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PHOSPHATIDATE PHOSPHATASE FROM DEVELOPING SEEDS AND MICROSPORE-DERIVED CULTURES OF *BRASSICA NAPUS**

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Key Word Index—*Brassica napus*; Cruciferae; oilseed rape; microspore-derived cultures; triacylglycerol biosynthesis; phosphatidate phosphatase; solubilization.

Abstract—Phosphatidate phosphatase (EC 3.1.3.4) was characterized in developing seeds (*Brassica napus* L. cv. Westar) and microspore-derived (MD) cultures of oilseed rape (*B. napus* L.). Differential centrifugation studies were conducted with homogenate prepared from developing seeds, MD embryos (*B. napus* L. cv. Reston) and an embryogenic MD culture (*B. napus* L. cv. Jet Neuf). Among the three tissue types, the level of microsomal phosphatidate phosphatase ranged from 11 to 17% of the total recovered enzyme activity whereas soluble phosphatidate phosphatase ranged from 25 to 61% of the total activity recovered. Microsomal phosphatidate phosphatase from developing seed displayed optimal activity in the range pH 6–7 whereas soluble phosphatidate phosphatase had a pH optimum of 5. The activity of phosphatidate phosphatase from microsomes of MD embryos exhibited a similar pH dependence. Activation energies for dephosphorylation of phosphatidate catalysed by phosphatidate phosphatase in microsomal and soluble fractions from developing seed were 15.6 and 9.4 kcal mol^{-1}, respectively. Assays with *p*-nitrophenyl phosphate as a substrate at pH 6.75 and 5 indicated that the overall character of phosphatase activity in the microsomal fraction was different from that of the enzyme in the soluble fraction. Tween 20 was used to solubilize phosphatidate phosphatase from microsomes of MD embryos (*B. napus* L. cv. Topas) with the most effective solubilization of enzyme occurring at a concentration of 0.4% (w/v) Tween 20 at a detergent to protein ratio of 1 : 1 (w/w). Solubilized microsomal phosphatidate phosphatase eluted within the sieving range of a Superose 6 column and displayed a minimum apparent M_r of *ca* 40 000. The solubilized fraction catalysed the hydrolysis of a number of forms of phosphatidate as well as various other phosphorylated compounds.

INTRODUCTION

The formation of triacylglycerol (TG) in developing oilseeds is catalysed by the membrane-bound enzymes of the Kennedy pathway [1, 2]. Identification of the genes encoding Kennedy pathway enzymes could contribute towards the improvement of oilseed crops, but molecular genetic studies have been impeded by the difficulties experienced in the solubilization and purification of these enzymes.

Phosphatidate (PA) phosphatase (EC 3.1.3.4) catalyses the penultimate step in the formation of TG by removal of phosphate from PA to generate *sn*-1,2-diacylglycerol (DG) [2]. PA phosphatase activity was first reported in plants by Kates [3]. In developing seeds, the DG formed by the PA phosphatase-catalysed hydrolysis of PA is used in both membrane and TG formation [4]. Both PA phosphatase [2] and diacylglycerol acyltransferase [5–7], which catalyses the final step in the TG synthesis, have been implicated in having a rate-limiting role in the formation of TG. PA phosphatase activity in extracts of seeds have been examined in castor bean [8], broad bean [9], mung bean [10] and safflower [11–16]. The activity of microsomal PA phosphatase in developing safflower seeds has been shown to be strongly dependent on Mg^{2+} [13, 14]. The alkaline PA phosphatase of spinach chloroplasts has also been partially characterized [16–19]. In studies with developing safflower seeds, Ichihara *et al.* [15] reported that a soluble form of PA phosphatase associates with the ER in response to increased oleate concentration, suggesting that PA phosphatase may be regulated by feedforward stimulation by this fatty acid. Soluble PA phosphatase from developing safflower seeds was shown to interact with sunflower microsomes in response to oleate, but there was no transfer of the soluble safflower enzyme to rapeseed microsomes [15]. PA phosphatase activity, in both developing safflower

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[15] and groundnut seeds [20], has been found to rise markedly during the rapid phase of lipid accumulation and decrease with seed maturation. The relative proportion of membrane-bound PA phosphatase in developing safflower seeds also increased during the rapid phase of lipid accumulation [15]. No soluble PA phosphatase was detected in studies with developing groundnut seeds [20]. Königs and Heinz [9] solubilized PA phosphatase from a 15000 *g* membrane fraction, prepared from broad bean leaves, by sonication in the presence of 0.2% Triton X-100. PA phosphatase from yeast [21] and porcine thymus [22] are the only forms of enzyme which have been purified to apparent homogeneity.

In the past few years, microspore-derived (MD) cultures of oilseed rape have proved very useful in the study of TG biosynthesis and associated enzymes [23–25]. The MD cultures also appear to be a convenient source of tissue for the purification of these enzymes. In the current study, PA phosphatase activity was characterized in developing seeds and MD cultures of oilseed rape. The microsomal enzyme from MD embryos of oilseed rape was solubilized and partially characterized.

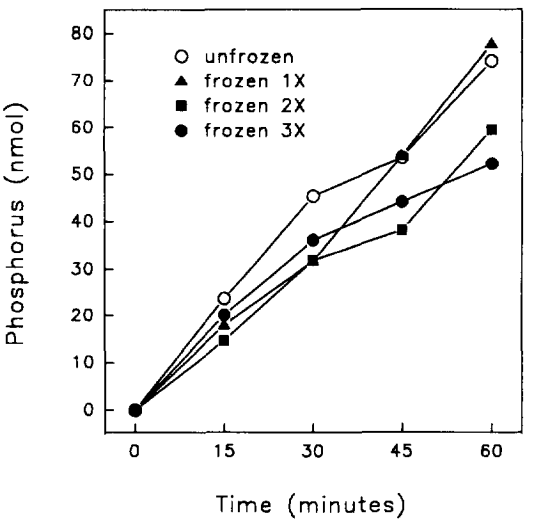

Fig. 1. Effect of freeze/thaw cycles on P_i release catalysed by PA phosphatase activity in homogenate of developing seed of *B. napus* L. cv. Westar at 30°. Data represent the means of triplicate assays with 300 μg protein per reaction mixture. Substrate was included in the reaction mixture in the form of a stabilized emulsion consisting of PA, PC and BSA.

RESULTS AND DISCUSSION

Assay and stability of PA phosphatase to freezing/thawing

PA was presented to PA phosphatase, in combination with egg yolk phosphatidylcholine (PC), in order to simulate the natural form of the substrate in the membrane [14, 15]. PA phosphatase activity was monitored by measuring the appearance of inorganic phosphate (Pi). Enzyme activity was assayed in a homogenate prepared from developing seeds (four weeks post-anthesis) of oilseed rape that were in the active phase of TG accumulation [25]. Time courses for the release of Pi catalysed by PA phosphatase activity in fresh seed homogenate and homogenate, subjected to repeated cycles of freezing and thawing, are shown in Fig. 1. The rate of Pi release was similar in each case suggesting that PA phosphatase activity was stable to at least three cycles of freezing and thawing. The enzyme reactions were linear for at least 60 min, a finding which was in agreement with time courses observed by Ichihara *et al.* [14] for PA phosphatase activity in extracts from developing safflower seeds. Enzyme assays conducted with seed homogenate, where PA was excluded from the reaction mixture, released Pi at about one-third the rate obtained using the complete reaction mixture. This observation suggested that endogenous phospholipase D activity may have resulted in the generation of PA to support some degree of PA phosphatase activity. Phospholipase D is widely distributed in plants [26]. In studies with rat liver fractions, Ide and Nakazawa [27] have indicated, however, that the release of Pi was the only reliable determination of PA phosphatase activity. In their studies, attempts to monitor DG formation were not reliable due to rapid hydrolysis of the product catalysed by lipase activities.

PA phosphatase activity in differential centrifugation fractions

PA phosphatase activity in fractions obtained following differential centrifugation of homogenates prepared from developing seed, MD embryos and an embryogenic cell suspension culture of oilseed rape is shown in Table 1. The data are based on the fractionation of homogenate from tissues which were frozen and thawed once prior to use. The non-differentiating embryogenic cell suspension system of winter oilseed rape (*Brassica napus* L. cv. Jet Neuf) was recently shown to contain 3–4% TG on a dry weight basis and produces a relatively constant level of diacylglycerol acyltransferase activity over a number of subcultures [25]. Total PA phosphatase activity recovered following fractionation of homogenate varied from *ca* 55–70% among the three tissue types. PA phosphatase activity in the 10000–100000 *g* microsomal fraction represented about 11, 17 and 12% of the recovered PA phosphatase activity, respectively, for developing seed, MD embryos and the cell suspension culture. Soluble PA phosphatase activity was 36, 61 and 25% of the recovered activity, respectively, for developing seed, MD embryos and the cell suspension culture. In all cases the lowest levels of recovered activity were associated with the fat layer. Ichihara *et al.* [14] recovered about 12 and 40% of the PA phosphatase activity, respectively, from microsomes and the 100000 *g* supernatant prepared from homogenate of developing safflower. The distribution of PA phosphatase activity in fractions obtained by differential centrifugation using MD embryos is shown for the cultivar Reston (Table 1). Similar results were obtained, however, following fractionation of homogenate prepared from the cultivar Topas.

Table 1. Distribution of PA phosphatase activity in fractions obtained by differential centrifugation of tissue homogenates

Cell fraction	Total protein (mg)	Specific activity (pkat mg protein^{-1})	Total activity (pkat)
Developing seed			
Homogenate	154.8	58.3	9030
1500 *g* pellet	44.4	45.0	2000
10000 *g* pellet	17.9	70.0	1250
100000 *g* pellet	13.9	51.7	719
100000 *g* supernatant	31.0	73.4	2280
Fat layer	6.4	23.3	149
MD embryos			
Homogenate	201.0	60.0	12100
1500 *g* pellet	25.0	20.0	500
10000 *g* pellet	5.2	86.7	451
100000 *g* pellet	17.6	63.4	1120
100000 *g* supernatant	60.3	66.7	4020
Fat layer	7.6	63.4	482
Cell suspension			
Homogenate	38.3	40.0	1530
1500 *g* pellet	13.0	38.3	498
10000 *g* pellet	2.7	36.7	99
100000 *g* pellet	4.4	26.7	118
100000 *g* supernatant	10.0	25.0	250
Fat layer	1.1	15.0	17

Tissue homogenization and subsequent fractionation were carried out as described in experimental, and in each case 5 g of tissue were used. *B. napus* L. cultivars of developing seed, MD embryos and cell suspension culture were, respectively, Westar, Reston and Jet Neuf. Assays were conducted in triplicate at pH 6.75 for 30 min at 28° and data represent the means. Floating fat layers, obtained by the centrifugation steps, were combined, and then washed 3 times by resuspending them in a volume of homogenizing buffer equal to one-half the tissue weight and centrifuging them at 10000 *g* for 20 min.

Fractionation studies with MD embryos that were not previously frozen resulted in slightly less PA phosphatase activity in the 100000 *g* supernatant with concomitant increase in enzyme activity in the 10000–100000 *g* fraction (data not shown). The results suggested that freezing resulted in some release of the particulate enzyme into a soluble form. Tissues could be stored frozen, however, for several months without a loss in total PA phosphatase activity. Thus, large quantities of tissue could be frozen for future purification of PA phosphatase.

Relationship between PA phosphatase activity and acid phosphatase activity

The effect of pH on PA phosphatase activity in the microsomal and soluble fraction (100000 *g* supernatant) from developing seeds is shown in Fig. 2A. Maximum release of Pi occurred at pH 6–7 and 5 for the microsomal and soluble enzyme, respectively. The pH optimum for PA phosphatase activity in microsomes from MD embryos (Fig. 2B) was similar to the pH optimum for the microsomal seed enzyme. The degree of experimental uncertainty depicted in the pH dependence plots in Fig. 2 was characteristic of other experiments. Our results are in good agreement with previous studies of pH effects on PA phosphatase activity using other types of plant tissues. Ichihara *et al.* [14] reported a pH optimum of pH 6.7 for the microsomal enzyme from safflower whereas Sukumar and Sastry [20] reported a pH optimum between 6 and 7 for the microsomal PA phosphatase from developing seeds of groundnut. Ichihara *et al.* [14], however, have not reported a pH optimum for the soluble enzyme from maturing safflower seed. Soluble PA phosphatase activity with acidic pH optima (pH < 6) have also been detected in germinating castor bean [8], broad bean leaves [9] and germinating mung bean cotyledons [10]. Soluble enzyme was not detected in developing groundnut seeds [20].

The more acidic pH optimum of the soluble PA phosphatase from developing seeds of oilseed rape suggested that this activity may be attributable to a different form of PA phosphatase or perhaps to a non-specific acid phosphatase. Acid phosphatases are characterized by pH optima for catalysis below pH 7 with most types identified having pH optima between 5 and 6 [28, 29]. For example, the major acid phosphatase in soybean leaf was shown to have optimal activity at pH 6 in acetate buffer [30]. Plant acid phosphatases do not usually exhibit

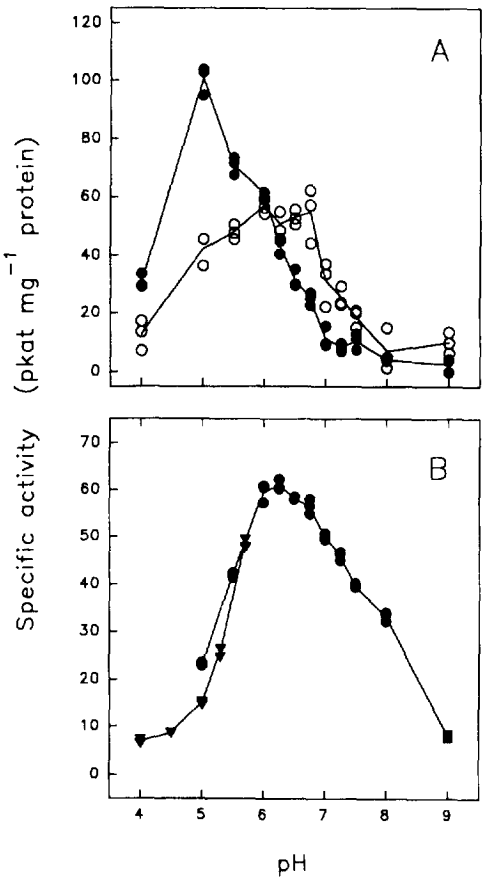

Fig. 2. (A) pH dependence of PA phosphatase activity in the microsomal fraction (10 000–100 000 *g*) (○) and dialysed soluble fraction (●) from developing seeds of *B. napus* L. cv. Westar. Buffers used were 50 mM acetate (pH 4.0), 50 mM Tris–50 mM maleic acid–NaOH (pH 5.0–8.0) and 50 mM Tris–HCl (pH 9.0). Assays were conducted in triplicate, at 30° for 30 min, using 150 μg and 200 μg protein for particulate and soluble fractions, respectively. (B) pH dependence of PA phosphatase activity using the microsomal fraction from MD embryos of *B. napus* L. cv. Topas. Assays were carried out at 30° for 30 min with 590 μg protein per reaction mixture. Buffers used were 50 mM acetate (▼), 50 mM Tris–50 mM maleic acid–NaOH (●) and 50 mM Tris–HCl (■). In both panels A and B, results of triplicate assays are shown with the plot lines connecting the means of the triplicate values.

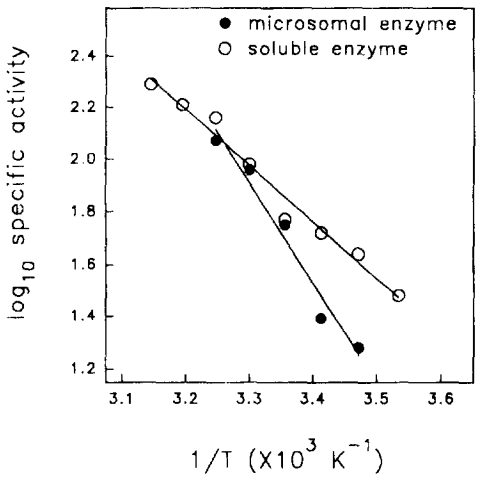

Fig. 3. Arrhenius plots for temperature dependence of microsomal and soluble PA phosphatase activity from developing seeds of *B. napus* L. cv. Westar. For the microsomal enzyme, assays were conducted at pH 6.75 with 123 μg protein per reaction mixture. The soluble enzyme (100 000 *g* supernatant) was dialysed against Tris maleic acid NaOH (pH 6.75) for 16 hr prior to assaying at pH 5.0 (Tris–maleic acid–NaOH buffer). Assays were carried out with 200 μg soluble protein per reaction mixture. Activity data represent the means of triplicate assays. Plot lines were generated by linear regression.

absolute substrate specificity and they occur in various subcellular locations including vacuoles, the cytoplasm and membrane fractions [29]. Wheat germ acid phosphatase has been shown to have activity towards PA [31]. The microsomal PA phosphatase from developing seeds of oilseed rape was more accurately called a 'neutral' phosphatase. Microsomal PA phosphatase is probably the important enzyme activity in terms of TG formation because the occurrence of TG biosynthesis in the ER is well documented [2]. It is possible, however, that some plant PA can be hydrolysed by the action of a non-specific acid phosphatase [4].

The effect of temperature on the PA phosphatase activity was also examined using microsomal and soluble fractions from developing seeds of oilseed rape under pH conditions that were optimal for enzyme activity. An activation energy of 15.6 kcal mol^{-1} was determined for microsomal PA phosphatase from the Arrhenius plot in Fig. 3. In contrast, temperature dependence studies of soluble PA phosphatase activity (Fig. 3) indicated an activation energy of 9.4 kcal mol^{-1}.

In order to characterize further microsomal and soluble phosphatase activity, enzyme assays were conducted using *p*-nitrophenyl phosphate (*p*NPP) as phosphatase substrate at pH 6.75 and 5 with enzyme extracts from both developing seeds and MD embryos (Table 2). *p*NPP is a synthetic substrate analogue which is routinely used to assay phosphatase activity [28, 29]. The time course for hydrolysis of *p*NPP catalysed by phosphatase activity in microsomal and soluble fractions was linear for at least 90 min (data not shown). At both pH 5 and 6.75, the rate of Pi release from *p*NPP catalysed by both microsomes and soluble fraction, shown in Table 2, was considerably greater than the rate of release of Pi from PA (see Fig. 2A). The microsomal fraction from both seeds and MD embryos was *ca* 30% more effective in catalysing the hydrolysis of *p*NPP at pH 5 than at 6.75. This was opposite to the trend described using PA as a substrate where enzyme activity was greater at pH 6.75 than at pH 5 (Fig. 2A and B). In contrast, the soluble fraction from both tissue sources was at least 70% more effective in catalysing the hydrolysis of *p*NPP at pH 5 than at 6.75. The soluble fraction from seed was about four times more effective in catalysing the hydrolysis of PA at pH 5 than

Table 2. Phosphatase catalysed hydrolysis of *p*NPP by microsomal and soluble fractions from MD embryos (*B. napus* L. cv. Topas) and developing seeds (*B. napus* L. cv. Westar) of oilseed rape

	Rate of *p*-nitrophenol formation (nkat mg protein^{-1})	
Cell fraction	pH 5.0	pH 6.75
MD embryos		
Microsomes	0.70	0.55
100000 *g* supernatant	4.56	2.48
Developing seeds		
Microsomes	1.47	1.15
100000 *g* supernatant	8.33	4.90

Data are the average of triplicate assays conducted at 30° in Tris–maleic acid–NaOH buffer using microsomes and dialysed soluble fractions obtained from MD embryos (*B. napus* L. cv. Topas) and developing seeds (*B. napus* L. cv. Westar) of oilseed rape. Enzyme assays were conducted with 5 mM *p*NPP and 2 μl of extract. Data represent the means of duplicate assays.

at 6.75 (Fig. 2A). In general, there was a greater increase in the rate of hydrolysis of *p*NPP by the soluble fraction in comparison with the particulate fraction when comparing reaction rates at pH 5–6.75. Thus, hydrolysis 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 fraction.

Since there was apparent PA phosphatase activity in the soluble fraction from developing seeds of oilseed rape, it is possible that the soluble enzyme may have a role in lipid metabolism in oilseeds. Indeed, Blank and Snyder [31] have suggested that acid phosphatase may play a role in lipid metabolism based on their observations that wheat germ acid phosphatase catalysed the hydrolysis of PA. Ichihara *et al.* [14] have described the existence of soluble PA phosphatase activity in developing safflower seeds and have further reported that soluble PA phosphatase can be made to interact with the microsomes following treatment with oleate [15]. Apart from mentioning that the thermolability of soluble PA phosphatase was similar to microsomal PA phosphatase, Ichihara *et al.* [14, 15], however, did not report on any further properties of soluble PA phosphatase.

Solubilization of microsomal PA phosphatase from MD embryos

Microsomal PA phosphatase 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 [2]. MD embryos, cultured for 14–21 days, were chosen as the tissue source of PA phosphatase since they were available on a nearly continuous basis. MD embryos of this age have previously been shown to accumulate TG rapidly [23]. For solubilization studies, microsomes were prepared from tissues which were not previously frozen.

A number of different detergents (cationic, anionic, zwitterionic and non-ionic) were screened for their ability to solubilize PA phosphatase from MD embryos of oilseed rape on the basis of two detergent concentrations (Table 3). Microsomes were pre-incubated with concentrations of 0.1 and 1% (w/v) detergent prior to separating solubilized proteins from insoluble material using centrifugation. Enzyme remaining in the supernatant following centrifugation at 105000 *g* for 60 min was defined as solubilized enzyme [32]. PA phosphatase activity was assayed in the total mixture before centrifugation and in the supernatant recovered after centrifugation. Assays of total PA phosphatase activity following detergent treatment gave an indication of the sensitivity of PA phosphatase activity to detergent type. Both cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) strongly inhibited PA phosphatase activity at concentrations of 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 effective in solubilizing more than 50% of the enzyme. Octyl-β-D-glucopyranoside (OGP) (1%) solubilized 74% of the PA phosphatase, but resulted in strong inhibition of enzyme activity.

Tween 20 was chosen for further solubilization studies since treatment of microsomes with this detergent resulted in reasonable solubilization of PA phosphatase with concomitant stimulation of enzyme activity. The effect of increasing Tween 20 concentration on the solubilization of PA phosphatase is shown in Fig. 4. The total activity of PA phosphatase following detergent treatment increased by more than 20% between 0 and 1% (w/v) Tween 20. It is possible that the carryover of detergent into the reaction mixture altered the dispersion of substrate, thus resulting in increased enzyme activity. Usually, 0–5% of the PA phosphatase was released from the microsomes without detergent (following resuspension of microsomes). The lowest concentration of Tween 20 required to solubilize PA phosphatase maximally was 0.4%, which corresponded to a detergent to protein ratio of about 1: 1 (w/w). The specific activity of solubilized PA phosphatase was greatest in the range 0.2–0.8% Tween 20. In general, the specific activity of PA phosphatase was increased by about two- to three-fold over that in the microsomes following solubilization with 0.4% Tween 20. Concentrations of Tween 20 above 1% were not effective in solubilizing more PA phosphatase activity. The higher concentrations of detergent also markedly decreased the specific activity of solubilized PA phosphatase due to further solubilization of impurities. Solubilized PA phosphatase appeared less stable than the microsomal form of the enzyme. For example, about half of the enzyme activity in the solubilized fraction was lost upon freezing and thawing. A similar activity loss was

Table 3. Solubilization of PA phosphatase by various detergents

Treatment	Detergent concentration (%, w/v)	Detergent/protein ratio (w/w)	Total activity of microsome (% detergent-free control)	Activity of solubilized enzyme (% total activity of microsome)
No detergent	0	—	100	0
CTAB	0.1	0.9	14	26
	1.0	9	0	0
CHAPS	0.1	0.9	77	17
	1.0	9	46	11
CHAPSO	0.1	0.9	74	14
	1.0	9.0	36	20
Sodium DOC	0.1	0.9	20	40
	1.0	9	26	61
DM	0.1	0.9	49	22
	1.0	9	0	0
SDS	0.1	0.8	13	75
	1.0	8	0	0
MEGA-8	0.1	0.8	112	4
	1.0	8	81	10
OGP	0.1	0.8	104	4
	1.0	8	19	74
Triton X-100	0.1	0.8	86	32
	1.0	8	86	31
Tween 20	0.1	0.3	113	33
	1.0	3	128	54

Microsomes obtained from MD embryos of *B. napus* L. cv. Topas were pre-incubated on ice for 1 hr at pH 6.75 with 0, 0.1 or 1% detergent. Data are the averages for triplicate exposures assayed at pH 6.75 for 30 min at 30°, with a TCA inactivated control for each exposure. Aliquots of 50 μl were assayed for enzyme activity. Data presented represent experiments conducted using four different preparations of microsomes. Control assays, without detergent, were run for each preparation of microsomes. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulphonate; CTAB, cetyltrimethylammonium bromide; DOC, deoxycholate; DM, *n*-dodecyl-β-D-maltoside; OGP, *n*-octyl-β-D-glucopyranoside.

experienced when the solubilized fraction was stored overnight at 4°.

In general, membrane proteins are most effectively solubilized at concentrations above the critical micellar concentration (CMC) of the detergent used for solubilization [33]. Microsomal PA phosphatase was partially solubilized above the CMC of Tween 20. The CMC of Tween 20 is 0.006% (w/v) [34]. Königs and Heinz [9] have used detergent treatment to solubilize PA phosphatase from the 15000 *g* pellet of broad bean leaves, which was enriched in mitochondria and microbodies. In the current study, isolation of PA phosphatase from the 10000 to 100000 *g* particulate fraction presumably helped in avoiding mitochondrial PA phosphatase.

Partial characterization of solubilized PA phosphatase

Gel filtration chromatography of solubilized PA phosphatase was conducted to confirm solubilization and to investigate the M_r properties of the solubilized enzyme. Various samples of solubilized PA phosphatase were subjected to gel filtration chromatography on a column of Superose 6 (HR 10/30) equilibrated with 0.2% (w/v) Tween 20. Solubilized fractions from two different batches of MD embryos were used. Identical samples were frozen and thawed once between consecutive runs. Elution profiles of PA phosphatase activity are shown for four experiments in Fig. 5. 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. The bulk of PA phosphatase activity eluted within the sieving range of the gel filtration column, thus confirming that the enzyme was solubilized effectively. PA phosphatase activity was distributed over a number of fractions. In contrast, the various standards used to calibrate the column eluted as considerably sharper peaks. With one of the samples, freezing and thawing appeared to cause a shift in the elution of the enzyme from higher to lower M_r. A minimum apparent M_r of about 40000 was observed for both preparations of solubilized PA phosphatase. The gel filtration chromatography experiments suggested that PA phosphatase may be subject to aggregation or there may be different forms of the enzyme. One of the solubilized fractions contained a larger proportion of PA phosphatase, which eluted at a M_r of about 600000. This

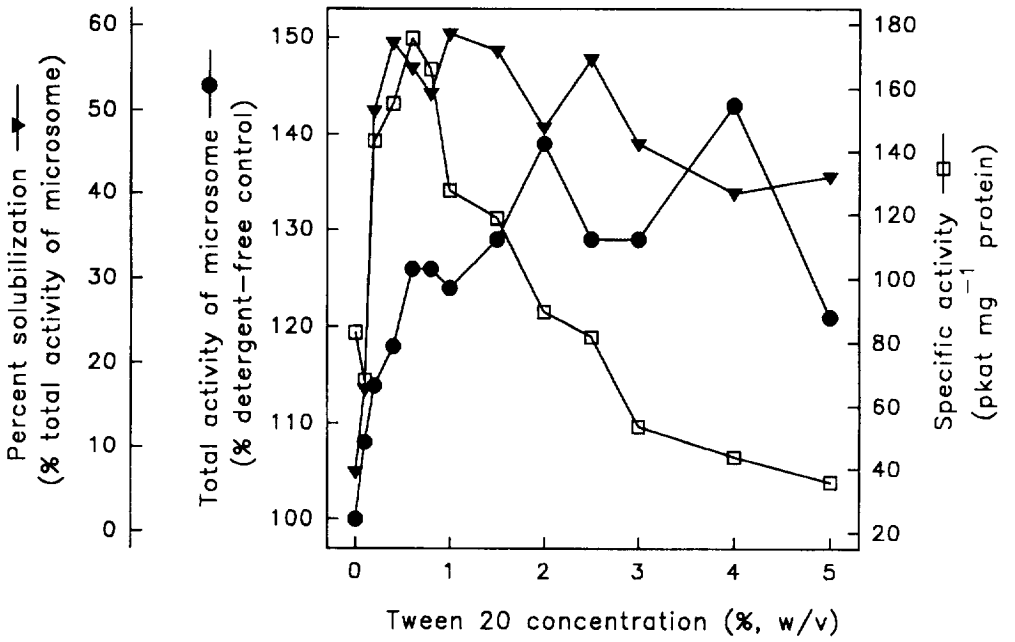

Fig. 4. Solubilization of PA phosphatase from microsomes of MD embryos of *B. napus* L. cv. Topas at various concentrations of Tween 20. The solubilized enzyme was recovered in the supernatant following centrifugation at 105 000 *g* for 60 min. Detergent treatments were in duplicate and each extract was in turn assayed in duplicate. Therefore, data represent the average of two experiments. Tween 20 concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0% (w/v) corresponded, respectively, to the following detergent to protein ratios: 0.2:1, 0.5:1, 1:1, 1.4:1, 1.9:1, 2.4:1, 3.5:1, 4.7:1, 5.9:1, 7:1, 9.4:1 and 11.7:1 (w/w).

may have been related to the two different batches of MD embryos used for preparation of these enzyme fractions.

The minimum apparent M_r of solubilized PA phosphatase from MD embryos was similar to the apparent M_r of 37 000 determined by gel filtration chromatography for soluble acidic PA phosphatase from germinating mung bean cotyledons [10]. Recently, a form of PA phosphatase involved in cell signalling was purified to homogeneity from porcine thymus membranes [22]. 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 PA phosphatase 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 [35]. In contrast, Superose 12 gel filtration chromatography of a Mg^{2+}-dependent PA phosphatase, solubilized from yeast microsomes, resulted in a M_r of 93 000 [21]. Chromatography of the yeast PA phosphatase was conducted in the presence of 1% sodium cholate. SDS–polyacrylamide gel electrophoresis indicated that this form of yeast PA phosphatase was a monomer.

The effect of pH on the activity of solubilized microsomal PA phosphatase is shown in Fig. 6. The pH dependence of the solubilized enzyme was similar to that of the particulate enzyme (Fig. 2B) with optimal activity occurring between pH 6 and 7.

The activity of solubilized PA phosphatase 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 (Fig. 7, lanes 'b' to 'e'). Under these conditions, *sn*-1,2-DG appeared to accumulate steadily in the reaction mixture for *ca* 90 min. Using the described solvent system, PA and PC remained at the origin of the TLC plate. The microsomes contained high levels of DG (lane 'a'), which interfered with attempts to monitor newly generated DG via the PA phosphatase reaction. Indeed, it has previously been shown that endogenous DGs can serve as effective substrates for particulate diacylglycerol acyltransferase [36]. In contrast, solubilized PA phosphatase 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 PA phosphatase 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 generated from PC as a result of 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

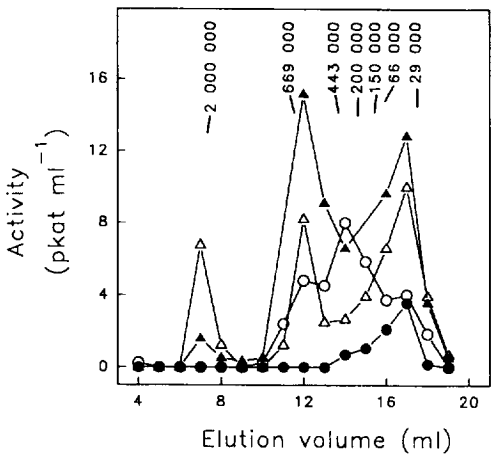

Fig. 5. Superose 6 gel filtration chromatography of solubilized PA phosphatase from MD embryos of *B. napus* L. cv. Topas. The column (HR 10/30, total bed vol. = 23.6 ml) was equilibrated with 0.2% (w/v) Tween 20 in 60 mM Tris–60 mM maleic acid–NaOH (pH 6.75) containing 0.1 M KCl, 0.12 mM EDTA and 2.5 mM $MgCl_2$. Samples of 200 or 500 μl were injected on to the column which was operated at a flow rate of 12 ml hr^{-1}. The following samples were applied to the column: batch 1—MD embryos, 426 μg solubilized enzyme exposed to one cycle of freezing/thawing (○); batch 1—embryos, 426 μg solubilized enzyme exposed to two cycles of freezing/thawing (●); batch 2—embryos, 720 μg freshly solubilized enzyme (△); batch 2—embryos, 1800 μg solubilized enzyme exposed to one cycle of freezing/thawing (▲). Fractions of 1 ml were collected and assayed immediately for PA phosphatase activity. From left to right M_r markers are as follows: Blue Dextran 2000, thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, BSA and carbonic anhydrase.

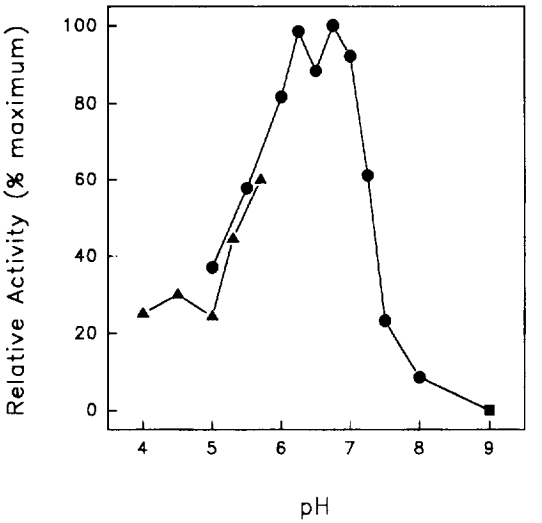

Fig. 6. pH dependence of solubilized PA phosphatase from MD embryos of *B. napus* L. cv. Topas. Assays were conducted with 100 μl of the 105 000 *g* supernatant per tube and were carried out for 30 min at 30°. Buffers used were 50 mM acetate (▲), 50 mM Tris–50 mM maleic acid–NaOH (●) and 50 mM Tris–HCl (■). Data points represent the means of triplicate assays.

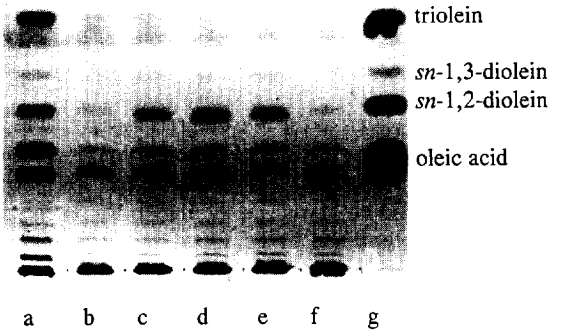

Fig. 7. Analysis by TLC of the products of PA hydrolysis catalysed by PA phosphatase activity in the solubilized fraction from MD embryos of *B. napus* L. cv. Topas. Lane (a): complete reaction mixture containing microsomes (740 μg protein), $t = 0$. Lanes (b)–(e): complete reaction mixture with solubilized PA phosphatase (90 μg protein) incubated for 0, 45, 90 and 135 min, respectively. Lane (f) solubilized PA phosphatase incubated for 135 min without PA (with PC and BSA). Lane (g): lipid standards.

solubilized PA phosphatase with PA and PC did not lead to accumulation of additional fatty acids in the reaction mixture (lanes 'c'–'e').

Based on studies with rat liver microsomes, Sturton and Brindley [37] have cautioned that PA phosphatase activity in crude extracts may be overestimated due to dephosphorylation of glycerol 3-phosphate generated by the deacylation of PA by phospholipases A and B. Deacylation of PA catalysed by solubilized oilseed rape PA phosphatase would have led to a build up of fatty acids in the reaction mixture, which would have been detected following TLC. As indicated previously, in studies with rat liver extracts, Ide and Nakazawa [27] found the DG was rapidly hydrolysed during its formation in PA phosphatase reaction mixtures. From the TLC results in Fig. 7, fatty acids did not appear to accumulate as DG was formed through PA phosphatase action, suggesting that DG was not deacylated in the microsomal system of MD embryos.

The specificity of the solubilized fraction was examined using various types of PA and other phosphorylated compounds (Table 4). The solubilized fraction was capable of catalysing the release of Pi from PC to some extent, suggesting interference due to phospholipase action on PC. This was in contrast to the results of analysis of DG by TLC in Fig. 7, which suggested that the solubilized fraction was free of phospholipase activity. The small amount of DG present in the solubilized fraction (Fig. 7, lane 'b') may have been sufficient to mask the limited generation of DG due to the action of phospholipase C. PA phosphatase was capable of accepting all forms of PA tested (Table 4), suggesting that the

Table 4. Dephosphorylation of various compounds catalysed by the solubilized fraction from MD embryos of *B. napus* L cv. Topas

Substrate	Relative specific activity (% maximum)
PC/BSA	2.5
Egg PA	7.2
Dioleoyl-PA	11.2
Dierucoyl-PA	4.9
Distearoyl-PA	2.9
Dipalmitoyl-PA	9.9
Erucoyl-LPA	4.1
sn-Glycerol 3-phosphate	21.6
Adenosine 5'-monophosphate	12.3
Phytate	6.2
*p*NPP	100.0

The reaction mixture (1 ml) contained 1 mM substrate with 0.5 mg PC ml^{-1} and 1 mg BSA ml^{-1} in the presence of in 50 mM Tris–50 mM maleic acid–NaOH buffer (pH 6.75) containing 0.1 mM EDTA and 2 mM $MgCl_2$. Reactions were allowed to proceed for 60 min using 165 μg solubilized protein per reaction mixture. The results represent the means of duplicate assays. An average M_r for egg yolk PA was determined based on the fatty acid composition reported by Ichihara [16].

enzyme could tolerate a wide range of modifications of the fatty acyl groups of PA. There was, however, preference for dioleoyl-PA over other forms of PA tested. Of all the PAs tested, the solubilized enzyme exhibited the least preference for distearoyl-PA. Ichihara [16] reported an enhanced and essentially equal preference for both dioleoyl-PA and dilinoleoyl-PA by PA phosphatase in microsomes from developing safflower seed. The ability of oilseed rape microsomal PA phosphatase to accept dierucoyl-PA is an important consideration in terms of genetic engineering projects directed towards the production of trierucin in developing seeds of oilseed rape [38]. Phosphatase activity in the solubilized fraction also catalysed the liberation of Pi from other Kennedy pathway intermediates including *sn*-glycerol 3-phosphate and erucoyl-lysophosphatidate (LPA) and a number of other phosphorylated compounds. At this stage it is difficult to ascertain whether this dephosphorylation activity was attributable to a mixture of membrane phosphatases or to a promiscuous PA phosphatase. The results of gel filtration chromatography of PA phosphatase may be taken to suggest that there may be more than one form of PA phosphatase in the solubilized fraction. It may also be argued that the specificity of microsomal PA phosphatase may not be that important a factor if the enzyme is limited to accepting PA generated via lysophosphatidate acyltransferase due to spatial constraints because of the enzyme's interaction with other proteins and lipids in the ER. Thus, the membrane-bound PA phosphatase of the Kennedy pathway may not be able to interact readily with other phosphate containing compounds in the cell.

CONCLUSIONS

The characteristics of microsomal and soluble PA phosphatase were determined in developing seeds of oilseed rape. Soluble PA phosphatase appeared to be due to the action of a non-specific acid phosphatase whereas microsomal PA phosphatase had a pH optimum near neutrality. Soluble PA phosphatase may have a role in lipid metabolism in developing seeds of oilseed rape, but investigators should exercise caution in ascribing a physiological role in TG biosynthesis to the soluble enzyme. Our further studies focused on microsomal PA phosphatase which was located in the same fraction as other Kennedy pathway enzymes. PA phosphatase was solubilized from the microsomes of MD embryos and partially characterized. The solubilization and characterization of PA phosphatase from the ER of plant tissue, involved in TG accumulation, are critical to the purification of this important Kennedy pathway enzyme.

EXPERIMENTAL

Chemicals. All lipids, detergents and other biochemicals were of the highest purity available.

Prepn of PA and LPA. Dierucoyl-PA and erucoyl-LPA were chemically synthesized by acylation of *sn*-glycerol 3-phosphate catalysed by TFA anhydride based on the procedure for acylation of glycerophosphocholine to form PC [39]. The di(monocyclohexylammonium) salt of glycerol 3-phosphate (50 μmol) was dissolved in 185 μl TFA and erucic acid (2 00 μmol) was dissolved in 56 μl TFA anhydride. The solns were combined in a screw-cap hydrolysis tube and the reaction was allowed to proceed at room temp. for 30 min. TFA was removed using a stream of N_2 gas, and the resulting lipid was redissolved with 200 μl $CHCl_3$–MeOH (2:1). Aliquots (40 μl) of the resulting soln were applied to 20 × 20 cm silica gel 60 G Al-backed TLC plates. PA and LPA were resolved using one ascension of EtOAc–isooctane–HOAc (9:3:2) as described in ref. [40]. A strip containing resolved lipids was cut from the side of the silica gel plate, dipped in 10% (w/v) $CuSO_4$ and 8% (w/v) H_3PO_4, and heated at 190° for 15 min to char the lipids [40, 41]. The strip containing charred lipids was then used as a guide for scraping PA (R_f *ca* 0.5) and LPA (R_f *ca* 0.1) from the remainder of the TLC plate. The two phospholipid classes were eluted from the silica with a modified soln [42] consisting of CH_2Cl_2–MeOH–1 M KCl (1:2:0.8) in 0.2 M maleic acid. Phases were sepd by adding 2 ml CH_2Cl_2 followed by 2 ml 1M KCl in 0.2 M maleic acid. The organic phase was removed and dried under a stream of N_2. The phospholipids were weighed and stored under N_2 gas at − 20°.

Plant material. Oilseed rape (*B. napus* L. cv. Westar) was field grown at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta. Oilseed rape flowers were tagged at anthesis and the developing siliques harvested after *ca* 4 weeks. Seeds were frozen in liquid N_2 and stored at − 20° for prepn of enzyme extracts.

Microspores of oilseed rape (*B. napus* L. cvs Reston and Topas) were isolated and induced to form MD embryos [23]. MD embryos at the early to mid-cotyledonary stage of development were washed with H_2O over a nylon sieve (60 μm), blotted with filter paper and the fr. wt determined. MD embryos were used either immediately or were frozen in liquid N_2 and then stored at $-20°$ prior to enzyme extraction. An embryogenic MD cell suspension culture of winter oilseed rape (*B. napus* L. cv. Jet Neuf) was provided by Dr J. Singh of the Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario. The cell suspension was maintained according to ref. [43]. These cells were collected, washed and stored as described for MD embryos.

Prepn and fractionation of homogenate. All procedures were conducted at 0–4° unless indicated otherwise. Frozen tissue was thawed on ice. Seeds were ground using a chilled mortar and pestle with 4 vol. of grinding medium per g of tissue. The grinding medium consisted of 0.5 M sucrose in 60 mM Tris–60 mM maleic acid–NaOH (pH 6.75) containing 0.12 mM EDTA and 2.5 mM $MgCl_2$. The concns of the buffer components were similar to those used in the PA phosphatase assay buffer described in ref. [14].

Tissue homogenate was fractionated into cellular components by differential centrifugation. The homogenate was centrifuged at 1500 *g* for 15 min to obtain the first particulate fr. and then at 10 000 *g* for 20 min to obtain a second particulate fraction. The microsomal fraction was prepd by centrifuging the 10 000 *g* supernatant for 2 hr at 100 000 *g*. Microsomes were usually washed once and then resuspended with grinding medium to a vol. equal to one-half of the tissue wt. The floating fat pad was collected by aspiration and washed 3 times with grinding medium. The 100 000 *g* supernatant was usually dialysed against assay buffer (without sucrose) for 16 hr to reduce the Pi background. Frs obtained by differential centrifugation were either assayed immediately or frozen as small aliquots using liquid N_2 and stored at $-20°$.

Enzyme assays and protein determination. Enzyme extracts were either assayed immediately or thawed on ice prior to activity determination. PA phosphatase activity was determined by monitoring the appearance of Pi according to the procedure described in ref. [14], which was based on the colorimetric method originally described in ref. [44]. The standard reaction mixt. (1 ml) contained 0.7 mg Na PA (derived from egg yolk PC), 0.5 mg PC and 1 mg BSA in 50 mM Tris–50 mM maleic acid–NaOH buffer (pH 6.75) containing 0.1 mM EDTA and 2 mM $MgCl_2$. Reactions were initiated with enzyme extract and usually allowed to proceed for 30 or 60 min at 30°. Reactions were corrected for the presence of endogenous Pi by substracting absorbance values resulting from treatment of extracts with TCA prior to addition of substrate.

The appearance of DG in the reaction mixt. was monitored by TLC according to ref. [14]. Reactions were carried out at 30° in 2 ml assay buffer containing 0.35 mg Na PA, 0.25 mg PC, 0.5 mg BSA and 90 μg of solubilized protein. Reactions were terminated by adding 30 μl 6 M HCl. Lipids were extracted from the reaction mixt. with 2 vol. of 1.5 ml $CHCl_3$ and the organic solvent phase was washed twice with 2 ml MeOH–H_2O (1:1). Lipids in the combined $CHCl_3$ layer were concd to dryness under N_2 gas. The lipid residues were resuspended in 100 μl $CHCl_3$, and 50 μl of each sample was applied to a silica gel 60 G Al-backed plate. The plate was developed with $CHCl_3$–MeOH–HOAc (195:4:1). The sepd lipid classes were visualized by spraying with 30% (w/w) H_2SO_4 and then heating at 145° for a few min.

Phosphatase activity was assayed by monitoring the release of *p*-nitrophenol from *p*NPP [45] using a modification of ref. [46]. The reaction buffer consisted of 50 mM Tris–50 mM maleic acid–NaOH (pH 6.75 or 5.0) containing 0.1 mM EDTA and 2 mM $MgCl_2$. Enzyme assays were conducted at 30° using 5 mM *p*NPP without BSA.

The protein content of plant tissue extracts was determined using the Bio-Rad protein microassay based on the method of ref. [47], with BSA as standard.

Solubilization of microsomal PA phosphatase from MD embryos. Resuspended microsomes were combined with 1 vol. of detergent soln prepd. in 50 mM Tris–50 mM maleic acid–NaOH buffer (pH 6.75) containing 0.1 mM EDTA and 2 mM $MgCl_2$. The resulting mixt. was vortexed for 2 min and then kept on ice for 60 min. An aliquot of the detergent–microsome mixt. was removed and centrifuged at 105 000 *g* for 60 min. The supernatant or solubilized fr. was assayed for PA phosphatase activity.

Gel filtration chromatography of solubilized PA phosphatase. PA phosphatase was solubilized from microsomes of MD embryos using 1% (w/v) Tween 20 at a detergent to protein ratio of 1:1. Gel filtration chromatography was conducted using a Superose 6 column (HR 10/30) (Pharmacia LKB) equilibrated with 0.2% (w/v) Tween 20 in 60 mM Tris–60 mM maleic acid–NaOH (pH 6.75) containing 0.1 M KCl, 0.12 mM EDTA and 2.5 mM $MgCl_2$. Samples (200 or 500 μl) of solubilized enzyme were applied to the column, operated at 12 ml hr^{-1} with an FPLC system (Pharmacia LKB). Column frs of 1 ml were collected and assayed for PA phosphatase activity. The column was calibrated with M_r markers in the absence or presence of 0.2% (w/v) Tween 20.

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