Once the contents approached dryness and began to char, the crucibles were placed in a drying oven (Isotemp, Fisher Scientific) for 12 h at 90°C and then for 8 h at 115°C. Crucibles were then placed in a cold Thermolyne (type 1500) muffle furnace and ignited to 600°C for 6 h. Heat was applied gradually up to full temperature to prevent rapid combustion and foaming. Cold ash was dissolved in 8 mL of 6 N H₂SO₄.

Total phosphorus content of homogenate was determined according to Chen *et al.* (1956) in a manner similar to that which was used to measure PAP activity. A 1 mL aliquot of appropriately diluted ash solution was mixed with 1 mL of 20% (w/v) TCA. A 1.5 mL portion of this mixture was added to 3 mL of color reagent C

incubated at 37°C for 1.75 h, and the absorbance at 820 nm was measured. The ash from each crucible was assayed in triplicate, and phosphorus content was determined from the mean absorbance value using KH_2PO_4 standards.

Solubilization of Microsomal PAP from MD Embryos

Resuspended microsomes obtained from MD embryos of *B. napus* L. cv Topas were combined with 1 volume of a detergent solution prepared in assay buffer (pH 6.75). The resulting mixture was vortexed for 2 min and then incubated on ice for 1 h. An aliquot of detergent-treated microsome was retained for PAP activity determination. The remainder of the mixture was centrifuged at 105,000 x g for 60 min. The supernatant, or solubilized fraction, was assayed for PAP activity and protein.

Identification of Reaction Products Following Hydrolysis of PA Catalyzed by Solubilized PAP

The appearance of DG in the reaction mixture was monitored by TLC according to Ichihara *et al.* (1989). Reactions were carried out at 30°C in 2 mL of assay buffer (pH 6.75) containing 0.7 mg sodium PA, 0.5 mg PC, 1 mg BSA, and 90 μg of solubilized microsomal protein. Reactions were terminated by adding 30 μL 6 N HCl. Lipids were extracted from the reaction mixture with 2 volumes of 1.5 mL chloroform and then washed twice with 2 mL of methanol/water (1:1, v/v). Lipids in the combined chloroform layer were concentrated to dryness under a stream of N_2 gas. The lipid residues were suspended in 100 μL of chloroform and 50 μL of each sample were applied to a Silica Gel 60 G aluminum-backed TLC plate. The plate was developed with chloroform/methanol/acetic acid (195:4:1, v/v/v) as the solvent system. The separated lipid classes were visualized by spraying with 30% (w/w) sulfuric acid and then heating at 145°C for a few minutes.

Gel Filtration of Solubilized PAP

Gel filtration chromatography was conducted at 4°C using a Pharmacia LKB FPLC system and a Superose 6 column (1.0 cm x 30.0 cm). The column was equilibrated with 60 mM Tris/60 mM maleic acid-NaOH buffer (pH 6.75) containing 2.5 mM MgCl₂, 0.12 mM EDTA, 0.1 M KCl, and 0.2% (w/v) Tween 20. Microsomal PAP from MD embryos of *B. napus* L. cv Topas was solubilized with 1% (w/v) Tween 20, at a 1:1 (w/w) detergent to protein ratio, by shaking the solubilization mixture on ice for 1.5 h at 200 rpm with an IKA-Labortechnik (type VXR S1) shaker. Solubilized protein (200 µL), recovered in the 105,000 x g supernatant following centrifugation for 60 min, was applied to the column and eluted at a flow rate of 0.2 mL/min. The column effluent was collected in 1 mL fractions which were assayed for PAP activity. The column was calibrated with M_r standards (Sigma Chemical Co.) using equilibration buffer without detergent.

Immunochemical Methods

Homogenate was prepared from MD embryos of *B. napus* L. cv Topas according to Weselake *et al.* (1992). Microsomes (10,000-100,000 x g particulate fraction) obtained by differential centrifugation of tissue homogenate were either treated with high salt concentration, glycerol, and MEGA-8, or were subjected to delipidation and SDS treatment. These preparations were used as the source of antigen to raise mouse antibodies against PAP.

To prepare native protein, the microsomes were shaken vigorously for 30 min at 4°C in 10 mM Tris-HCl buffer (pH 8.0) containing 2 M NaCl, 20% (w/v) glycerol, and 1% (w/v) MEGA-8 at a detergent to protein ratio of about 2:1 (w/w). The solubilized native protein, recovered in the supernatant following centrifugation at 105,000 x g, was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% (w/v) MEGA-8 and 20% (w/v) glycerol to remove DTT and induce protein

aggregation. The dialyzed protein was centrifuged at 105,000 x g for 1 h and the sediment was resuspended, for immunization, in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl.

For the preparation of delipidated, denatured protein, microsomes were treated with 75% (v/v) cold acetone (-20°C) and centrifuged for 10 min at 10,000 x g. The pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 2% (w/v) SDS and 1% (v/v) β-mercaptoethanol, and then boiled for 5 min. The boiled sample was treated with a potassium salt to reduce the concentration of SDS (Suzuki and Terada, 1988). One molar KH₂PO₄ was added to the sample to give a final concentration of 50 mM. The sample was centrifuged at 15,900 x g (Beckman Microfuge E clinical centrifuge) for 5 min. The supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, and used for immunization.

Mouse polyclonal antisera were raised according to Harlow and Lane (1988). Immunizations, animal care, and bleedings were carried out by Greg Tiffin, Animal Diseases Research Institute, Agriculture and Agri-Food Canada, Lethbridge. Mice (female BALB/c), 14 weeks of age, received primary intraperitoneal injections containing complete Freund's adjuvant and either native or delipidated, SDS-treated microsomal protein (50-100 μg protein per injection). Two more intraperitoneal injections containing the antigen and incomplete Freund's adjuvant were administered 21 and 43 days after the initial injection. A final intravenous injection was given in the tail without Freund's adjuvant on day 57. Individual mice were immunized using the same protein preparation for all 4 injections, except one mouse inadvertently received 3 injections containing denatured protein and 1 final injection of native protein. Mice were bled by the ocular method on day 60. Test serum was collected from an uninjected mouse of the same age reared under the same conditions.

Blood was incubated at 27-30°C for 1 h, to allow clotting, and then centrifuged for 30 min at 1,500 x g. The cell pellets were discarded, and the sera were dialyzed at 0-4°C against Tris-maleic acid-NaOH assay buffer (pH 6.75) for 16 h to decrease the levels of endogenous P_i. All sera were simultaneously dialyzed against the same container of buffer so that P_i backgrounds were identical. Antisera were tested for PAP inhibition by preincubating the 10,000-100,000 x g particulate fraction from MD embryos of oilseed rape (cv Topas) with antiserum for 30 min at 30°C and then assaying for PAP activity.

Protein Determination

The protein content of plant tissue extracts was determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure, with BSA as the standard. Absorbance measurements at 280 nm were used to detect protein in fractions eluted from the Superose 6 gel filtration column.

RESULTS AND DISCUSSION

Assay for Phosphatidate Phosphatase Activity and Stability of Enzyme to Freezing/Thawing

In the past, assays for plant PAP activity have been based on the release of P_i , which can be measured spectrophotometrically as the molybdate complex (Moore *et al.*, 1973; Ichihara *et al.*, 1989) or, if a ^{32}P -substrate is used, as radioactivity in the water-soluble fraction (Herman and Chrispeels, 1980; Sukumar and Sastry, 1987). PA labelled in its fatty acid moiety is now commercially available (Stobart and Stymne, 1990), and this may be useful for the assay of PAP in oilseed preparations. Additionally, PA can be synthesized from radiolabelled PC by employing phospholipase D (Kates and Sastry, 1969; Königs and Heinz, 1974), or it can be generated using a modification of the organic synthesis described by Kanda and Wells (1981), where either of the radiolabelled precursors, G3P or fatty acid, can be employed. When possible, it is much more convenient and practical to use non-radioactive substrates, partly because of the potential hazards associated with radiolabelled compounds, and partly because of the high cost of purchasing these chemicals. When unlabelled substrates are employed, however, it is important that precautions are taken to insure that the product being measured has in fact resulted from the enzymic activity of interest (Reynolds *et al.*, 1991). This is particularly important when crude extracts are being used, since an extensive mixture of active enzymes and endogenous compounds will usually be present.

In the current study, non-radioactive substrate was presented to PAP in the form of a stabilized emulsion consisting of egg yolk PA, egg yolk PC, and BSA (Ichihara *et al.*, 1989). PA was presented with the predominant eukaryotic phospholipid, PC, to simulate the natural environment of the physiologically active form of PAP (Stobart and Stymne, 1990), and BSA was included because of its stimulatory effect on the microsomal enzyme (Ichihara *et al.*, 1989). Following the addition of TCA to the

reaction mixtures, PAP activity was determined by measuring the amount of P_i in the aqueous supernatant using a colorimetric method involving the acid reduction of the phosphomolybdate complex (Chen *et al.*, 1956). The linear standard curve relating the increase in absorbance at 820 nm to μg phosphorus is shown in Figure 2.

The role of TCA in the assay procedure was not only to quench the enzyme reactions, but also to facilitate the precipitation of lipids and proteins and, therefore, to aid in the clarification of the supernatant during the centrifugation step which occurred just prior to color development. Increasing the concentration of TCA from 10% (w/v) to 20% (w/v) had no effect on the absorbance values of the KH_2PO_4 standards used to prepare the standard curve (Figure 2). This minor modification of the procedure described by Chen *et al.* (1956) did, however, increase the rate with which the precipitate sedimented and decreased the time it took to centrifuge the quenched reactions, especially at high pH. That is, supernatant clarification was hampered by alkaline assay conditions, but this was mitigated by the increase in TCA concentration.

Using the assay described above, enzyme activity was monitored in the homogenate obtained from *Brassica napus* L. cv Westar seeds harvested 3-4 weeks post-anthesis, which were in the active phase of TG accumulation (Weselake *et al.*, 1993). Time courses for the release of P_i catalyzed by PAP activity in the fresh homogenate obtained from frozen seeds, and the same homogenate subjected to repeated cycles of freezing and thawing, are shown in Figure 3. The rate of P_i release was similar in each case, suggesting that PAP activity was stable to at least 3 cycles of freezing and thawing. Additionally, the enzyme reactions were linear for at least 60 min, which was in agreement with time courses observed by Ichihara *et al.* (1989) for PAP activity in extracts from developing safflower (*Carthamus tinctorius* L.) seeds. In the present study, the addition of 1 mM DTT to the homogenizing buffer did not stimulate PAP activity, or appear to be necessary for the preservation of PAP activity

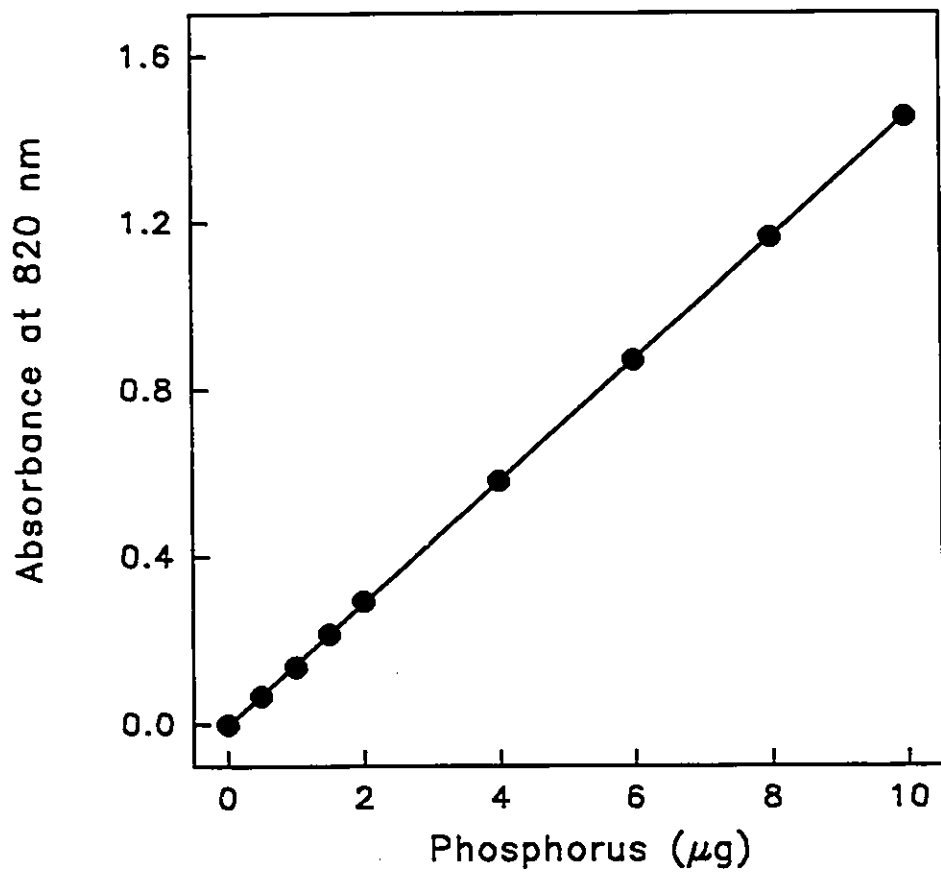


Figure 2. KH_2PO_4 standard curve used for the microdetermination of phosphorus.

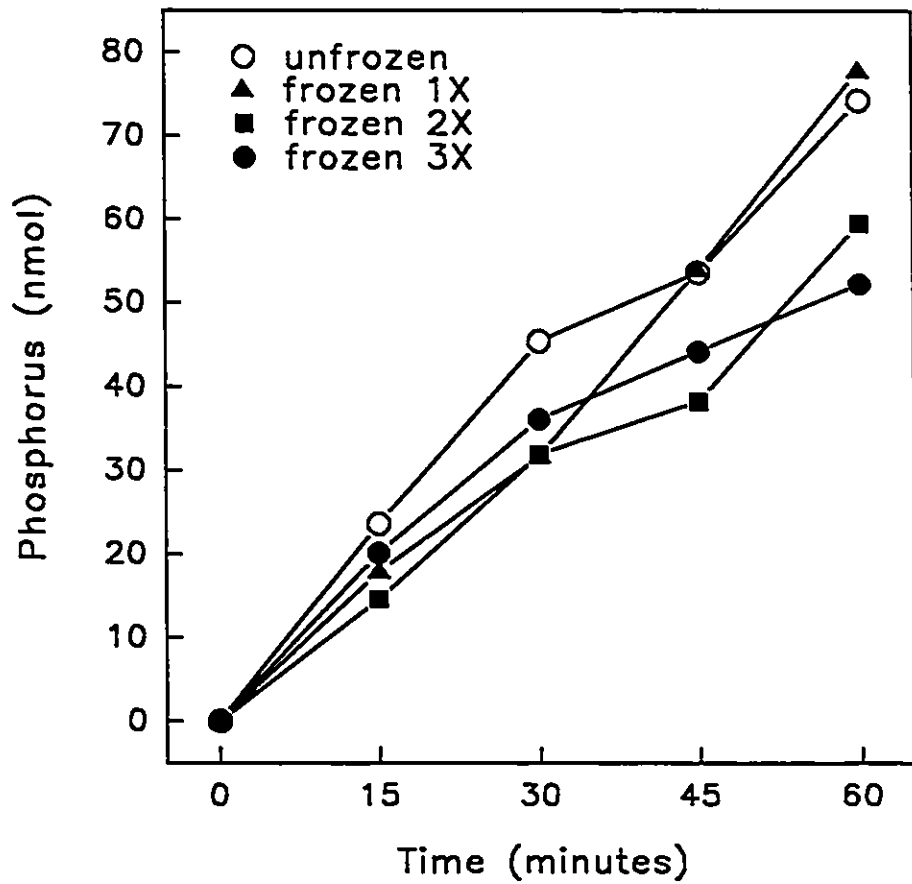


Figure 3. Effect of freeze/thaw cycles on P_i release catalyzed by PAP activity in homogenate of developing seeds of *Brassica napus* L. cv Westar at 30°C. Data represent the means of triplicate assays using 300 μg protein per reaction mixture. Substrate was included in the reaction mixture in the form of a stabilized emulsion consisting of PA, PC, and BSA.

in fresh or frozen homogenates obtained from the developing seeds of *B. napus*. Therefore, this reducing agent was not included in the preparation of enzyme extracts.

Enzyme assays conducted with seed homogenate, where PA was excluded from the reaction mixture, resulted in the release of P_i at about one-third the rate obtained using the complete reaction mixture (Table I). Given the ubiquitous nature of phospholipase D (Gurr and Harwood, 1991; Lambrecht and Ulbrich-Hofmann, 1992), especially in plants (Kates, 1954; Davidson and Long, 1958; Heller, 1978), this observation suggested that endogenous phospholipase D activity may have been present in the developing seed homogenate, and could have resulted in the generation of PA to support some degree of PAP activity. The PC used in this particular experiment was derived from soybean, whereas the substrate, PA, was obtained from egg yolk. Since PC may contribute to the PA pool as a result of endogenous phospholipase D activity, at least in this crude extract, egg yolk PC was employed in all of the other experiments described here. This was done so that any PA formed by the hydrolysis of PC would have an acyl composition similar to that of the exogenously supplied substrate. Of course, the ultimate remedy for possible complications associated with the presence of phospholipase D is, if possible, to remove this enzyme or to render it inactive. In addition to demonstrating the probable existence of phospholipase D in the homogenate, this experiment also showed that there was no endogenous PA in this tissue, since incubating the extract without any substrate components did not result in the evolution of P_i (Table I). Also, this indicated that under the assay conditions described, there were no contributions of P_i from the hydrolysis of endogenous phosphate-containing compounds.

In order to evaluate the total potential contribution of P_i from all endogenous sources, a single 2 mL aliquot from each of three separate preparations of developing seed homogenate was ashed according to the micromethod of Isaac (1990). Dissolved ash from each aliquot was assayed in triplicate using, in each reaction mixture, an

Table I. Release of P_i catalyzed by maturing canola (*B. napus* L. cv Westar) seed homogenate using different reaction mixture components. PA was derived from egg yolk lecithin and PC was prepared from soybean. Absorbance values represent the mean value of triplicate assays which were carried out at 28°C, for 30 min, at pH 6.75, and included 25 μ L of homogenate (206 μ g of protein) per reaction mixture.

Reaction Mixture	Absorbance at 820 nm		
	water	PC/BSA	PA/PC/BSA
Reagent Blanks	0.000	0.013	0.015
Controls*	0.304	0.294	0.301
Samples	0.297	0.326	0.383

PA = phosphatidic acid

PC = phosphatidylcholine

BSA = bovine serum albumin

* TCA added prior to addition of extract

amount of ash equivalent to 25 μL of homogenate. Based on the three averages obtained from these triplicate assays, the total phosphorus content in 25 μL of typical oilseed rape seed homogenate was 6.6-6.7 μg . Additionally, using each of the three preparations of seed homogenate, and based on the three averages obtained from triplicate assays employing 25 μL of TCA-inactivated homogenate per reaction mixture, typical developing seed homogenate contained 2.2-2.3 μg of phosphorus in the form of soluble inorganic orthophosphate. This represented one-third of the total phosphorus content. Also, PAP activity assays were conducted at 28°C for 30 min by, once again, assaying in triplicate each of the homogenate preparations described above, and employing 25 μL of homogenate per reaction mixture. Activity data showed that 20-23% of the soluble phosphorus present was the result of PA-hydrolase activity.

Characterization of PAP Activity in Microsomal and Soluble Fractions of Developing Seeds

The effect of pH on PAP activity in the microsomal (10,000-100,000 \times g sediment) and soluble (100,000 \times g supernatant) fractions from developing seeds of *B. napus* L. cv Westar is shown in Figure 4. Based on the release of P_i , microsomal PAP activity from the developing seeds of *B. napus* L. cv Westar exhibited a broad pH optimum between pH 5 and pH 6.75, with maximum activity occurring at pH 6. These results are in good agreement with previous studies of the pH effects on PAP activity using other plant tissues. For example, Ichihara *et al.* (1989) reported a pH optimum of pH 6.75 for the microsomal enzyme from developing seeds of safflower, and Sukumar and Sastry (1987) reported a broad pH optimum between pH 6 and pH 7 for the microsomal PAP from developing seeds of groundnut.

In contrast to the microsomal PAP activity, PA-hydrolase activity associated with the dialyzed cytosolic fraction from the developing seeds of *B. napus* L. cv Westar exhibited a sharp peak at pH 5 (Figure 4). To insure that the pH optimum for the

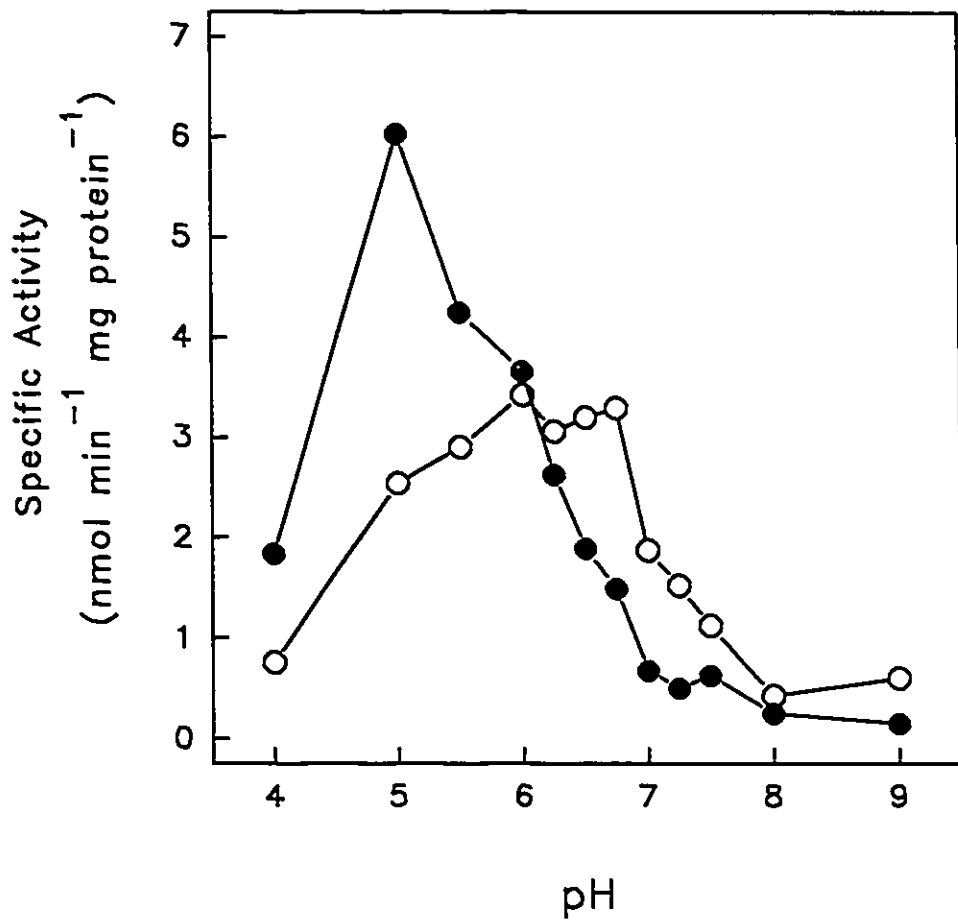


Figure 4. pH dependence of PAP activity in the microsomal (hollow circle) and dialyzed soluble (shaded circle) fractions from developing seeds of *B. napus* L. cv Westar. Buffers used were 50 mM acetate (pH 4.0), 50 mM Tris/50 mM maleic acid-NaOH (pH 5.0-8.0), and 50 mM Tris-HCl (pH 9.0). Assays were conducted at 30°C for 30 min, using 150 μ g and 200 μ g of protein for particulate and soluble fractions, respectively. Data points represent the means of triplicate assays.

soluble activity from developing seeds of *B. napus* was in fact distinctly at pH 5, and that the decrease in activity at pH 4 was not a buffer species effect resulting from employing acetate buffer at pH 4, the experiment was repeated over a smaller pH range employing only acetate buffers (Figure 5). The pH dependence activity data shown in Figure 5 confirm that the soluble PA-hydrolase activity from developing seeds of oilseed rape exhibited a distinct optimum at pH 5. This finding is in agreement with studies of the pH effects on soluble PA-hydrolase activities using other types of plant tissues. For example, soluble PAP activities with acidic pH optima (< pH 6) have also been detected in germinating castor beans (Moore *et al.*, 1973), broadbean leaves (Königs and Heinz, 1974), and germinating mung bean cotyledons (Herman and Chrispeels, 1980). Although soluble activity was also detected in the maturing seeds of safflower (Ichihara *et al.*, 1989, 1990), the pH optimum for cytosolic PA-hydrolase activity from this tissue has not yet been reported. No soluble enzyme was detected in the developing seeds of groundnut (Sukumar and Sastry, 1987). For the pH profiles shown in Figures 4 and 5, the soluble fractions were dialyzed against Tris-maleate-NaOH assay buffer (pH 6.75) before being assayed to reduce interference due to endogenous P_i .

The effect of temperature on PAP activity was also examined using microsomal and dialyzed soluble fractions from developing seeds of oilseed rape under pH conditions which were optimal for enzyme activity. At pH 6.75, the activity of microsomal PAP was optimal at 40°C (Figure 6A). Additionally, a denaturation temperature of 40°C and activation energy of 15.6 kcal/mol were determined for microsomal PAP from the Arrhenius plot shown in Figure 6B. According to Lin and Carman (1989), activation energy was 11.9 kcal/mol for the 91-kDa form of PAP purified from microsomes of *Saccharomyces cerevisiae*. The yeast enzyme appeared to be labile at temperatures above 30°C. In agreement with the freeze/thaw data obtained using developing seed homogenate (Figure 3), microsomal PAP assayed at 28°C had

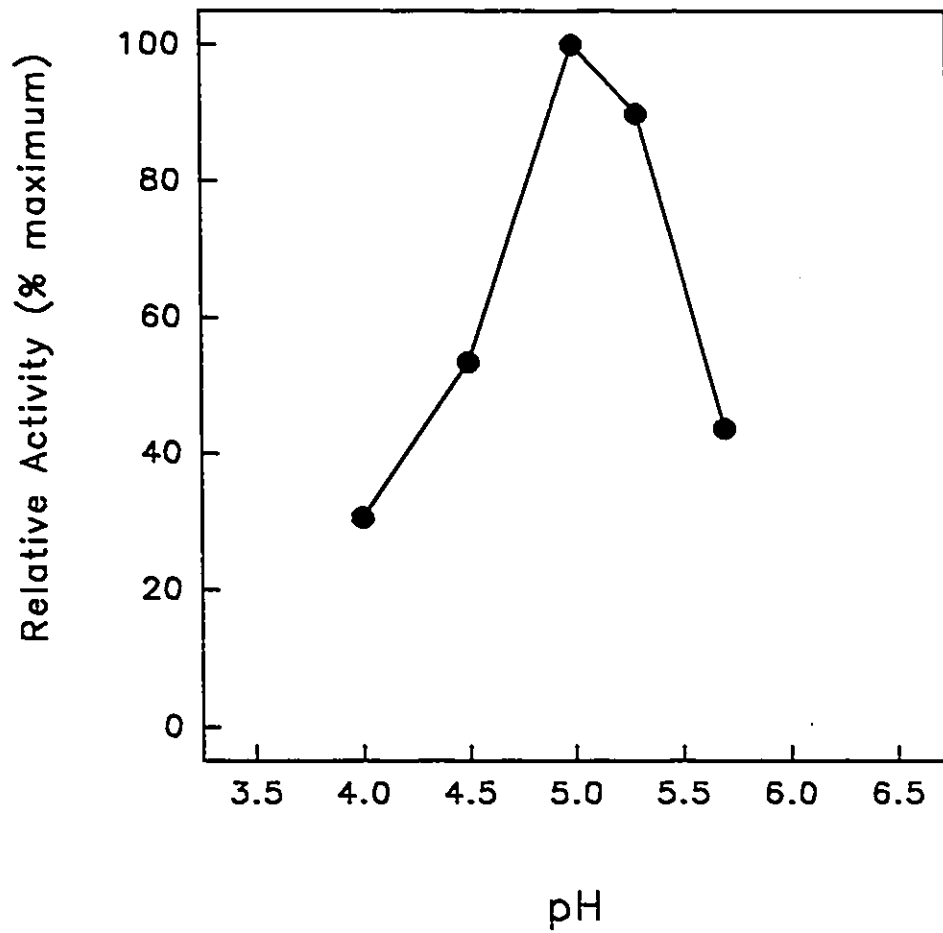


Figure 5. pH dependence of PAP activity in the dialyzed soluble fraction (100,000 x g supernatant) from the developing seeds of *B. napus* L. cv Westar assayed in the presence of 50 mM acetate buffers. Assays were conducted at 30°C, for 30 min, using 200 μ g of protein per reaction mixture. Data points represent the means of triplicate assays.

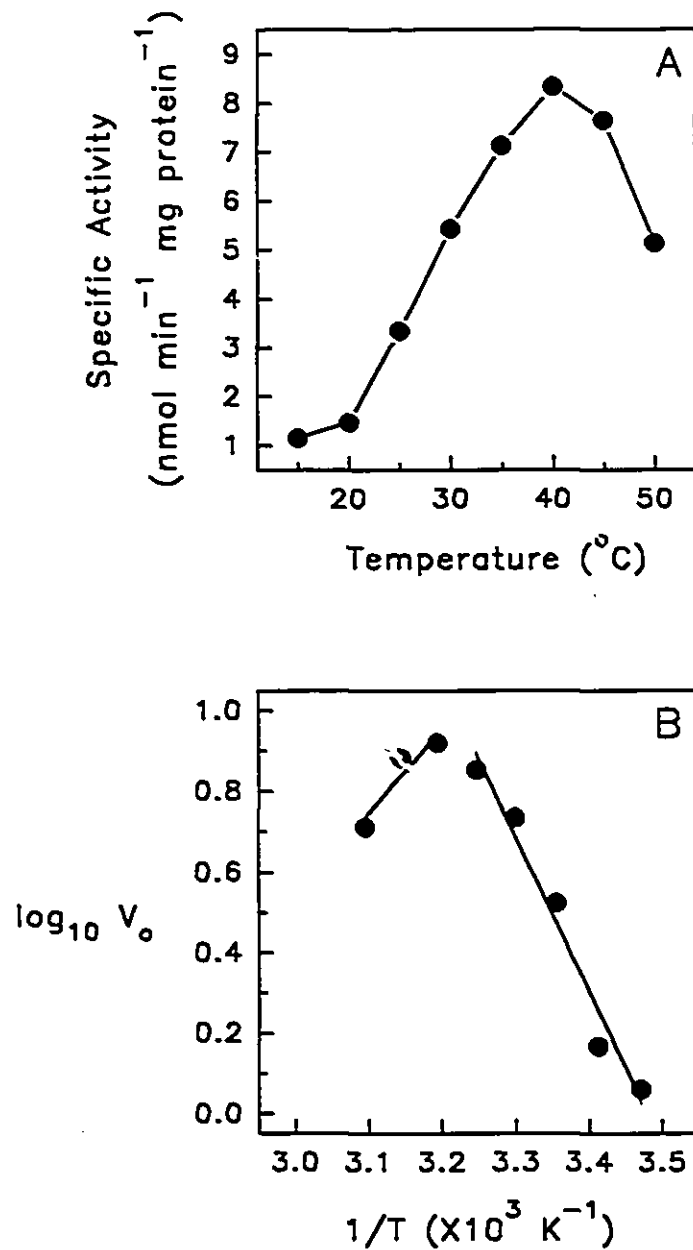


Figure 6. (A) Microsomal PAP activity versus temperature for microsomal PAP from developing seeds of *B. napus* L. cv Westar. Assays were conducted at pH 6.75 (Tris-maleic acid-NaOH buffer), for 30 min, using 123 μg protein per reaction mixture. Activity data represent the means of triplicate assays. (B) Arrhenius plot for microsomal PAP from developing seeds.

the same activity regardless of whether the microsomal preparation obtained from frozen seeds was assayed fresh, or after being flash frozen in liquid N₂ and stored at -20°C for several weeks. Thus, a rationale for the preservation of PAP in the form of frozen microsomal preparations was established. Ichihara *et al.* (1989) found that PAP activity was stable for at least 3 weeks when the microsomal preparations from maturing safflower seeds were stored at -20°C.

When assayed at pH 5, soluble PAP activity from the developing seeds of *B. napus* was optimal at 50°C (Figure 7A). Based on the Arrhenius plot shown in Figure 7B, soluble PAP exhibited a denaturation temperature of 45°C and activation energy of 9.4 kcal/mol. Thus, when compared at optimal reaction conditions, microsomal PAP from *B. napus* was more heat-labile than the soluble form of the enzyme. Ichihara *et al.* (1989) reported that the thermolabilities of microsomal and soluble PAP from developing safflower seeds were similar to each other. The dialyzed soluble fraction from *B. napus* could also be stored at -20°C for several weeks without the loss of any PA-hydrolase activity.

PAP Activity in Differential Centrifugation Fractions

PAP activity in fractions obtained following differential centrifugation of homogenates prepared from developing seeds, MD embryos, and an embryogenic MD cell suspension culture of oilseed rape is shown in Table II. The data are based on the fractionation of homogenate from tissues which were frozen and thawed once prior to use. The nondifferentiating embryogenic cell suspension culture of winter oilseed rape (*B. napus* L. cv Jet Neuf) was recently shown to contain 3-4% TG on a dry weight basis, and produce a relatively constant level of DGAT activity over a number of subcultures (Weselake *et al.*, 1993). Total PAP activity recovered following fractionation of homogenate varied from 54-71% among the three tissue types. The percent distribution of total PAP activity recovered in the crude subcellular fractions,

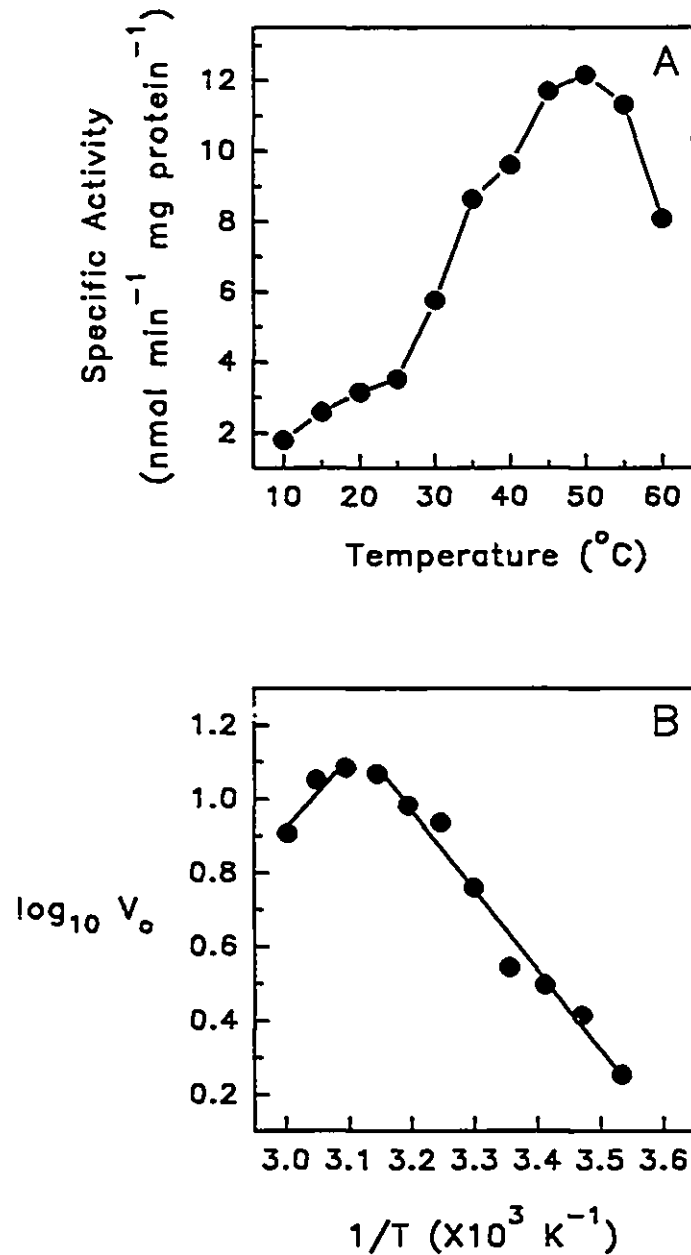


Figure 7. (A) Soluble PAP activity versus temperature for soluble PAP from developing seeds of *B. napus* L. cv Westar. The 100,000 x g supernatant was dialyzed against Tris-maleic acid-NaOH buffer (pH 6.75) for 16 h prior to assaying at pH 5.0 (Tris-maleic acid-NaOH buffer), for 30 min, using 200 μ g soluble protein per reaction mixture. Activity data represent the means of triplicate assays. (B) Arrhenius plot for soluble PAP from developing seeds.

Table II. Distribution of PAP activity in crude subcellular fractions obtained by the differential centrifugation of homogenate from 3 different types of TG forming *B. napus* tissues. *B. napus* cultivars of developing seeds, MD embryos, and MD cell suspension culture were Westar, Reston, and Jet Neuf, respectively. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**, and in each case 5 g of tissue were used. Assays were conducted at 28°C, for 30 min, at pH 6.75, and activity data represent the means of triplicate assays. Floating fat layers, obtained by the centrifugation steps, were combined, and washed 3 times by resuspending in a volume of homogenizing buffer equal to one-half the tissue weight followed by centrifugation at 10,000 x g for 20 min. The particulate fractions were not washed.

Cell Fraction	Resuspended Volume (mL)	Total Protein (mg/5g fresh weight)	Specific Activity (nmol/min/mg protein)	Total Activity (nmol/min/total protein)
DEVELOPING SEED				
Homogenate	20.0	154.8	3.5	542
1500xg p	3.8	44.5	2.7	120
10,000xg p	3.2	17.9	4.2	75
100,000xg p	2.5	13.9	3.1	43
100,000xg s	17.0	30.8	4.4	136
Fat layer	2.7	6.5	1.4	9
MD EMBRYOS				
Homogenate	22.5	201.2	3.6	724
1500xg p	3.4	25.2	1.2	30
10,000xg p	2.9	5.1	5.2	27
100,000xg p	2.5	17.6	3.8	67
100,000xg s	20.0	60.2	4.0	241
Fat layer	2.9	7.6	3.8	29
CELL SUSPENSION				
Homogenate	21.5	38.3	2.4	92
1500xg p	3.8	13.1	2.3	30
10,000xg p	2.8	2.9	2.2	6
100,000xg p	2.6	4.4	1.6	7
100,000xg s	19.0	9.7	1.5	15
Fat layer	2.9	0.9	0.9	1

p = precipitate

s = supernatant

for each type of TG forming *B. napus* tissue, is shown in Figure 8. PAP activity in the 10,000-100,000 x g microsomal fraction represented 11%, 17%, and 12% of the recovered PAP activity, respectively, for developing seeds, MD embryos, and the MD cell suspension culture. Also, soluble PAP activity was 36%, 61%, and 25% of the recovered activity, respectively, for developing seeds, MD embryos, and the MD cell suspension culture. In all cases the lowest levels of recovered activity were associated with the fat layer. In differential centrifugation studies by Ichihara *et al.* (1989) using the developing seeds of safflower, about 12% and 40% of the recovered PAP activity was found in the microsomal fraction and 100,000 x g supernatant, respectively, which compares favorably with the results obtained using the developing seeds of *B. napus* L. cv Westar (Figure 8). When assayed at 28°C, total activity per gram fresh weight was 108 nmol/min for developing seeds, 145 nmol/min for MD embryos, and only 18 nmol/min for the MD cell suspension culture. However, the ease with which the MD cell suspension was maintained, combined with the presence of a reasonable level of microsomal PAP activity in cells which had been stored frozen, suggests that this nondifferentiating system can be employed as a reliable source of PAP for biochemical investigations. The ability to produce and store copious amounts of cells makes this cell culture system particularly well suited for the production of large amounts of biological material necessary for scaled-up attempts at enzyme purification.

It is uncertain, based on the information shown in Table II and Figure 8, whether differences observed in the distribution of PAP activity in crude subcellular fractions obtained from different tissues of *B. napus* were the result of tissue-specific variation or, alternatively, because of varietal differences, since each of the tissues employed was from a different cultivar. In order to address this issue, PAP activity was assayed in fractions obtained following differential centrifugation of homogenates prepared from developing seeds and MD embryos of the low erucic acid *B. napus* cultivar, Topas (Table III). These tissues were frozen and thawed once prior to

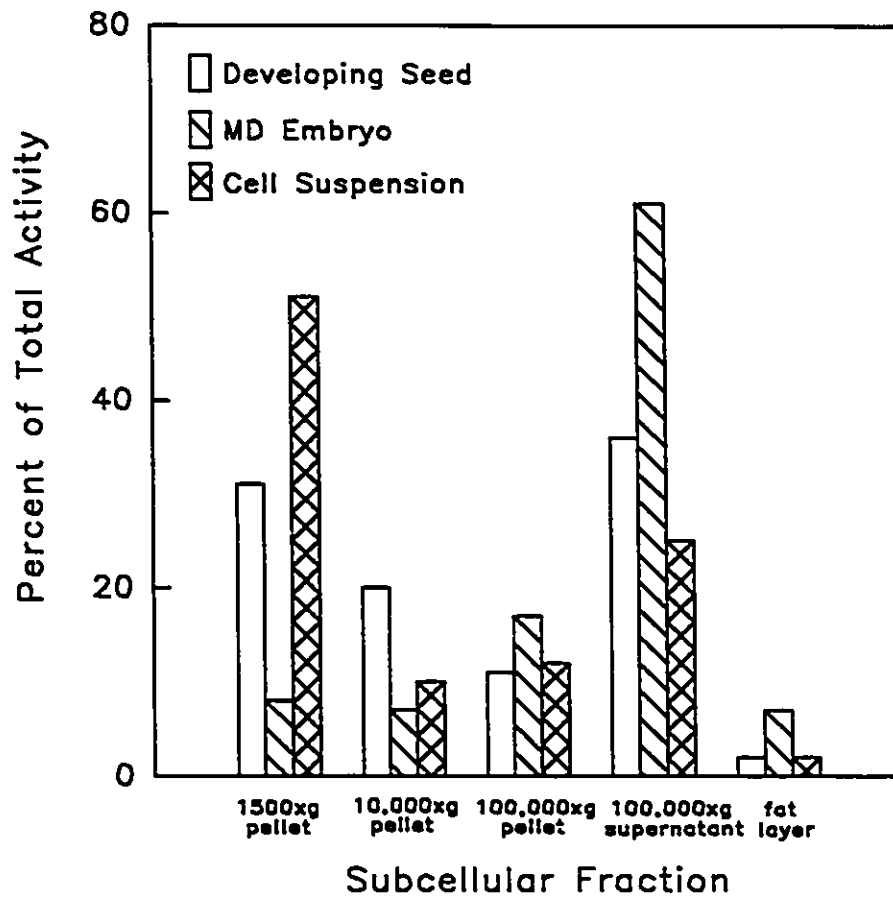


Figure 8. Percent distribution of total PAP activity recovered in the crude subcellular fractions prepared from 3 different types of TG forming *B. napus* tissues. *B. napus* cultivars of developing seeds, MD embryos, and MD cell suspension culture were, respectively, Westar, Reston, and Jet Neuf. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**, and in each case 5 g of tissue were used. Assays were conducted at 28°C, for 30 min, at pH 6.75, and data represent the means of triplicate assays.

Table III. Distribution of PAP activity in crude subcellular fractions obtained by the differential centrifugation of homogenate from frozen seeds, frozen MD embryos, and fresh MD embryos of *B. napus* L. cv Topas. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**, and the amount of tissue homogenized is indicated for each case. Assays were conducted at 28°C, for 30 min, at pH 6.75, and activity data represent the means of triplicate assays. Floating fat layers, obtained by the centrifugation steps, were combined, and washed 3 times by resuspending in a volume of homogenizing buffer equal to one-half the tissue weight followed by centrifugation at 10,000 x g for 20 min. The particulate fractions were not washed.

Cell Fraction	Resuspended Volume (mL)	Total Protein (mg per fresh weight)	Specific Activity (nmol/min/mg protein)	Total Activity (nmol/min/total protein)
FROZEN DEVELOPING SEED (FW = 0.91g)				
Homogenate	20.5	18.5	3.17	58.7
1500xg p	3.6	7.0	1.83	12.8
10,000xg p	3.0	0.9	2.37	2.1
100,000xg p	2.5	1.7	1.04	1.8
100,000xg s	18.0	6.1	2.34	14.3
Fat layer	2.5	0.1	1.43	0.1
FROZEN MD EMBRYOS (FW = 4.39g)				
Homogenate	22.0	184.8	5.17	955.4
1500xg p	3.0	18.3	2.12	38.8
10,000xg p	2.7	5.7	5.39	30.7
100,000xg p	2.8	27.7	2.47	68.4
100,000xg s	19.5	100.4	4.61	462.8
Fat layer	4.2	4.6	4.07	18.7
FRESH MD EMBRYOS (FW = 5.00g)				
Homogenate	22.0	82.5	5.30	437.3
1500xg p	2.9	20.7	2.06	42.6
10,000xg p	2.7	6.3	8.65	54.5
100,000xg p	2.5	9.5	11.45	108.8
100,000xg s	20.0	40.0	0.99	39.6
Fat layer	3.2	5.4	7.49	40.4

p = precipitate

s = supernatant

homogenization. Additionally, PAP activity was assayed in fractions obtained from the homogenate prepared from fresh MD embryos of *B. napus* L. cv Topas (Table III), as a preliminary investigation into the effects that freezing of whole tissues may have had on the distribution of PAP activity in the crude fractions obtained from tissue homogenates. In comparison to frozen MD embryos of *B. napus* L. cv Topas, fractionation of homogenate from MD embryos which were not previously frozen appeared to result in lower specific PAP activity in the 100,000 x g supernatant, with a concomitant increase in the specific activities associated with the mitochondrial (1,500-10,000 x g sediment) and microsomal fractions, suggesting that some amount of organelle rupture may have occurred during tissue freezing. Although differential centrifugation is the simplest method for the separation of homogenate into different subcellular fractions (Graham, 1989), the particulate fractions are in fact composite membrane fractions containing all the particles which can pellet from the supernatant at a given centrifugal force. The rupture of mitochondria and plastids, along with shearing of the Golgi apparatus and ER, will alter the distribution of PAP in crude fractions obtained by differential centrifugation of tissue homogenate. Additionally, the apportionment of PAP activity can be altered, not only because membrane-bound forms of PAP associated with the ER, plastids, and, most likely, mitochondria are distributed differently, but because organelle lysis may cause the release of soluble hydrolytic enzymes capable of dephosphorylating PA. It is also possible that freezing may simply result in the release of membrane-associated PAP into the putative cytosolic form of the enzyme.

In this case, fresh and frozen MD embryos of *B. napus* L. cv Topas were obtained from different cultures. The rationale for the use of embryos from different cultures was based upon the assumption that it is possible to make generalizations about different cultures grown under identical conditions in much the same way that generalizations are made about different plants grown under the same, highly

controlled, conditions. In agreement, the specific activities associated with homogenates of fresh and frozen MD embryos were similar (Table III).

The total PAP activity recovered following the fractionation of homogenates prepared from the tissues of *B. napus* L. cv Topas was 65% for both the frozen and fresh MD embryos, and 53% for frozen seeds. The percent distribution of total PAP activity recovered in the crude subcellular fractions prepared from frozen seeds, frozen MD embryos, and fresh MD embryos of *B. napus* L. cv Topas is shown in Figure 9. PAP activity in the 10,000-100,000 x g particulate fraction represented 6%, 11%, and 38% of the recovered PAP activity, respectively, for frozen seeds, frozen MD embryos, and fresh MD embryos. Soluble PAP activity was 46%, 75%, and 14% of the recovered PAP activity, respectively, for frozen seeds, frozen MD embryos, and fresh MD embryos. Comparison of the distribution profiles in Figure 8 and Figure 9 show that the distribution of PAP activity in crude subcellular fractions was similar for frozen *B. napus* L. cv Topas seeds and frozen seeds of the low erucic acid *B. napus* cultivar, Westar. Additionally, the distribution of PAP activity was similar for fractions obtained from frozen MD embryos of *B. napus* L. cv Topas and frozen MD embryos of the high erucic acid *B. napus* cultivar, Reston. Thus, the distribution of PAP activity in crude subcellular fractions obtained by the differential centrifugation of tissue homogenate appears to be tissue-specific. In all cases where frozen MD embryos or frozen seeds were used (Figures 8 and 9), the highest levels of recovered activity were associated with the soluble fraction. For fresh MD embryos of *B. napus* L. cv Topas, the highest level of recovered activity was associated with the microsomal fraction (Figure 9). For frozen Jet Neuf cells, the plastidular/nuclear fraction (1,500 x g sediment) represented the highest level of recovered activity (Figure 8).

In determining the subcellular distribution of PAP, sand was always employed as an abrasive in the tissue homogenization process. To investigate further the effects of freezing on the subcellular distribution of PAP, and to evaluate the possible effect of

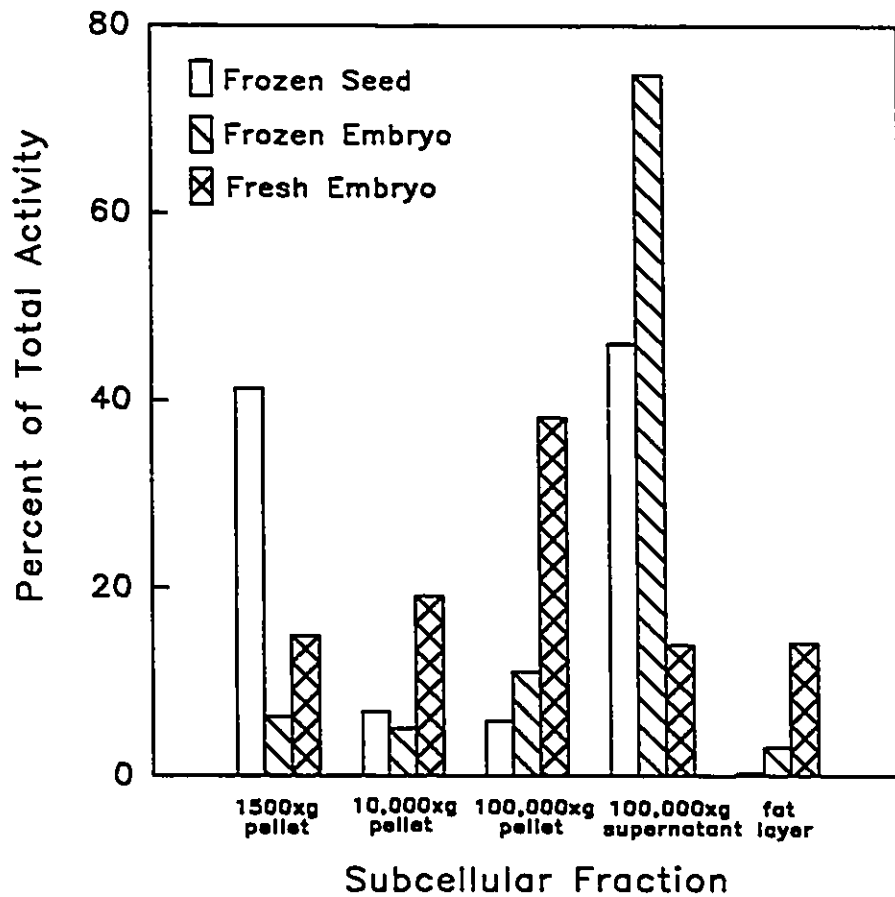


Figure 9. Percent distribution of total PAP activity recovered in the crude subcellular fractions prepared from frozen seeds, frozen MD embryos, and fresh MD embryos of *B. napus* L. cv Topas. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**. The amount of tissue used was 0.91 g, 4.39 g, and 5.00 g for developing seeds, frozen MD embryos, and fresh MD embryos, respectively. Assays were conducted at 28°C, for 30 min, at pH 6.75, and data represent the means of triplicate assays.

abrasion on organelle rupture and the apportionment of PAP activity in crude subcellular fractions obtained by differential centrifugation of tissue homogenate, fresh *B. napus* L. cv Topas MD embryos from the same culture were partitioned out into three 2.5 g aliquots and subjected to variations in tissue processing. Prior to the fractionation of tissue homogenate, each aliquot was either homogenized without sand while still fresh, homogenized with sand while still fresh, or subjected to one freeze/thaw cycle and then homogenized without sand (Table IV). Specific PAP activities associated with each of the tissue homogenates were similar to one another, indicating that, as was the case with developing seeds (Figure 3), freezing of MD embryos prior to tissue homogenization did not alter total PA-hydrolase activity. Total PAP activity recovered following fractionation of homogenate varied from 65-73% among the three different tissue homogenization treatments. The percent distribution of total PAP activity recovered in crude subcellular fractions prepared from homogenates of MD embryos of *B. napus* L. cv Topas ground fresh without sand, fresh with sand, and without sand after one freeze/thaw cycle is shown in Figure 10. PAP activity in the 10,000-100,000 x g particulate fraction represented 32%, 24%, and 20% of the recovered PAP activity, respectively, for MD embryos homogenized fresh without sand, fresh with sand, and without sand after one freeze/thaw cycle. Also, PAP activity in the soluble fraction represented 31%, 33%, and 43% of the recovered PAP activity, respectively, for MD embryos homogenized fresh without sand, fresh with sand, and without sand after one freeze/thaw cycle. The microsomal fraction represented the highest level of recovered PAP activity when fresh MD embryos were homogenized without sand, but only marginally. When fresh MD embryos of *B. napus* L. cv Topas were homogenized with sand, and when frozen MD embryos obtained from the same culture were subjected to a single freeze/thaw cycle and then homogenized without sand, the highest levels of recovered PAP activity were associated with the 100,000 x g supernatant, suggesting that both freezing of tissues

Table IV. Distribution of PAP activity in crude subcellular fractions obtained by the differential centrifugation of tissue homogenate prepared from MD embryos of *B. napus* L. cv Topas using fresh tissue ground with no abrasive (sand), fresh tissue ground with abrasive, and frozen tissue ground with no abrasive. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**, and in each case 2.5 g of MD embryos were used. Assays were conducted at 28°C, for 30 min, at pH 6.75, and activity data represent the means of triplicate assays. Floating fat layers, obtained by the centrifugation steps, were combined, and washed 3 times by resuspending in a volume of homogenizing buffer equal to one-half the tissue weight followed by centrifugation at 10,000 x g for 20 min. The particulate fractions were not washed.

Cell Fraction	Resuspended Volume (mL)	Total Protein (mg/2.5g fresh weight)	Specific Activity (nmol/min/mg protein)	Total Activity (nmol/min/total protein)
FRESH MD EMBRYOS GROUND WITHOUT SAND				
Homogenate	9.4	58.3	3.99	232.6
1500xg p	1.7	16.5	1.70	28.0
10,000xg p	1.3	4.7	3.87	18.2
100,000xg p	1.3	10.4	5.16	53.7
100,000xg s	8.4	21.8	2.43	53.0
Fat layer	1.8	2.1	7.91	16.6
FRESH MD EMBRYOS GROUND WITH SAND				
Homogenate	9.4	63.9	3.29	210.2
1500xg p	1.8	19.0	1.51	28.7
10,000xg p	1.4	5.9	3.12	18.4
100,000xg p	1.3	9.6	3.44	33.0
100,000xg s	8.4	24.4	1.84	44.9
Fat layer	1.8	2.2	5.35	11.8
FROZEN MD EMBRYOS GROUND WITHOUT SAND				
Homogenate	9.4	80.8	4.55	367.6
1500xg p	1.7	20.3	1.55	31.5
10,000xg p	1.4	5.6	5.74	32.1
100,000xg p	1.3	11.7	4.27	50.0
100,000xg s	8.4	30.2	3.52	106.3
Fat layer	1.8	4.7	5.96	28.0

p = precipitate

s = supernatant

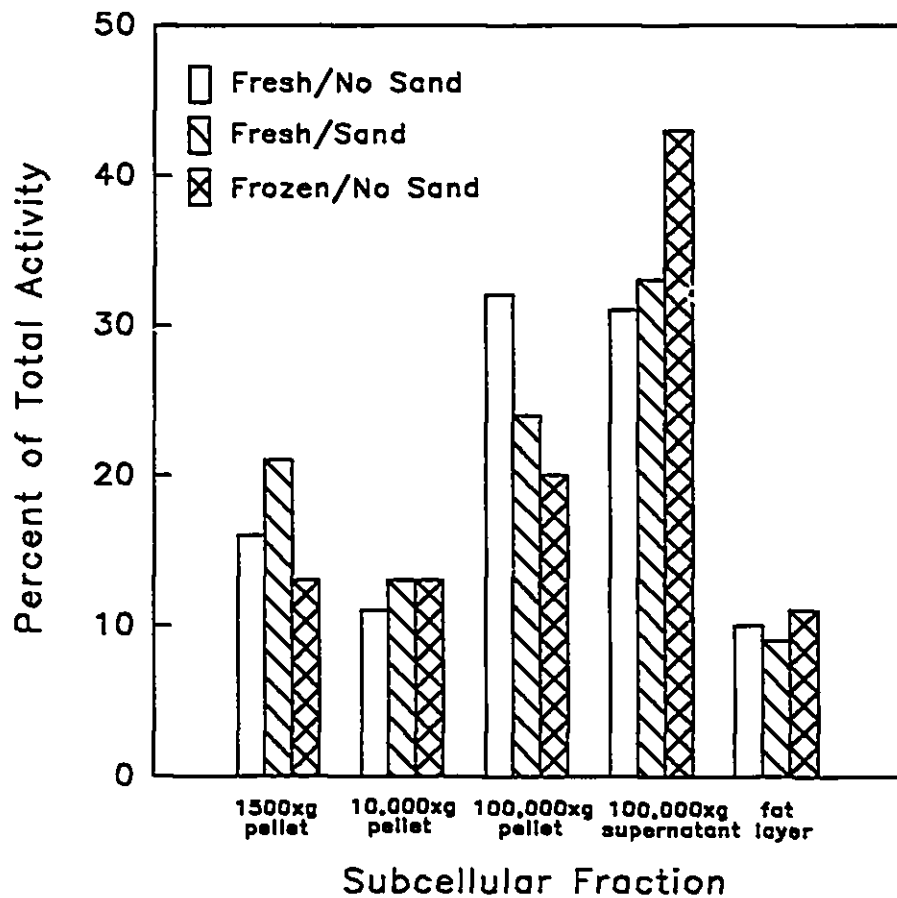


Figure 10. Effect of abrasive (sand) and freezing on the percent distribution of total PAP activity recovered in the crude subcellular fractions prepared from MD embryos of *B. napus* L. cv Topas. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**, and in each case 2.5 g of tissue were used. Assays were conducted at 28°C, for 30 min, at pH 6.75, and data represent the means of triplicate assays.

and the use of abrasive resulted in organelle lysis and a change in the apportionment of PAP activity in crude subcellular fractions obtained by differential centrifugation of tissue homogenate. Although the PAP activity distribution data in Figures 9 and 10 demonstrate that freezing may have resulted in an increase in soluble PA-hydrolase activity at the expense of particulate PAP activity, all of the tissues described above could be stored frozen for several months without a loss in total PAP activity. Thus, large quantities of developing seeds, MD embryos, and *Set Neuf* cells can be frozen for future studies of PAP, which would be advantageous in terms of preparative biochemistry. Furthermore, it appears that generalizations based on comparisons made between MD embryos from different cultures, but generated under the same culture conditions, are justified.

Relationship Between PAP Activity and Acid Phosphatase Activity

The more acidic pH optimum of the PAP activity associated with the 100,000 x g supernatant obtained from the homogenate of developing seeds of oilseed rape (Figures 4 and 5), in relation to the broad pH optimum between pH 5 and pH 6.75 for the microsomal fraction (Figure 4), suggested that this activity may be attributable to a different form of PAP, or perhaps a nonspecific acid phosphatase (orthophosphoric monoesterphosphohydrolase, acid optimum; EC 3.1.3.2). Acid phosphatases are ubiquitous in nature (Schmidt, 1961; Fujisawa *et al.*, 1993), but their physiological function still remains largely unknown (Van Etten and Waymack, 1991). In plants, they are generally thought to be somehow involved in the production, transport, and recycling of P_i (Duff *et al.*, 1994). In a number of plant tissues, conditions of phosphorus deficiency result in the induction of acid phosphatases (Waymack and Van Etten, 1991; Theodorou and Plaxton, 1994). Acid phosphatases are categorized based on pH optima for catalysis below pH 7, with most types identified having pH optima between pH 5 and pH 6 (Vincent *et al.*, 1992; Duff *et al.*, 1994). Recently, the major

acid phosphatase in soybean leaf was shown to have optimal activity at pH 6 in acetate buffer (Staswick *et al.*, 1994). Plant acid phosphatases do not usually exhibit absolute substrate specificity and they occur in various subcellular locations including the cytoplasm, vacuoles, sphaerosomes, and membrane fractions (Waymack and Van Etten, 1991; Duff *et al.*, 1994). Purified wheat germ acid phosphatase has been shown to have activity towards LP and other phospholipid intermediates, including PA (Blank and Snyder, 1970). Additionally, Ching *et al.* (1984) have provided strong evidence for an acidic PAP in the seeds of triticale. In oilseed species, microsomal PAP is probably the important enzyme activity in terms of TG formation since the occurrence of TG biosynthesis in the ER is well documented (Stymne and Stobart, 1987). It is possible, however, that some plant PA can be hydrolyzed by the action of a nonspecific acid phosphatase (Harwood and Price-Jones, 1988).

The synthetic substrate analogue, *p*NPP (Sommer, 1954), is the most commonly utilized substrate for the *in vitro* estimation of acid phosphatase activity in plant extracts (Duff *et al.*, 1994). It has long been known, however, that the hydrolysis of *p*NPP is not an absolute measure of nonspecific phosphatase activity, since many enzymes have been found that readily hydrolyze this substrate but have little or no activity on naturally occurring substrates which are actively hydrolyzed by nonspecific phosphatases (Stadtman, 1961). In an attempt to discern possible differences in soluble and microsomal PAP activities in the developing seeds of *B. napus*, and to investigate the possibility that soluble PAP activity may be attributable to a nonspecific acid phosphatase, enzyme assays were conducted at pH 5, using 5 mM *p*NPP as the phosphatase substrate (Kilsheimer and Axelrod, 1957; Moss, 1984), with the undialyzed 100,000 x *g* supernatant and microsomal fraction from developing seeds of *B. napus* L. cv Westar as sources of enzyme (Figure 11). Both fractions hydrolyzed *p*NPP in the presence of acetate buffer and initial time courses for both fractions were linear for at least 35 min. Specific activities for the hydrolysis of *p*NPP were 600

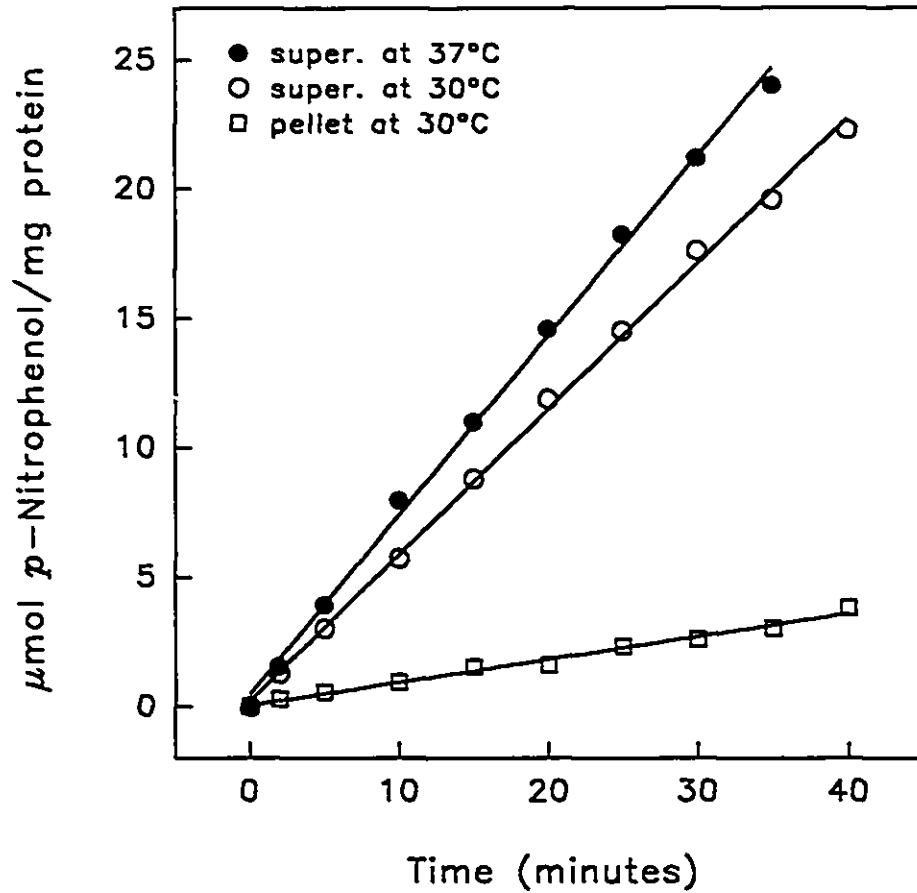


Figure 11. Release of *p*-nitrophenol, resulting from the hydrolysis of *p*NPP, catalyzed by the undialyzed soluble fraction (100,000 x *g* supernatant) and unwashed microsomal fraction (10,000-100,000 x *g* pellet) prepared from the developing seeds of *B. napus* L. cv Westar. Assays were carried out in duplicate at pH 5.0 (1 M sodium acetate buffer), and data points represent the means. The particulate fraction was assayed at 30°C, and the soluble fraction was assayed at both 30°C and 37°C. Assays were conducted with 5 mM *p*NPP, using 23 µg and 16 µg of protein per reaction mixture for the soluble and particulate fractions, respectively.

nmol/min/mg protein and 706 nmol/min/mg protein for the soluble fraction assayed at 30°C and 37°C, respectively. In comparison, specific activity for the hydrolysis of *p*NPP was only 95 nmol/min/mg protein for the microsomal fraction when assayed at 30°C. Based on the differential rates of *p*NPP hydrolysis at 30°C, these preliminary data suggest that a nonspecific acid phosphatase may exist in the soluble fraction obtained from the developing seeds of *B. napus* L. cv Westar. A soluble acid phosphatase, if present, could be responsible for at least some of the PA-hydrolase activity observed in the 100,000 x *g* supernatant obtained from oilseed rape seed homogenate, and may account for the acidic pH optimum associated with the soluble fraction.

It has been suggested that TG formation in oilseeds may be regulated by the intracellular translocation of an ambiquitous form of PAP (Ichihara *et al.*, 1990) in a feedforward manner similar to that which has been established for animal systems (Brindley, 1984, 1988). That is, in the developing seeds of safflower, TG biosynthesis appeared to be regulated by a mechanism involving the translocation of PAP from the cytosol to the microsomal membranes (Ichihara *et al.*, 1990). This intracellular translocation appeared to occur in response to elevated levels of free fatty acids. Ichihara *et al.* (1989) have stressed the importance of insuring that the hydrolysis of PA is not due to a nonspecific acid phosphatase when assays are conducted with crude extracts. Although microsomal PAP has been characterized in the developing seeds of safflower (Ichihara *et al.*, 1989, 1990; Ichihara, 1991), the pH optimum for the cytosolic enzyme has never been reported. In addition, Ichihara *et al.* (1989) showed that the microsomal enzyme was specific for PA, but the substrate specificity of the soluble PA-hydrolase activity from developing safflower seeds was never investigated. In light of the differences in the character of the phosphatase activities associated with the soluble and microsomal fractions obtained from the developing seeds of oilseed rape (Figures 4, 5, 6A, 6B, 7A, 7B, and 11), and the lack of information on the

character of the soluble PA-hydrolase activity in developing seeds of safflower, crude subcellular fractions obtained by differential centrifugation of tissue homogenate were prepared from the developing seeds of safflower and assayed for acid phosphatase activity (Figure 12). At 30°C and in the presence of acetate buffer, the 1,500 x g supernatant hydrolyzed *p*NPP at a rate of 698 nmol/min/mg protein, suggesting the presence of an acid phosphatase. Under the same assay conditions, the rates of *p*NPP hydrolysis catalyzed by the 10,000-100,000 x g particulate and 100,000 x g soluble fractions were 77 nmol/min/mg protein and 764 nmol/min/mg protein, respectively. These specific activities were similar to those obtained for the microsomal and soluble fractions from the developing seeds of *B. napus* L. cv Westar. Clearly, based on the distribution of activity between the 100,000 x g supernatant and 10,000-100,000 x g sediment (Figure 12), *p*NPP-hydrolase activity associated with the 1,500 x g supernatant from developing safflower seeds was predominantly soluble. Thus, as was determined for the developing seeds of oilseed rape (Figure 11), a soluble nonspecific acid phosphatase may also be associated with developing seeds of safflower.

In terms of PA-hydrolase activity in crude subcellular fractions obtained from developing safflower seeds, high activity was again apparent in the soluble fraction when assayed at pH 5 (Figure 13). When assays were conducted at pH 6.75 the activity of apparent soluble PAP was diminished by about 5-fold. In contrast, the apparent PAP activity in the microsomal fraction decreased only by about 2-fold. If microsomal PAP had a pH optimum near neutrality as described by Ichihara *et al.* (1989), then one may have expected an increase in PAP activity in this fraction at pH 6.75. The particulate fraction, however, was not washed and thus the higher level of PAP activity associated with the microsomal fraction at pH 5 may be attributable to nonspecific acid phosphatase activity. The current observations of soluble phosphatase activity in developing safflower seeds suggest that the quantitation of apparent soluble PAP activity by Ichihara *et al.* (1990) may have been complicated by interference from

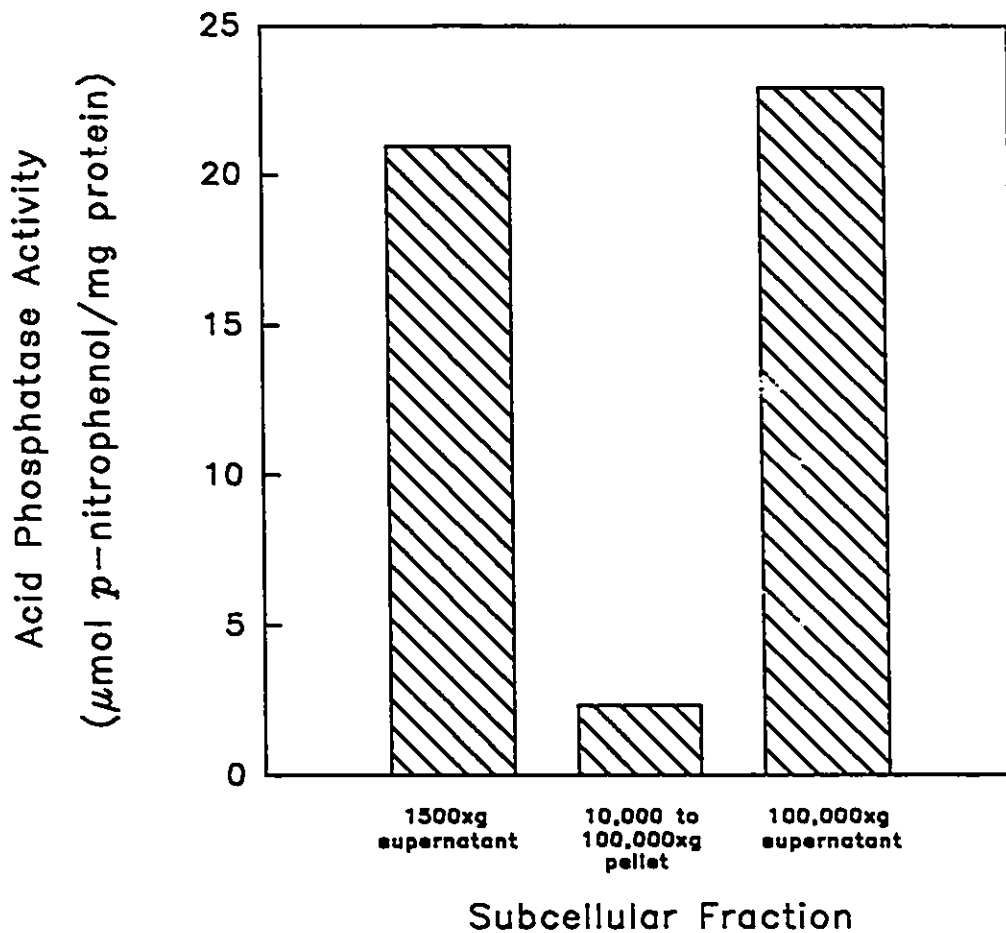


Figure 12. Acid phosphatase activity in crude subcellular fractions obtained by the differential centrifugation of tissue homogenate prepared from the developing seeds of safflower. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**. Assays were conducted at 30°C, for 30 min, at pH 5.0 (sodium acetate buffer), and activity data represent the means of triplicate assays. Assays were initiated by the addition of extract to the reaction mixture, using 3 μg, 49 μg, and 11 μg of protein for the 1,500 x g supernatant, 10,000-100,000 x g pellet, and 100,000 x g supernatant, respectively.

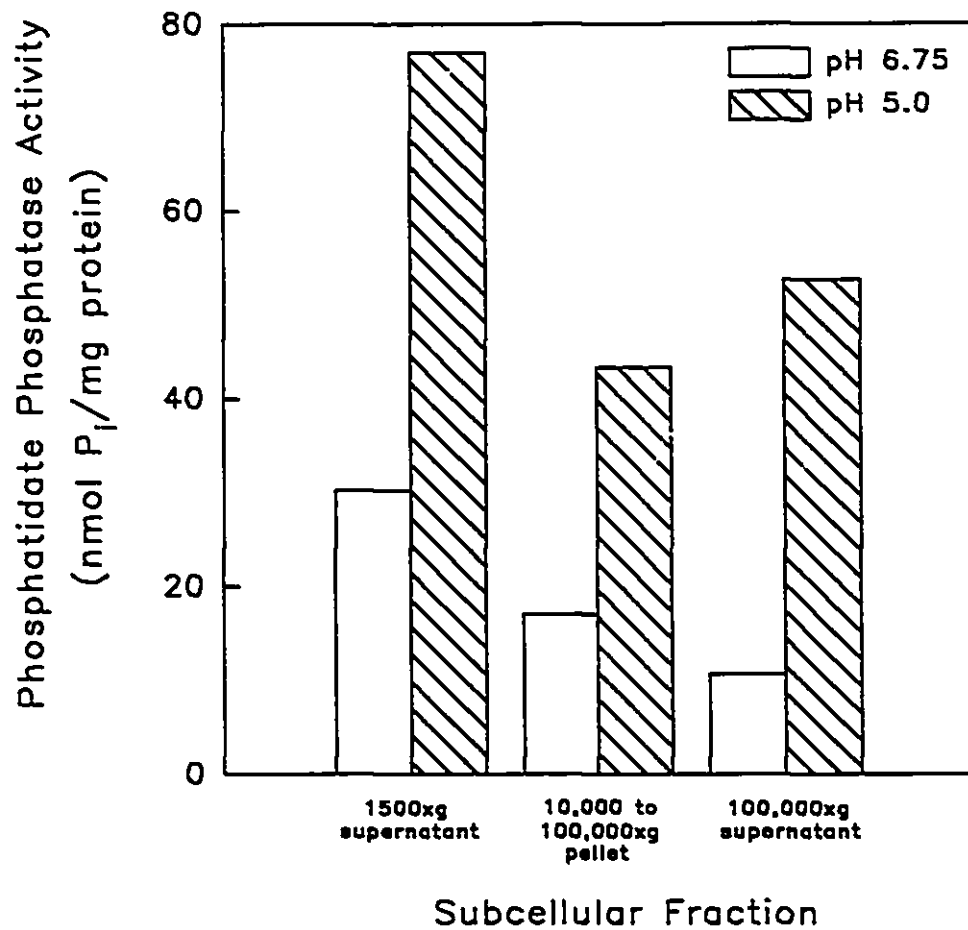


Figure 13. PA-hydrolase activity in crude subcellular fractions obtained by the differential centrifugation of tissue homogenate prepared from the developing seeds of safflower. Tissue homogenization and subsequent fractionation were carried out as described in **MATERIALS AND METHODS**. Assays were conducted at 30°C, for 30 min, at pH 6.75 and pH 5.0 using Tris-maleic acid-NaOH buffers. Assays were initiated by the addition of extract to the reaction mixture, using 66 μg , 49 μg , and 265 μg of protein for the 1,500 x g supernatant, 10,000-100,000 x g pellet, and 100,000 x g supernatant, respectively. Activity data represent the means of triplicate assays.

nonspecific acid phosphatase activity. These preliminary results, however, should be investigated further using freshly harvested seeds to prepare particulate fractions which have been washed to remove residual soluble enzyme activity.

One of the best ways to discern possible differences in the character of two or more phosphatases is to compare their activities towards both the physiologically relevant substrate and the synthetic substrate, *p*NPP (Yupsanis *et al.*, 1993). Although both substrates may be hydrolyzed by each of the enzymes, the enzyme which shows greatest activity towards the physiologically important substrate, in relation to *p*NPP, may be considered to show specificity for the natural substrate. In order to further characterize the microsomal and soluble phosphatase activities in *B. napus*, assays were conducted at both pH 6.75 and pH 5 using *p*NPP and PA as phosphatase substrates.

Time courses for the hydrolysis of *p*NPP, at pH 6.75 and pH 5, catalyzed by the washed microsomal fraction and dialyzed soluble fraction prepared from developing seeds of *B. napus* L. cv Westar are shown in Figure 14. In every case the release of *p*-nitrophenol was linear for at least 90 min. At 30°C, the specific activities for the hydrolysis of *p*NPP catalyzed by the microsomal fraction were 70 nmol/min/mg protein and 98 nmol/min/mg protein at pH 6.75 and pH 5, respectively. In contrast, the specific activities for the hydrolysis of *p*NPP catalyzed by the soluble fraction, at 30°C, were 293 nmol/min/mg protein and 502 nmol/min/mg protein at pH 6.75 and pH 5, respectively. Preincubating the soluble fraction, which had previously been dialyzed against Tris-maleate-NaOH buffer (pH 6.75), at pH 5 for 30 min prior to being assayed had no effect on the rate of *p*NPP hydrolysis. Although pH had little effect on the *p*NPP-hydrolase activity associated with the microsomal fraction, the relatively high rate of *p*NPP hydrolysis catalyzed by the soluble fraction was 71% greater at pH 5 than at pH 6.75.

Time courses for the hydrolysis of PA, at pH 6.75 and pH 5, catalyzed by the washed microsomes and dialyzed soluble fraction prepared from the developing seeds

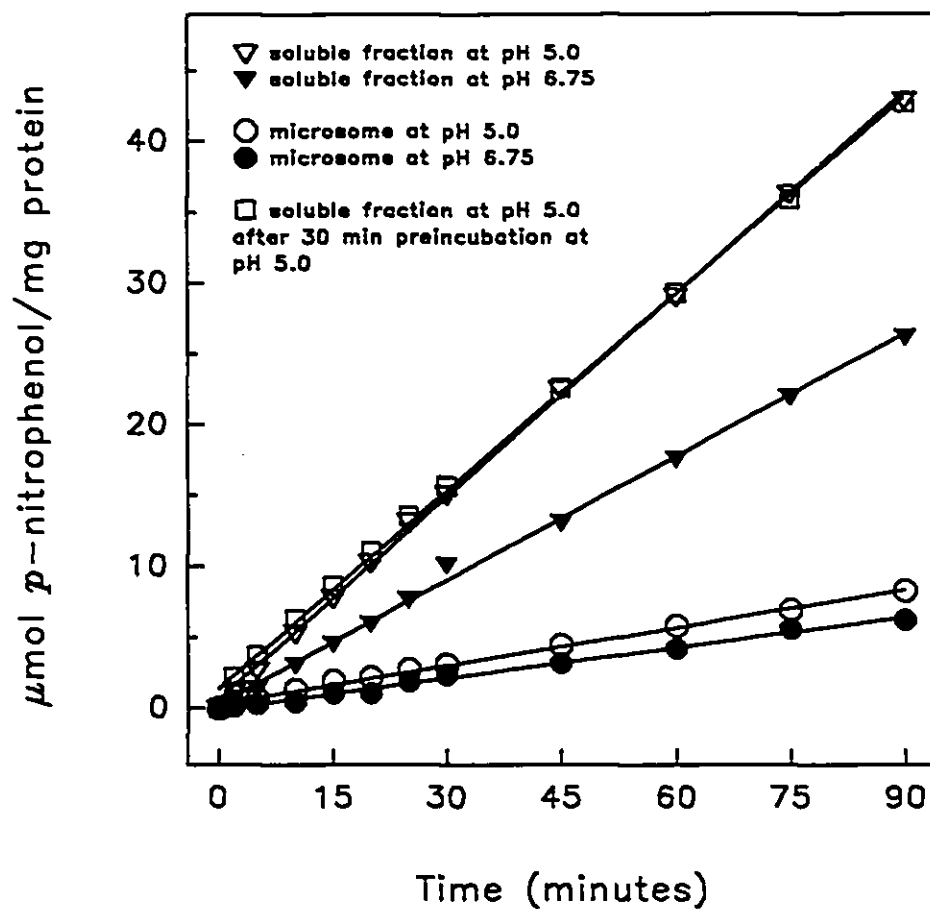


Figure 14. Release of *p*-nitrophenol, resulting from the hydrolysis of *p*NPP at pH 5.0 and pH 6.75, catalyzed by the dialyzed soluble fraction and washed microsomal fraction prepared from the developing seeds of *B. napus* L. cv Westar. Assays were carried out in triplicate at 30°C, and data points represent the means. The soluble fraction was also assayed at pH 5.0 after a 30°C, 30 min preincubation at pH 5.0. Assays were conducted with 5 mM *p*NPP, using 4 µg and 7 µg of protein per reaction mixture for the soluble and particulate fractions, respectively.

of *B. napus* L. cv Westar are shown in Figure 15. The release of P_i catalyzed by the microsomal fraction was linear for at least 90 min at both pH 6.75 and pH 5. Additionally, the hydrolysis of PA catalyzed by the soluble fraction was linear for at least 90 min at pH 6.75. In contrast, the release of P_i catalyzed by the soluble fraction at pH 6.75 was curvilinear. This relationship may possibly have resulted because of substrate depletion associated with the high rate of hydrolysis. At 30°C, the specific activities for the hydrolysis of PA catalyzed by the microsomal fraction was 2.9 nmol/min/mg protein at both pH 6.75 and pH 5. The specific activity, at 30°C, was 2.8 nmol/min/mg protein for the hydrolysis of PA catalyzed by the soluble fraction at pH 6.75. Based on the initial, linear portion of the curve, the specific activity for the hydrolysis of PA catalyzed by the soluble fraction at pH 5 and 30°C was 12.8 nmol/min/mg protein. The soluble fraction from developing seeds of oilseed rape was 4 times more effective in catalyzing the hydrolysis of *p*NPP at pH 5 than at pH 6.75.

Time courses for the hydrolysis of *p*NPP, at pH 6.75 and pH 5, catalyzed by the washed microsomal fraction and dialyzed soluble fraction prepared from MD embryos of *B. napus* L. cv Topas are shown in Figure 16. In each case the release of *p*-nitrophenol was linear for at least 90 min. At 30°C, the specific activities for the hydrolysis of *p*NPP catalyzed by the microsomal fraction were 22 nmol/min/mg protein and 43 nmol/min/mg protein at pH 6.75 and pH 5, respectively. In comparison, the specific activities for the hydrolysis of *p*NPP catalyzed by the soluble fraction, at 30°C, were 147 nmol/min/mg protein and 271 nmol/min/mg protein at pH 6.75 and pH 5, respectively. The relatively high rate of *p*NPP hydrolysis catalyzed by the soluble fraction was 84% greater at pH 5 than at pH 6.75. Although pH had some effect on the *p*NPP-hydrolase specific activity associated with the microsomal fraction, in terms of total activity the effect was very small. The overall relationship between pH and the hydrolysis of *p*NPP catalyzed by the microsomal and soluble fractions from MD embryos of *B. napus* L. cv Topas (Figure 16) was very similar to that observed

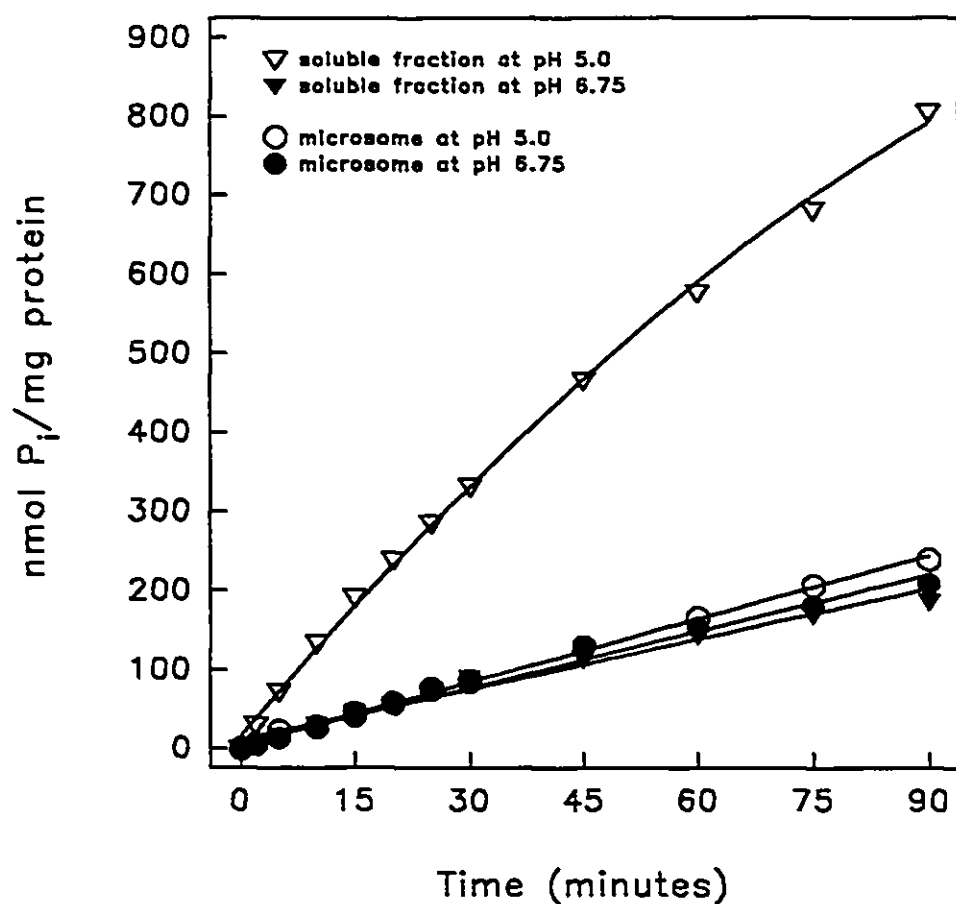


Figure 15. Release of P_i , resulting from the hydrolysis of PA at pH 5.0 and pH 6.75, catalyzed by the dialyzed soluble fraction and washed microsomal fraction prepared from the developing seeds of *B. napus* L. cv Westar. Assays were conducted at 30°C, using 190 μg and 90 μg of protein per reaction mixture for the soluble and particulate fractions, respectively. Data points represent the means of triplicate assays.

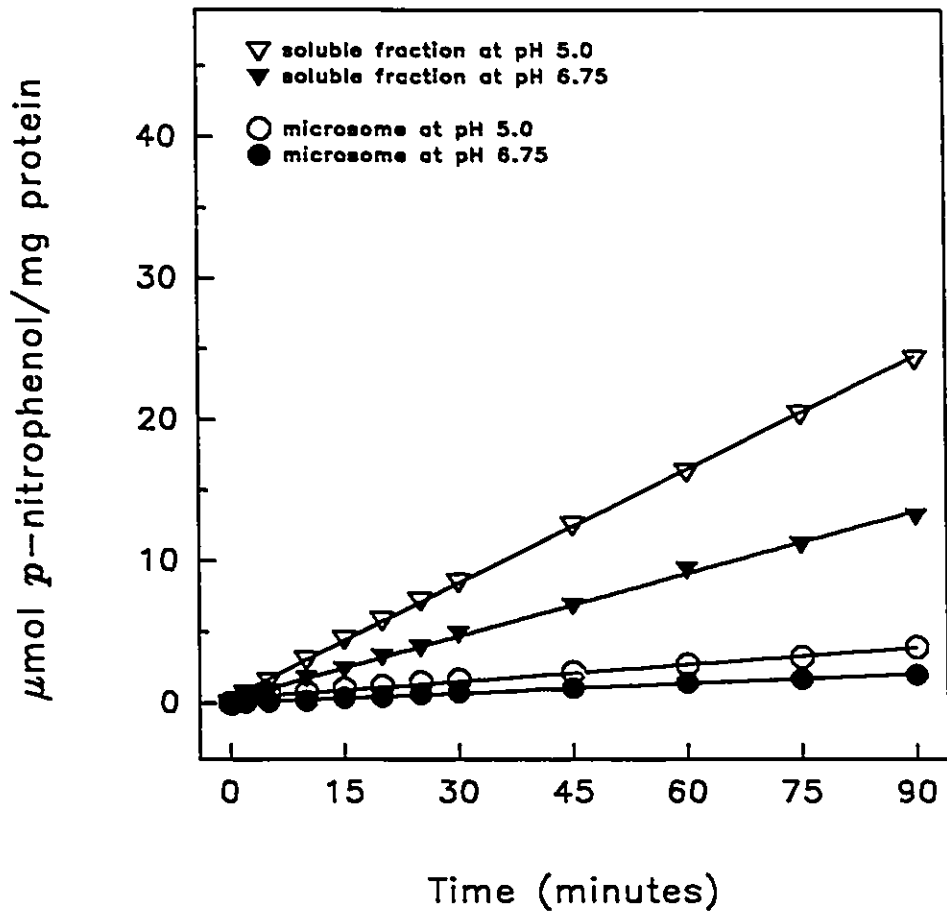


Figure 16. Release of *p*-nitrophenol, resulting from the hydrolysis of *p*NPP at pH 5.0 and pH 6.75, catalyzed by the dialyzed soluble fraction and washed microsomal fraction prepared from MD embryos of *B. napus* L. cv Topas. Assays were conducted at 30°C, with 5 mM *p*NPP, using 3 μg and 11 μg of protein per reaction mixture for the soluble and particulate fractions, respectively. Data points represent the means of triplicate assays.

when using the same crude subcellular fractions prepared from developing seeds of *B. napus* L. cv Westar (Figure 14).

Finally, shown in Figure 17 are time courses for the hydrolysis of PA, at pH 6.75 and pH 5, catalyzed by the washed microsomes and dialyzed soluble fraction prepared from MD embryos of *B. napus* L. cv Topas. In every case the release of P_i was linear for at least 90 min. At 30°C, specific activities for the hydrolysis of PA catalyzed by the microsomal fraction were 4.6 nmol/min/mg protein and 1.4 nmol/min/mg protein at pH 6.75 and pH 5, respectively. In comparison, specific activities for the hydrolysis of PA catalyzed by the soluble fraction, at 30°C, were 0.7 nmol/min/mg protein and 0.9 nmol/min/mg protein at pH 6.75 and pH 5, respectively. The microsomal fraction obtained from MD embryos of oilseed rape was at least 3 times more effective in catalyzing the hydrolysis of PA at pH 6.75 than at pH 5.

In general, there was a greater increase in the rate of hydrolysis of *p*NPP by the soluble fraction in comparison to the particulate fraction when comparing reaction rates at pH 5 to those at pH 6.75. Thus, the hydrolysis of *p*NPP at two different pH values was used to demonstrate that there were differences in the overall character of phosphatase activity between the microsomal and soluble fractions prepared from *B. napus* tissues. Given the higher pH optimum and apparent specificity of the microsomal enzyme for PA in relation to *p*NPP, it is most likely that, if there are indeed different enzymes, the microsomal enzyme represents the Mg^{2+} -dependent PAP important to glycerolipid metabolism.

Solubilization of Microsomal PAP from MD Embryos of Oilseed Rape

Microsomal PAP was chosen for solubilization and further characterization because this form of the enzyme would most likely participate in the formation of TG within the ER where other Kennedy pathway enzymes are located (Stymne and Stobart, 1987). MD embryos, cultured for 14-21 days, were chosen as the tissue source of PAP

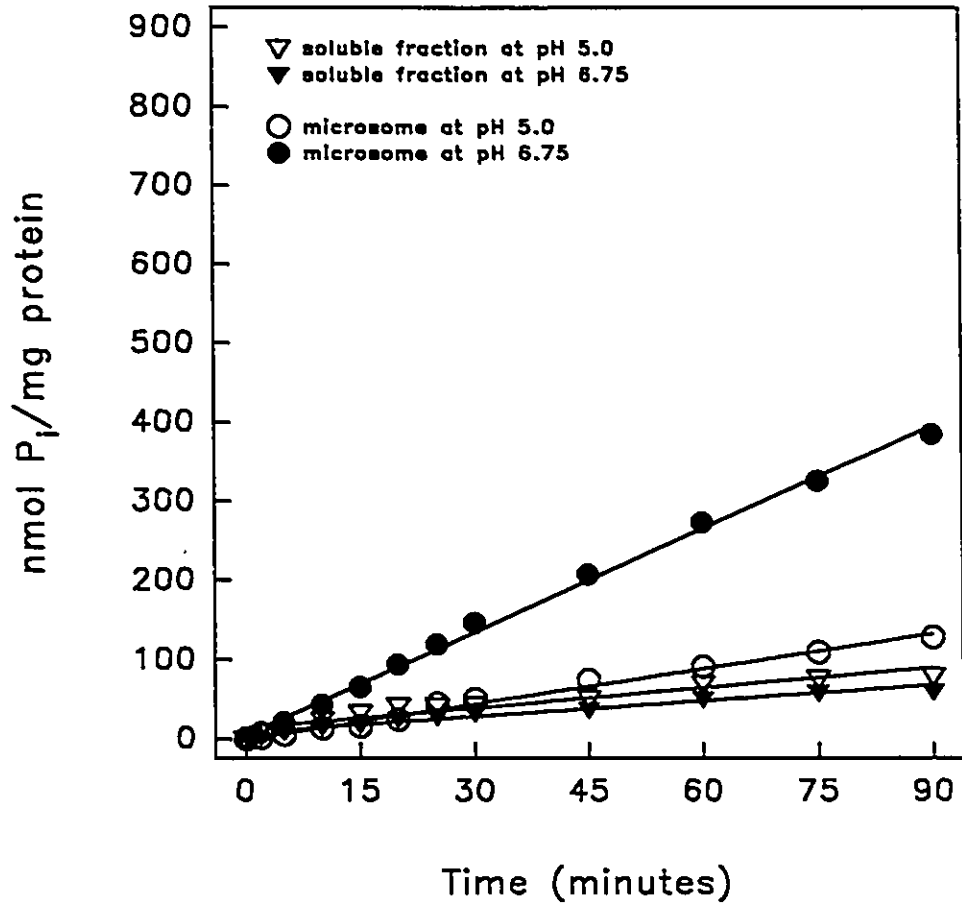


Figure 17. Release of P_i , resulting from the hydrolysis of PA at pH 5.0 and pH 6.75, catalyzed by the dialyzed soluble fraction and washed microsomal fraction prepared from MD embryos of *B. napus* L. cv Topas. Assays were conducted at 30°C, using 170 μg and 135 μg of protein per reaction mixture for the soluble and particulate fractions, respectively. Data points represent the means of triplicate assays.

since they were available on a nearly continuous basis. MD embryos of this age have previously been shown to rapidly accumulate TG (Pomeroy *et al.*, 1991). In the past few years, MD cultures of oilseed rape have proven very useful in the study of TG biosynthesis and associated enzymes (Pomeroy *et al.*, 1991; Taylor *et al.*, 1990, 1991, 1992a, 1992c; Weselake *et al.*, 1991, 1993; Little *et al.*, 1994). The MD cultures also appear to be a convenient source for the purification of these enzymes since they are easily manipulated and maintained in culture. The pH optimum for PAP activity in washed microsomes from MD embryos (Figure 18) was similar to the pH optimum determined for the microsomal form of the seed enzyme (Figure 4). The soluble fraction from MD embryos was avoided in further studies of PAP due to the possible presence of nonspecific acid phosphatase activity .

A number of different detergents (cationic, anionic, zwitterionic and nonionic) were screened for their ability to solubilize PAP from MD embryos of oilseed rape (Table V). Microsomes were preincubated with concentrations of 0.1% (w/v) and 1% (w/v) detergent prior to separating solubilized proteins from insoluble material using centrifugation. Enzyme remaining in the supernatant following centrifugation at 105,000 x g for 60 min was defined as solubilized enzyme (Hjelmeland, 1990). PAP activity was assayed in the total mixture before centrifugation and in the supernatant recovered after centrifugation. Assays of total PAP activity following detergent treatment gave an indication of the sensitivity of PAP activity to detergent type. Both cetyltrimethylammonium bromide (CTAB) and SDS strongly inhibited PAP activity at concentrations of both 0.1% and 1% whereas Tween 20 was somewhat stimulatory at both concentrations. Octanoyl-N-methylglucamide (MEGA-8) appeared stimulatory at a concentration of 0.1%. Tween 20 (1%) was also effective in solubilizing more than 50% of the enzyme. Octyl- β -D-glucopyranoside (1%) solubilized 74% of the PAP but resulted in strong inhibition of enzyme activity.

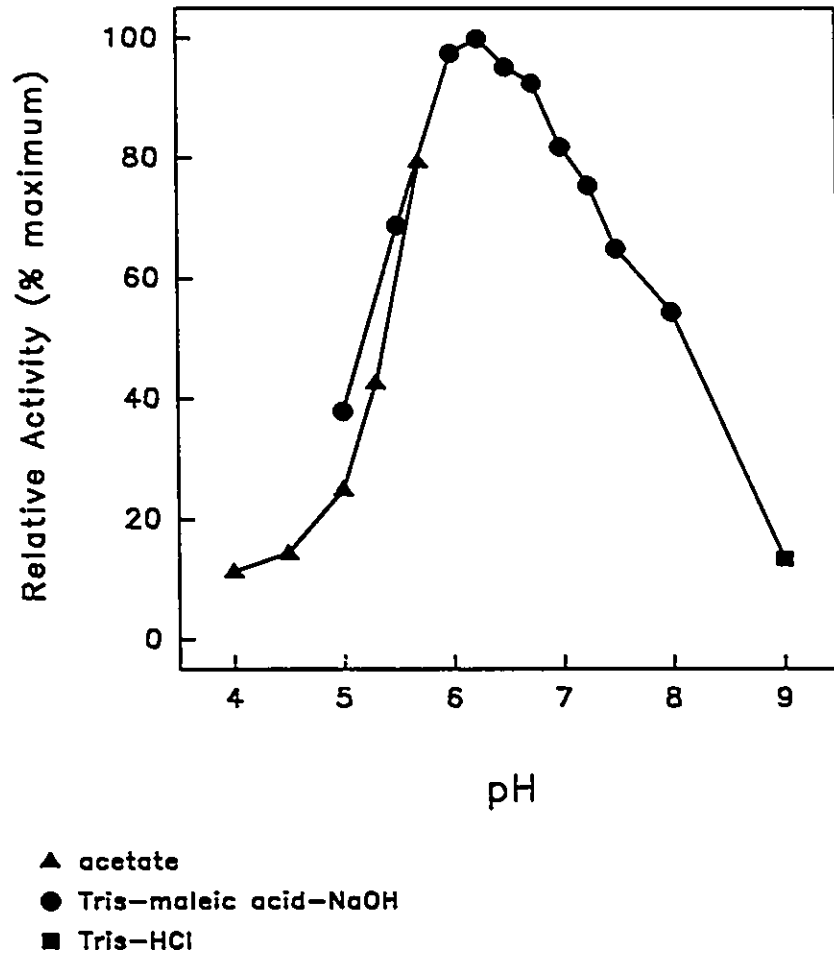


Figure 18. pH dependence of PAP activity using the microsomal fraction from MD embryos of *B. napus* L. cv Topas. Assays were carried out at 30°C, for 30 min, with 590 μg protein per reaction mixture. Buffers used were 50 mM acetate, 50 mM Tris/50mM maleic acid-NaOH, and 50 mM Tris-HCl. Data points represent the means of triplicate assays.

Table V. Solubilization of phosphatidate phosphatase by various detergents. Washed microsomes obtained from MD embryos of *B. napus* L. cv Topas, as described in **MATERIALS AND METHODS**, were preincubated on ice for 1 h at pH 6.75 with 0, 0.1, and 1% detergent. Data are the average for triplicate exposures assayed at pH 6.75 for 30 min at 30°C, with a TCA inactivated control for each exposure. In each reaction tube, 50 μ L of detergent treated microsomal fraction or 105,000 x g supernatant were used.

Treatment	Detergent Concentration	Total Activity of Microsome	Activity of Solubilized Enzyme	Relative Solubilization
	% (w/v)	% detergent-free control	% activity of microsome	% total activity of detergent-free microsome
no detergent	0	100	0	0
CTAB	0.1	14	26	4
	1	0	0	0
CHAPS	0.1	77	17	13
	1	46	11	5
CHAPSO	0.1	74	14	10
	1	36	20	7
Sodium DOC	0.1	20	40	8
	1	26	61	16
n-Dodecyl B-D-maltoside	0.1	49	22	11
	1	0	0	0
SDS	0.1	13	75	10
	1	0	0	0
MEGA-8	0.1	112	4	4
	1	81	10	8
n-Octyl B-D-glucopyranoside	0.1	104	4	4
	1	19	74	14
Triton X-100	0.1	86	32	28
	1	86	31	27
Tween 20	0.1	113	33	37
	1	128	54	69

Tween 20 was chosen for further solubilization studies since treatment of microsomes with this detergent resulted in reasonable solubilization of PAP with concomitant stimulation of enzyme activity. The effect of increasing Tween 20 concentration on the solubilization of PAP is shown in Figure 19. The total activity of PAP following detergent treatment increased by more than 20% between 0 and 1% (w/v) Tween 20. It is possible that the carry-over of detergent into the reaction mixture altered the dispersion of substrate thus resulting in increased enzyme activity. Usually, 0-5% of the PAP was released from the microsomes without detergent (following resuspension of microsomes). The lowest concentration of Tween 20 required to maximally solubilize PAP was 0.4% which corresponded to a detergent to protein ratio of about 1:1 (w/w). The specific activity of solubilized PAP was greatest in the range of 0.2% to 0.8% Tween 20. In general, the specific activity of PAP was increased by about 2 to 3-fold over the microsome following solubilization with 0.4% Tween 20. Concentrations of Tween 20 beyond 1% were not effective in solubilizing more PAP activity. The higher concentrations of detergent also markedly decreased the specific activity of solubilized PAP due to further solubilization of impurities.

In general, membrane proteins are most effectively solubilized at concentrations above the critical micellar concentration (CMC) of the detergent used for solubilization (Thomas and McNamee, 1990). Microsomal PAP was partially solubilized above the CMC of Tween 20. The CMC of Tween 20 is 0.006% (w/v) (Findlay, 1990). Königs and Heinz (1974) have used detergent-treatment to solubilize PAP from a 15,000 x g fraction of broad bean leaves which was enriched in mitochondria and microbodies. In the current study, isolation of PAP from the 10,000 to 100,000 x g particulate fraction presumably helped in avoiding mitochondrial PAP.

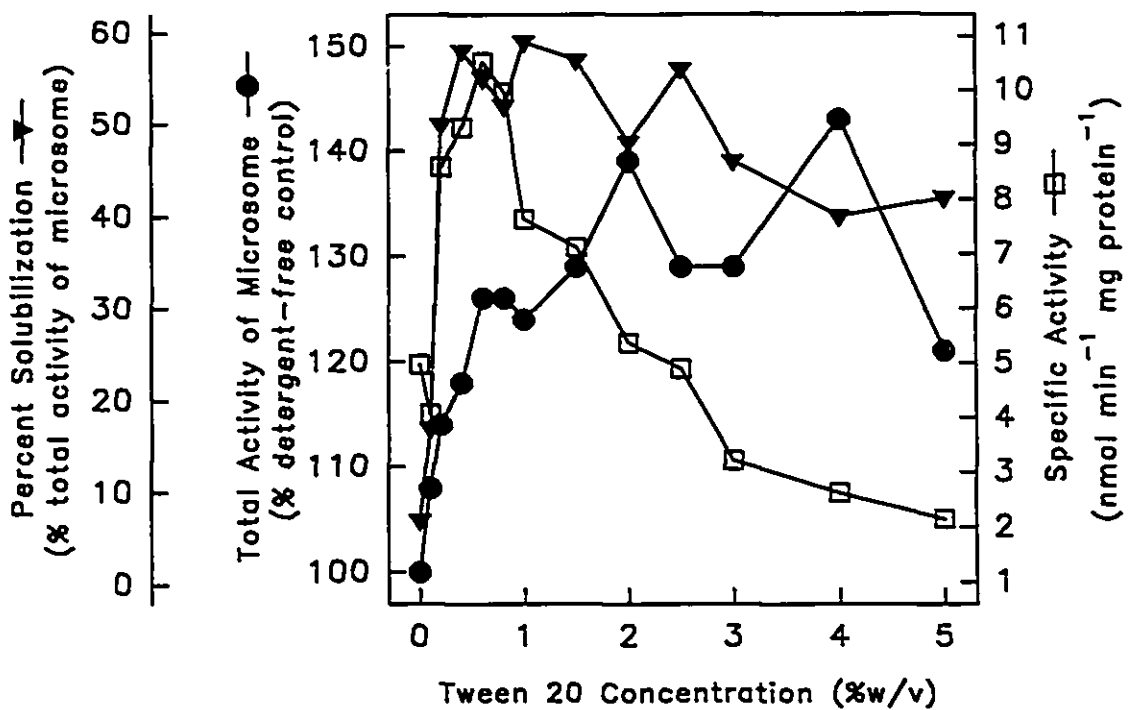


Figure 19. Solubilization of PAP from microsomes of MD embryos of *B. napus* L. cv Topas at various concentrations of Tween 20. The solubilized enzyme was recovered in the supernatant following centrifugation at 105,000 x *g* for 60 min. Detergent treatments were in duplicate and each extract was in turn assayed in duplicate. Therefore, data represent the average of two experiments. When determining the total PAP activity of the microsome, as a function of Tween 20 concentration, 213 μ g of protein were used per reaction mixture.

Characterization of Solubilized PAP

The effect of pH on the activity of solubilized microsomal PAP is shown in Figure 20. The pH dependence of the solubilized enzyme was similar to that of the particulate enzyme (Figure 18) with optimal activity occurring between pH 6 and 7.

The assay of solubilized PAP activity was further characterized by monitoring the appearance of DG in the reaction mixture. The formation of DG was examined by TLC at intervals over a 135 min reaction period (Figure 21, lanes b to e). Under these conditions, *sn*-1,2-DG appeared to steadily accumulate in the reaction mixture for about 90 min. Using the described solvent system, PA and PC remained at the origin of the TLC plate. The microsome contained high levels of DG (lane a) which interfered with attempts to monitor newly generated DG via the PAP reaction. Indeed, a previous study by Weselake et al. (1991) has indicated that endogenous DGs can serve as effective substrates for particulate diacylglycerol acyltransferase. In contrast, solubilized PAP contained a considerably lower level of DG as indicated by the lipid status at the start of the reaction (lane b). The lipid profile obtained following incubation (135 min) of solubilized PAP without addition of exogenous PA (lane f) was identical to the lipid profile obtained with PA at the start of the reaction (lane b). This result suggested that PA was not converted to DG due to phospholipase D activity. The possible action of phospholipase C on PC was also ruled out because DG was not generated from PC. In addition, incubation of solubilized PAP with PA and PC did not lead to accumulation of additional fatty acids in the reaction mixture (lanes c to e). Based on studies with rat liver microsomes, Sturton and Brindley (1978) have cautioned that PAP activity in crude extracts may be overestimated due to dephosphorylation of G3P generated by the deacylation of PA by phospholipases A and B. Deacylation of PA catalyzed by the solubilized fraction would have led to a build up of fatty acids in the reaction mixture which would have been detected following TLC.

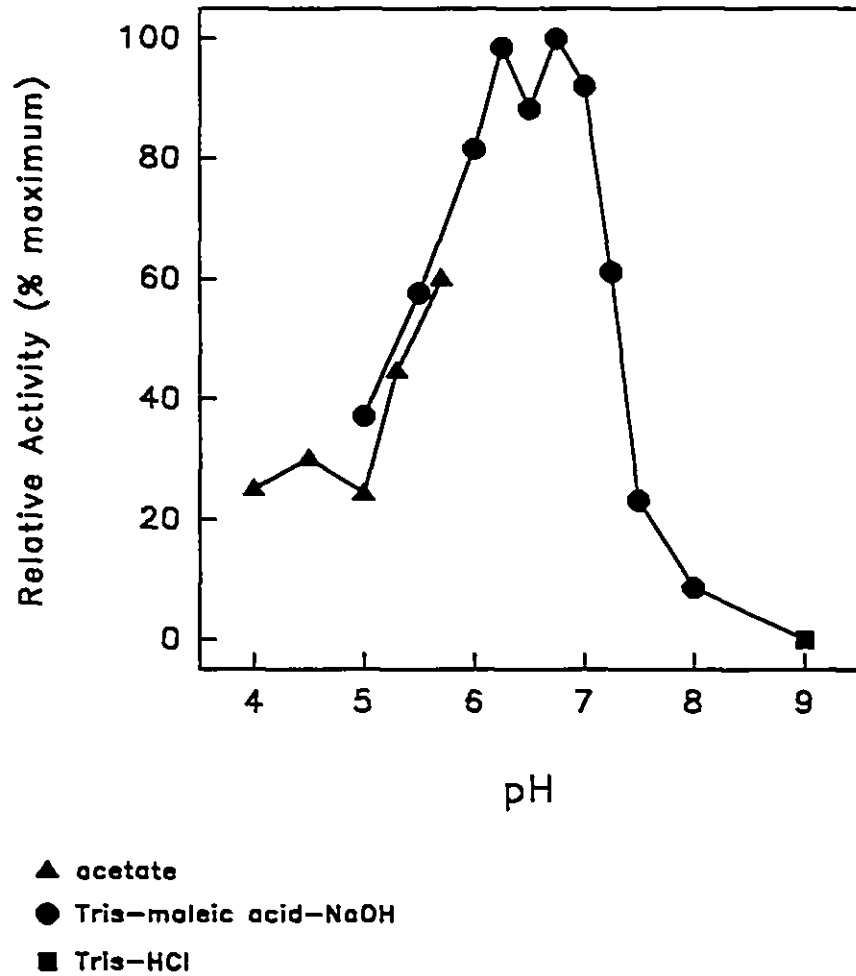
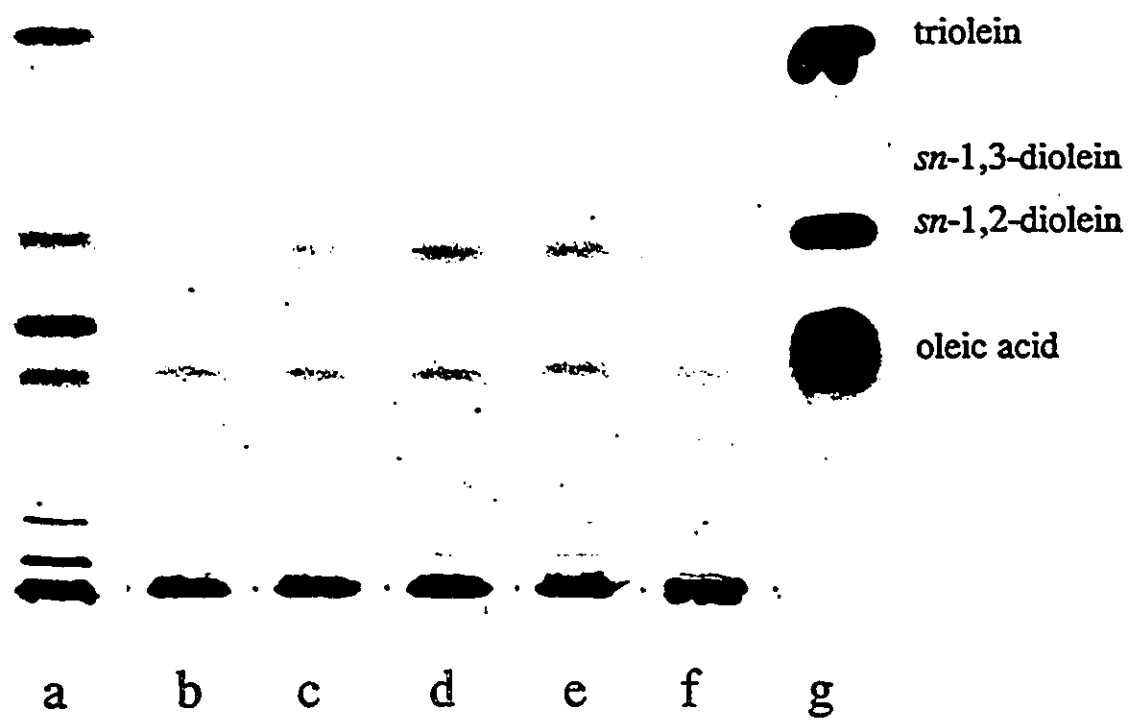


Figure 20. pH dependence of solubilized PAP from MD embryos of *B. napus* L. cv Topas. Assays were conducted with 100 μ L of 105,000 \times g supernatant per reaction tube, and were carried out for 30 min at 30°C. Buffers used were 50 mM acetate, 50 mM Tris/50 mM maleic acid-NaOH, and 50 mM Tris-HCl. Data points represent the means of triplicate assays.

Figure 21. Analysis by TLC of the products of PA hydrolysis catalyzed by PAP activity in the solubilized fraction from MD embryos of *B. napus* L. cv Topas. Enzyme reactions and extraction of lipids were conducted as described in **MATERIALS AND METHODS**. The TLC plates were irrigated with one ascent of chloroform/methanol/acetic acid (195:4:1, v/v/v). Lipids were visualized by spraying the plates with 30% (w/w) sulfuric acid followed by heating to 145°C for a few minutes. Lane (a): complete reaction mixture containing microsomes (740 µg protein), t = 0. Lanes (b) to (e): complete reaction mixture with solubilized PAP (90 µg protein) incubated for 0, 45, 90, and 135 min, respectively. Lane (f): solubilized PAP incubated for 135 min without PA (with PC and BSA). Lane (g): lipid standards.



The M_r of solubilized PAP was examined by gel filtration chromatography on a column of Superose 6 (HR 10/30). The elution profile of PAP activity and absorbance at 280 nm are shown in Figure 22. The calibration plot generated by passing M_r markers through the column is shown in Figure 23. The elution volume of PAP corresponded to an apparent M_r of about 300,000. The elution volumes of the standards remained the same regardless of whether chromatography was conducted in the absence or presence of 0.2% (w/v) Tween 20. Gel filtration chromatography confirmed that microsomal PAP was solubilized since the enzyme eluted within the sieving range of Superose 6.

Recently, a form of PAP involved in cell signalling was purified to homogeneity from porcine thymus membranes (Kano et al., 1992). The native M_r of the porcine enzyme was estimated to be about 218,000 by Superose 12 gel filtration chromatography in the presence of 1% (w/v) Triton X-100. Gel filtration chromatography of a Mg^{2+} -dependent PAP solubilized from rat liver microsomes resulted in an apparent M_r of about 500,000 when chromatography was conducted in the presence of 0.075% Tween 20 (Ide and Nakazawa, 1985). In contrast, Superose 12 gel filtration chromatography of a Mg^{2+} -dependent PAP, solubilized from yeast microsomes, resulted in M_r of 93,000. Chromatography of the yeast PAP was conducted in the presence of 1% sodium cholate. SDS-PAGE indicated that this form of yeast PAP was a monomer.

Inhibition of Microsomal PAP Activity by Polyclonal Antibodies

Polyclonal antibodies, raised in mice, against crude extracts of microsomes prepared from MD embryos of oilseed rape were previously shown to inhibit DGAT activity (Weselake, 1994). In an effort to develop an immunological probe for PAP, the same antisera were used to test for inhibition of PAP activity in microsomes of MD embryos. Preparation of the crude native antigen involved extraction of membranes

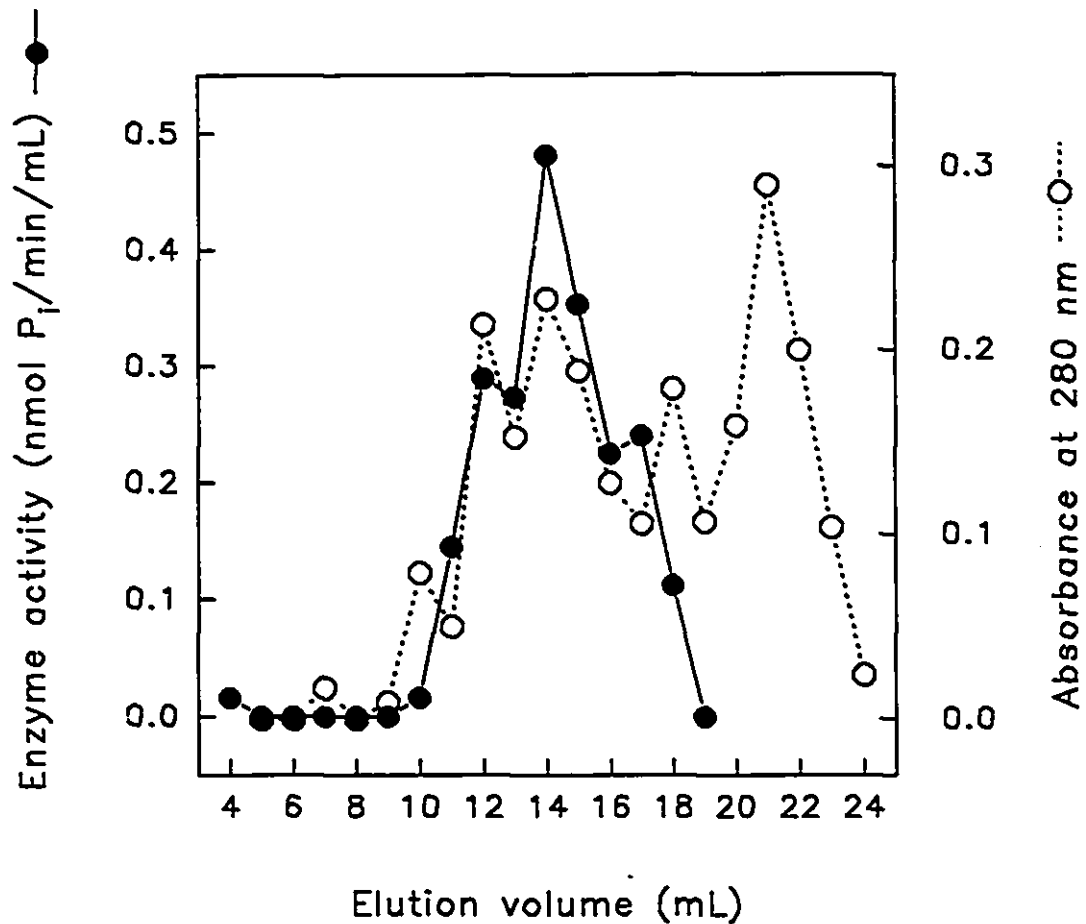


Figure 22. Superose 6 gel filtration chromatography of solubilized PAP from MD embryos of *B. napus* L. cv Topas. The column was equilibrated with 60 mM Tris/60 mM maleic acid-NaOH buffer (pH 6.75) containing 2.5 mM MgCl₂, 0.12 mM EDTA, 0.1 M KCl, and 0.2% (w/v) Tween 20. Solubilized microsomal PAP (200 μ L containing 420 μ g of protein) was injected on to the column which was eluted at a flow rate of 0.2 mL/min using an FPLC system. Fractions of 1 mL were collected and assayed for PAP activity.

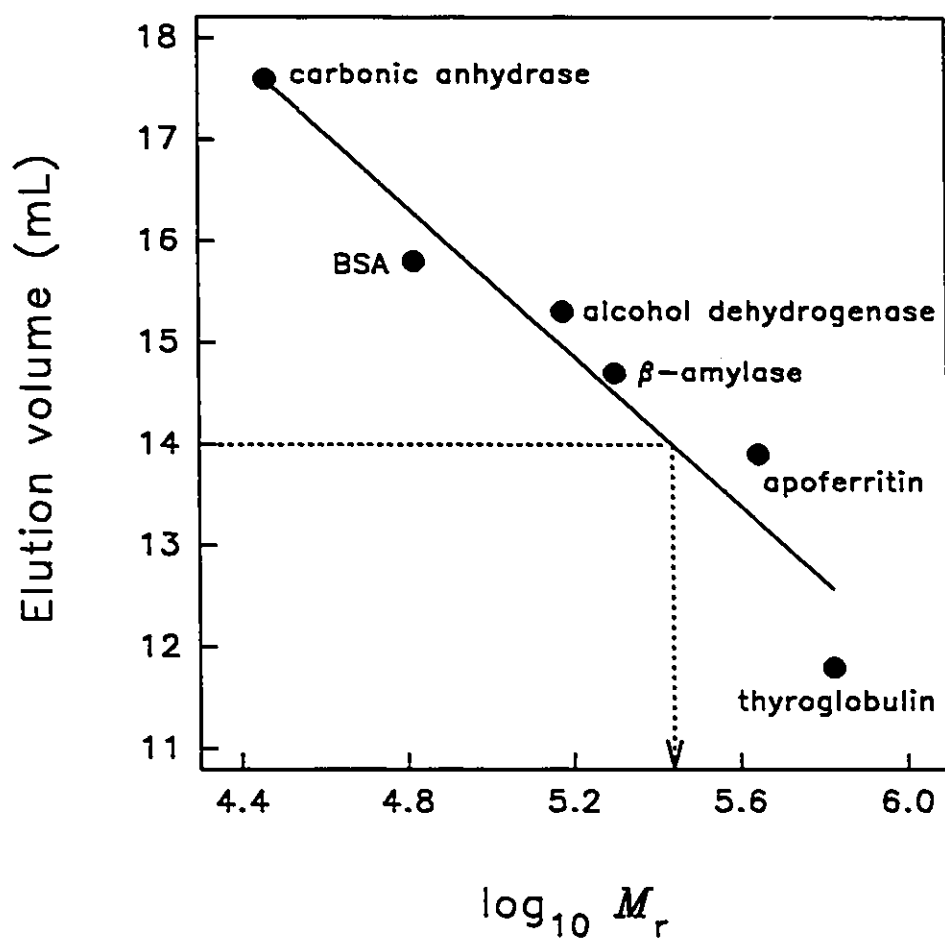


Figure 23. Superose 6 gel filtration selectivity curve showing elution volume versus $\log_{10} M_r$ for molecular weight standards. Also shown is the the $\log_{10} M_r$ corresponding to the elution position obtained for PAP solubilized from the microsomal membranes prepared from MD embryos of oilseed rape (*B. napus* L. cv Topas).

with 1% (w/v) MEGA-8 in the presence of 20% (w/v) glycerol and 2 M NaCl. It was previously shown that a concentration of 1% (w/v) MEGA-8 solubilized about 10% of the PAP from the microsomes of MD embryos (Table V). The solubilization experiments shown in Table V, however, were conducted at pH 6.75 and did not include glycerol or a high concentration of NaCl in the solubilization mixtures. Therefore, it is possible that the crude antigen used for immunization of mice contained more PAP than indicated by the solubilization experiments depicted in Table V.

Polyclonal antibodies raised against the MEGA-8 extract of microsomes resulted in about 20% inhibition of microsomal PAP activity (Table VI, antiserum A). Polyclonal antibodies raised against delipidated/SDS-treated microsomes resulted in less inhibition of microsomal PAP activity (antiserum B). Mice immunized with 3 injections of delipidated/SDS-treated membranes and a final injection of native antigen resulted in the production of antiserum which inhibited PAP activity to the same extent as antiserum derived from immunization with only MEGA-8 extract (antiserum C). Additional control experiments (data not shown), under the described assay conditions, ruled out possible interference by serum phosphatase and other serum proteins. It would have been useful to conduct inhibition tests over a range of antiserum concentrations but the small volume of mouse antiserum obtained by ocular bleed limited the number of tests that could be performed. These preliminary results, however, indicated that polyclonal antibodies inhibiting PAP activity could be developed using crude extracts of microsomes. Fusion of spleen cells from these mice with myeloma cells to produce hybridoma cells (Kohler and Milstein, 1975) might lead to the development of inhibitory monoclonal antibodies which in turn could be used for screening a cDNA expression library for clones encoding PAP. If inhibitory monoclonal antibodies were developed, it might also be possible to further purify solubilized PAP using immobilized antibodies.

Table VI. Inhibition of microsomal phosphatidate phosphatase by mouse antisera. Washed microsomes (10 μ L containing 74 μ g of protein), prepared from MD embryos of *B. napus* L. cv Topas, were preincubated for 30 min at 30°C (pH 6.75) with 5 μ L of mouse antiserum obtained as described in **MATERIALS AND METHODS**, and then assayed for PAP activity. Prior to preincubation, antisera were dialyzed against Tris-maleic acid-NaOH buffer (pH 6.75) to decrease the levels of endogenous P_i . Antisera were obtained from a control mouse (Control), a mouse which received 4 injections of solubilized native microsomal protein (A), a mouse which received 4 injections of delipidated, SDS-treated microsomal protein (B), and a mouse which received 3 injections of the SDS-treated protein and a final injection of the native protein (C). Assays were initiated by the addition of substrate to the incubation mixture, and were conducted at 30°C, for 60 min, at pH 6.75. Data represent the means of triplicate assays.

Antiserum	Net Absorbance at 820 nm	PAP Activity (% Control)
Control	0.091	100
A	0.075	82
B	0.084	92
C	0.075	82

CONCLUSIONS AND FUTURE DIRECTIONS

The characteristics of microsomal and soluble PAP were determined in developing seeds of oilseed rape. Soluble PAP appeared to be due to the action of a nonspecific acid phosphatase whereas microsomal PAP had a pH optimum near neutrality. The occurrence of apparent soluble PAP with an acidic pH optimum suggests that investigators should be cautious in ascribing a physiological role to the soluble enzyme in TG biosynthesis. The soluble enzyme, however, does utilize PA as a substrate and therefore it is possible that soluble PAP may have a role in lipid metabolism in developing seeds of oilseed rape. Further studies focused on microsomal PAP which was located in the same fraction as other Kennedy pathway enzymes. PAP was solubilized from the microsomes of MD embryos of oilseed rape using Tween 20. The solubilized enzyme produced DG without interference due to endogenous phospholipase action.

Solubilization of PAP from the ER of plant tissue, involved in TG accumulation, is a critical step in the purification of this important Kennedy pathway enzyme. Purification of microsomal PAP will eventually lead to the identification of the gene encoding PAP. As well, development of monoclonal antibodies, which are inhibitory to PAP, may serve as probes to identify cDNA encoding the enzyme thereby overcoming the need for extensive enzyme purification. Insights into the regulation of PAP at the biochemical level will contribute to our understanding of seed oil formation. Both crude solubilized PAP and purified PAP will be useful in the further characterization of the enzyme. For example, it will be important to conduct specificity and selectivity studies of PAP in order to determine the substrates most effectively utilized by the enzyme. The possible effects of oleate on promoting the interaction of solubilized PAP with microsomes may provide insights into the biochemical regulation of PAP and also contribute to the development of a procedure

for reconstitution of PAP with membrane compartments. Knowledge of the biochemical properties of PAP will in turn facilitate effective biotechnological strategies for the modification of seed oil quality and/or content. Increasing PAP and/or DGAT production may lead to increased seed oil content. In addition, seed oil quality might be altered by modifying the gene encoding PAP. For example, one might envisage the development of a transgenic plant producing seeds which express a form of PAP which preferentially accepts specific forms of PA. Providing DGAT effectively accepts the resulting DG, the seed oil formed would have an intentionally altered fatty acid composition. Modification of oil quality could contribute to product diversification, such as the production of oils suited to industry.

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