Partial Purification and Properties of Phosphatidic Acid Phosphohydrolase-1 from Rat Liver

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Abstract :

The phosphatidic acid phosphohydrolase-1 (EC 3.1.3.4) which catalyses the conversion of phosphatidic acid to diacylglycerol was purified from the soluble fraction (cytosolic fraction) of rat liver. After poly(ethylene glycol) 8000 precipitation, the enzyme was solubilised with Triton X-100 and stabilised by addition of phosphatidic acid and diacylglycerol. The purification was then achieved by chromatography in the presence of Triton X-100 with Hydroxyapatite, Mono Q, Heparin and CDP-diacylglycerol-Sepharose. The highly purified fraction showed two major bands after silver detection at 110 kDa and 55 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Phosphatidic acid phosphohydrolase-1 activity was located in those gel slices containing a single band protein of 110 kDa. The purified enzyme was sensitive to thioreactive reagents like N-ethylmaleimide and slightly stimulated by Mg^{2+}. The specific enzyme activity was 2850 ± 100 nmol/min/mg of protein when assayed using phosphatidic acid as Triton X-100 micelles. The Km for phosphatidic acid was 0.14 ± 0.02 mM. The present procedure of purification allowed us for the first time to purify the phosphatidic acid phosphohydrolase-1 (soluble form) from animal tissue with a specific activity more than 10,000-fold over that of homogenate fraction. This work would provide a valuable basis for further characterisation of phosphatidic acid phosphohydrolase-1 in order to elucidate the molecular properties of this enzyme.

Keywords: *Phosphatidic acid phosphohydrolase; Purification; Rat Liver.*

Abbreviations:

***BSA**, bovine serum albumin; **DAG**, diacylglycerol; **ER**, endoplasmic reticulum; **NEM**, N-ethylmaleimide; **PA**, phosphatidic acid; **PAP**, phosphatidic acid phosphohydrolase; **TAG**, triacylglycerol*

Phosphatidic acid phosphohydrolase (PAP) plays an important role in the lipid metabolism in eucaryotic cells. This enzyme catalyses the dephosphorylation of phosphatidate (PA) to *sn*-1,2-diacylglycerol (DAG), a substrate for the synthesis of the nitrogenous phospholipids, phosphatidycholine and phosphatidylethanolamine, as well as a direct precursor of

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triacylglycerol (TAG). PA can also be converted to CDP-DAG, which is the precursor for the anionic phospholipids, phosphatidylinositol, phosphatidylglycerol and cardiolipids [1,2].

In mammalian cells, two forms of PAP activity have been identified [3-5]. PAP-2, the plasma membranous form (for which at least three forms exists [6,7]) is Mg2+-independent and N-ethylmaleimide (NEM)-insensitive and is thought to be mainly involved in cell signalling [8,9]. On the other hand, PAP-1, stimulated by Mg^{2+} and inhibited by NEM, is distributed between the cytosol and endoplasmic reticulum (ER) and is regarded as the metabolic form, involved in the regulation of glycerolipid synthesis. The metabolic expression of PAP-1 activity is thought to be regulated by the ability of the cytosolic form to translocate to the ER, where its substrate is generated mainly by the acylation of glycerol-3-phosphate [5,10].

Regulation of PAP-1 activity can therefore be expected to play a crucial role in determining the rate and direction of lipid metabolism as evidenced recently by the in vitro regulation of PAP-1 by fatty acids and their acyl-CoA esters [11]. These studies, however, have been confined to the use of crude enzyme preparations, and hence, the molecular properties of animal PAP-1 have remained entirely unknown.

PAP-1 from mammalian sources has yet to be purified, which has considerably hampered the study of biological role of this enzyme in metabolism and cell activation. This mainly due to the lack of pure or semi-purified enzyme PAP-1, the soluble form. However, there seems little doubt that control of PAP-1 activity by both hormones and substrate supply is important in the regulation of glycerolipid synthesis. In the present investigation, we described the purification to nearly homogeneity of the soluble PAP-1 for the first time from animal tissues.

Materials and Methods

Animals: Male wistar rats (150 - 200 g) were obtained from Newcastle University Comparative Biology Centre (United Kingdom). They were allowed free access to food and water prior to sacrifice by cervical dislocation, after which rat livers were immediately removed and stored at -20°C until use. Phosphatidic acid, 1,2-dioleoyl-sn-glycerol, bovine serum albumin (BSA) (fatty acid free), Triton X-100, and polyethylene glycol (MW 8,000) were from Sigma Chemical (Poole, Dorset, United Kingdom).

Purification procedure of the soluble phosphatidic acid phosphohydrolase-1 (PAP-1)
All operations were done at 4°C.

Step1: Preparation of cytosol fraction - Livers from rats were suspended at 4°C in 4-5 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 0.2 mM dithiothreitol or 10 mM β-mercaptoethanol (buffer A) and minced with scissors before homogenisation with a Teflon-glass homogeniser. The crude homogenate was then centrifuged at 4°C for 20 min at 10,000 x g and the supernatant (homogenate) collected. The homogenate was then centrifuged at 150,000 x g for 90 min at 4°C, the resulting supernatant being recentrifuged for a further 60 min at 150,000 x g to produce a cytosolic fraction. Fractions were stored at -20°C or used immediately. Previous work in our laboratory using this methodology, coupled with marker enzymes, has shown that the cytosolic fraction contains essentially PAP-1 (over 85%) [4].

Step 2: Solid PEG_{8000} fractionation - Cytosol fraction was diluted in homogenate buffer (buffer A) to give a final protein concentration of 6-7 mg protein per ml, and then solid polyethylene glycol (MW 8,000) was added (6 g per 100 ml of cytosol). The mixture was stirred for 1 hour at 4 °C and then centrifuged at 5,000

x g for 15 min. The precipitate was washed three times with buffer A containing 3% polyethylene glycol (MW 8,000). Then PAP activity was resuspended in 10% of the original volume in 20 mM Tris-HCl (pH 7.5) containing 10% glycerol, 0.3% Triton X-100, 0.2 DTT or 5 mM β-mercaptoethanol, 1 mM $MgCl_2$, 50 mM NaCl, 0.005 mM of PA and 0.005 mM DAG (Buffer B). After overnight incubation with stirring, the mixture was centrifuged at 40,000 x g for 30 min to recover the solubilised enzyme activity as the supernatant. Contamination of this fraction with PAP-2 was negligible, as judged by the complete sensitivity of the activity to NEM.

Step 3: Hydroxyapatite Column Chromatography - Hydroxyapatite gel was equilibrated with Buffer B. The soluble enzyme fraction from the previous step was passed through a short column of hydroxyapatite (5 mg of protein per ml of gel). The pass-through fractions were pooled.

Step 4: Mono Q Column Chromatography - A anion exchange Mono Q column was equilibrated with Buffer B. The hydroxyapatite pass-through enzyme fraction was applied to the column (1 mg of protein per ml of gel) at flow rate 0.5 ml/min. The column was washed with 5 column volumes of Buffer B and PA phosphohydrolase was eluted with the same buffer B containing 0.2 M NaCl, and 2-ml fractions were collected. The active fractions were combined.

Step 5: Heparin Column Chromatography - A heparin column (2 ml) was equilibrated with buffer B. Mono-Q purified enzyme was diluted in buffer B minus NaCl to reduce NaCl concentration to 50 mM, then applied to the column at flow rate 0.5 ml/min. The column was washed with 20 ml of buffer B containing 50 mM NaCl. PAP activity was then eluted from the column in 1-ml fractions with 10 column volumes of a linear NaCl gradient (0.05 - 1 M) in Buffer B. Two peaks of PAP activity were eluted from this column. The first peak (peak 1) of activity eluted from the column at NaCl concentration of about 0.4 M while the second peak (peak 2) of activity eluted at NaCl concentration of about 0.6 M.

Step 6: CDP-DAG-Sepharose Affinity Chromatography – The NaIO4-oxidised derivative CDP-DAG was covalently attached to Sepharose 4B via an adipic acid dihydrazine spacer arm as described previously [12]. CDP-DAG-Sepharose (3 ml) was equilibrated with buffer C (buffer B minus PA and DAG). The purified enzyme (peak 2) from the previous step was diluted with buffer C (5 times) and applied to the column at flow rate 0.2 ml/min. The column was washed with 10 column volumes of buffer C, and PAP activity was eluted from the column in 1-ml fractions with same buffer C containing 1 M NaCl. The active fractions were combined.

Facultative Step 7: a second Heparin Column Chromatography or Hi-performance mono-Q chromatography are useful to get PAP activity in small volume of elution buffer. The experimental procedure is the same as described above, step 4 or 5.

Determination of PAP activity. This was measured by the production of water soluble [^{32}P] inorganic phosphate from the substrate [^{32}P] PA [4,6,11]. Substrate [^{32}P]PA was synthesised as reported previously [13]. PA was dispersed by sonication (bath water sonicator) in buffer A until the solution became clear. Unless otherwise specified, 0.1 ml of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (fatty acid free), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol, 0.125 M sucrose (basic assay system). Then 10 to 20 µl of enzyme preparation containing 0.3% TX-100 was added to the reaction mixture. PAP activity

was assayed following addition of 0.025 ml of 2 mM [^{32}P]PA (3,000 – 10,000 cpm/nmol). After 10 minutes incubation at 37 °C, the reaction was terminated by the sequential addition of 0.25 ml of 1 M HCl in methanol, 0.25 ml chloroform, and 0.25 ml of 1 M $MgCl_2$. After vortexing, the phases were separated by centrifugation (10,000 x g, 30 s) and a 0.3 ml aliquot of aqueous layer was transferred to a scintillation vial and radioactivity was counted in 4 ml of liquid scintillation. The time of the reaction and the amount of enzyme protein added to the reaction mixture were chosen to ensure that no more than 10% of PA was hydrolysed to DAG. One unit of PAP activity was defined as the release of 1 nmol of [^{32}P] inorganic phosphate per minute.

Identification of PAP activity on SDS-page. The highly purified PAP fraction was used to identify PAP enzyme after SDS-polyacrylamide gel electrophoresis. Purified enzyme (0.2 µg) was subjected to 10% polyacrylamide gel electro-phoresis under reducing conditions at 7°C. One lane was stained with silver and showed two major bands at 110 kDa and 50 kDa. A second lane was cut into 0.3 cm slices, and each slice was minced with a razor blade in assay buffer containing 0.5 mM PA and 0.1% Triton X-100 and incubated overnight at 7°C. The samples were assayed for PAP activity using [^{32}P]PA as substrate. Positions of myosin (220 kDa) and phosphorylase b (97 kDa) on a parallel lane are indicated.

Results

Phosphatidic acid phosphohydrolase (PAP) activity has been detected in the membrane and soluble fractions of a variety of mammalian tissues [3-5]. The membranous form (PAP-2) has been purified from yeast and animal tissue membranes [6, 14-17], but the purification of animal PAP-1 (soluble form) was so far achieved only to a very modest extent.

Therefore, we tried to purify the phosphatidic acid phosphohydrolase-1 from the soluble fraction of rat liver tissue. The purified enzyme was incubated with an aqueous dispersion of [^{32}P] phosphatidate as substrate under the standard assay conditions and the water soluble product inorganic [^{32}P] was analysed. Table I summarises a typical purification of PAP-1 enzyme from the soluble fraction of rat liver.

The soluble enzyme was first precipitated from cytosol fraction with solid polyethylene glycol (PEG), then the precipitate was re-suspended in buffer containing TX-100, PA and DAG as indicated under 'Method section'. It was critical at this step and during the following purification procedure the maintain TX-100, PA and DAG in the buffer in order to overcome any inhibition of PAP-1 activity *via* aggregation phenomenon (Elabbadi and al. Submitted, [18]). The PEG precipitate fraction was applied to the hydroxyapatite column and PAP activity was collected as the pass-through fraction. This step, which gave 13-fold purification over the previous step, was necessary to obtain highly purified enzyme. The pass-through fraction was then fractionated by Mono Q chromatography. The PAP activity was eluted from the column at 0.2 mM NaCl, diluted and directly applied to the heparin column.

Elution of the enzyme in two peaks from the heparin chromatography was invariably reproduced in several experiments. One was eluted at about 0.4 M NaCl (peak1) with 633-fold purification, and the second peak (peak 2) was reproducibly observed at 0.6 M NaCl with 1516-fold purification. The peak 1 was not purified further. The peak 2 from the previous step was further purified on CDP-DAG-Sepharose chromatography. This final step of purification gave about 3-fold purification above the previous step.

SDS-polyacrylamide gel electrophoresis of the

Purification procedure	Specific activity *Units/mg*	Purification *-fold*
1. Homogenate	-	-
2. Cytosol	0.6	1
3. PEG precipitation (6%)	3	5
4. Hydroxyapatite	40	66
5. Mono Q	-	-
6[a]. Heparin (peak 1)	380	633
6[b]. Heparin (peak 2)	910	1516
7. CDP-diacyglycerol-Sepharose	2820	4700

Table I : Purification of phosphatidic acid phosphohydrolase (PAP-1) from rat liver cytosol fraction. PAP was purified from the soluble fraction of rat liver as described in the text. Typical result of the purification procedure is presented. PAP activity was determined under standard assay conditions.

highly purified enzyme fraction under reducing conditions (Fig. 1) showed two major bands at 110 kDa and 50 kDa.

In order to confirm that these 110 kDa and 50 kDa proteins represented PAP enzyme, we analysed directly the PAP activity inside the gel. Indeed, The counts of water-soluble inorganic ^{32}Pi hydrolysed from [^{32}P]PA were 38,000 cpm above the background (200 cpm) in those gel slices which contained the protein migrating close to the 110 kDa (Fig. 1). Thus, upon SDS-polyacrylamide gel electrophoresis, the 110 kDa band appeared in parallel to the PAP activity, confirming at least that this protein represented the enzyme. As summarised in Table I, PAP-1 enzyme (soluble form) was purified for the first time from animal tissue, rat liver, with a specific activity more than 10,000-fold over that of homogenate fraction.

PAP-1 activity versus PA concentration exhibited typical hyperbolic function of Michaelis-Menten saturation kinetics (Fig. 2). Experiments carried out under standard conditions using mixed micelle substrates of PA and TX-100 (which was held at 0.03 to 0.06%) showed that PAP activity was entirely dependent on the presence of PA. From Lineweaver-Burk representation (Fig. 2 insert), the Km value was 0.14 ± 0.02 mM and the specific activity of the final enzyme preparation was 2850 ± 100 nmol/min/mg protein under the standard assay conditions.

At least two forms of PAP-1 activity were found in the rat liver cytosol fraction as evidenced by heparin column fractionation and the sensitivity to the NEM and Mg^{2+}. Indeed, the highly purified PAP enzyme which appeared unaffected by 1 mM EDTA + 1 mM EGTA was inhibited by NEM. When Mg^{2+} was added to the reaction mixture at 10 mM, the PAP-1 activity was slightly stimulated (Table II).

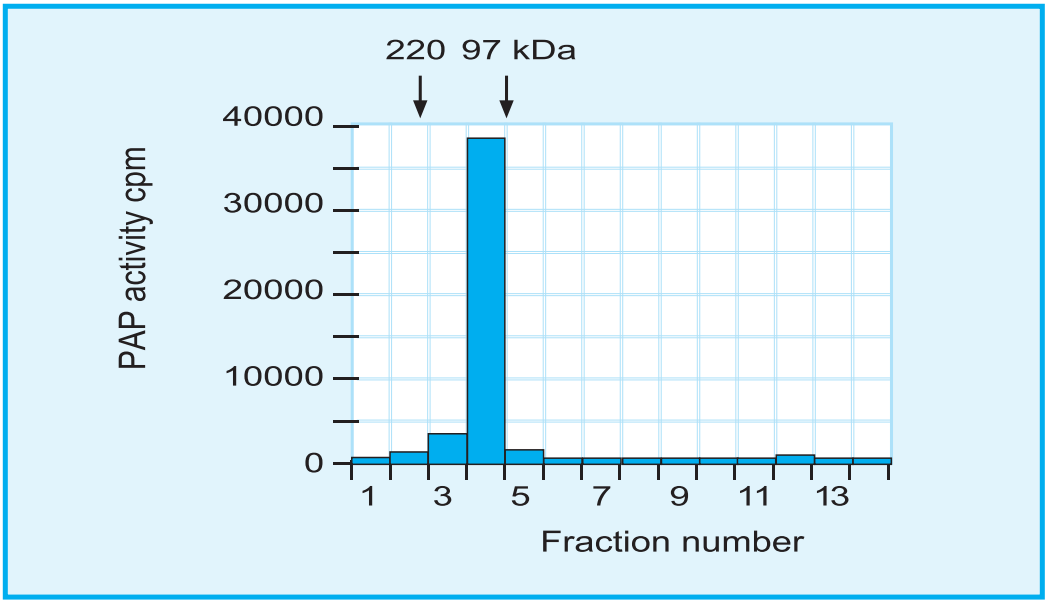

Figure 1 : Identification of cytosol phosphatidic acid phosphohydrolase activity after SDS-poly-acrylamide gel electrophoresis.

On the other hand, the partially purified PAP (peak 1 from Heparin chromatography) was insensitivity to the NEM, but showed a slight stimulation (1.5-fold) when 1 mM EDTA + 1 mM EGTA was added to the reaction mixture. This stimulation was completely cancelled by 10 mM Mg^{2+} (Table II). These results showed that the soluble PAP is consisted of at least two isoforms, which were differently affected by Mg^{2+} and NEM.

Discussion

In mammalian cells, phosphatidic acid phosphohydrolase (PAP) is consisted of several isoforms, which can be distinguished from each other with respect to subcellular localisation, cation dependence, and sensitivity to various inhibitors [3,19]. Phasphatidic acid phosphohydrolase-1 (PAP-1) activity is distributed between the cytosol and endoplasmic reticulum and is regarded as the metabolic form, involved in the glycerolipid metabolism. In previous studies, the role of this enzyme was assessed by treating cells or homogenates with several enzyme inhibitors such as propranolol [20-23], noradrenaline [24], sphingosine[19, 25-27] or fatty acids and their acyl-CoA [11,28]. These studies, however, have been confined to use of crude enzyme preparations, and hence, the molecular properties of animal phosphatidic acid phosphohydrolase, particularly the soluble form PAP-1, have remained entirely unknown. The membranous form PAP-2 has been purified from yeast and animal tissue membranes [6, 14-17], but the purification of animal PAP-1 was so far achieved only to a very modest extent. This is mainly due to the instability of PAP-1 enzyme activity and to its capacity to aggregate as we have recently reported *(Elabbadi and al. Submitted)*. Indeed, all measurable PAP-1 activity was lost when the purification steps were carried out in absence of PA, DAG and Triton X-100. Our work represents an initial purification to nearly homogeneity of animal PAP-1 from the soluble fraction. Upon SDS-polyacrylamide gel electrophoresis, the PAP-1 activity was located directly in those gel slices

Relative activity expressed as % of control (standard assay conditions = 100%)		
	Purified PAP	Heparin peak 1
20 mM NEM	59 ± 4	98 ± 5
1 mM EDTA + 1 mM EGTA	103 ± 5	157 ± 6
1 mM EDTA + 1 mM EGTA + 5 mM Mg^{2+}	97 ± 4	117 ± 5
1 mM EDTA + 1 mM EGTA + 10 mM Mg^{2+}	130 ± 5	**100 ± 4**

Table II : Effects of N-ethymaleimide (NEM) and Magnesium (Mg^{2+}) on the purified PAP enzyme. PAP activity was measured under standard assay conditions. Results are expressed as % of control. Each value is the mean of ± S.E.M. for triplicate determinations.

containing a single band protein of 110 kDa (Fig.1), thus confirming that this protein represented the enzyme. Although the enzyme preparation was not homogeneous, this is the most purified preparation of PAP-1 about 10,000-fold over that of homogenate fraction that has been described to date.

In mammalian cells, Mg^{2+} has been described to stimulate PAP-1 activity using cytosol fraction or crude homogenate [3-5]. Mg^{2+} was also a nearly absolute requirement for the activity of PAP-1, partially purified from yeast [29]. Our results showed that the highly purified PAP-1 enzyme was not affected by 1 mM EGTA and 1 mM EDTA, but remained sensitive to thioreactive reagents like NEM. When the Mg^{2+} was added to the assay mixture at 10 mM, the activity was stimulated but to a limited extent (Table II). On the other hand, the partially purified PAP (peak 1 from heparin chromatography) was insensitive to the NEM but stimulated by 1 mM EGTA and 1 mM EDTA (Table II). As expected, the addition of 10 mM Mg^{2+} cancelled this stimulation. However, caution must be used when interpreting these data, since bivalent cations including Mg^{2+} which would interact with membranes or phospholipids rather then directly with enzyme itself. This is particularly true when the experiments were carried out using cytosol or crude homogenate fractions. In addition, other factors such as enzyme aggregation or its oligomeric structure as we have previously suggested ([11] *Elabbadi Submitted*) may be responsible for this discrepancy, and further work is required to confirm the significance of the present finding. On the other hand, the apparent Km value for the highly purified PAP was 0.14 ± 0.02 mM, within the range of values reported in rat liver (0.13-0.17 mM [11] and in yeast (0.05 mM) [29].

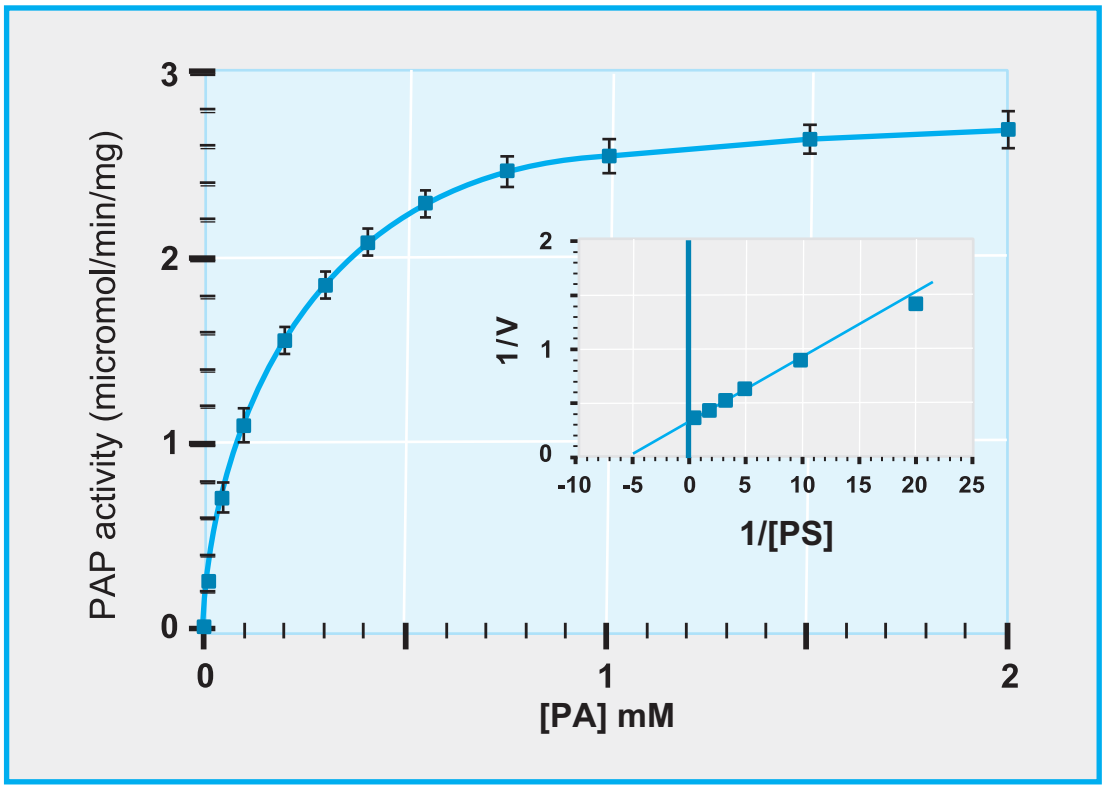

Figure 2 : Effect of phosphatidic acid (PA) concentration on the initial velocity of phosphatidic acid phosphohydrolase-1 (PAP-1) activity

In the present study, we have succeeded in purifying to nearly homogeneity of animal PAP-1 (soluble form), which can be involved in glycerolipid biosynthesis in mammalian cells. Recently, we showed that the inhibition of PAP-1 by fatty acids and their acyl-CoA esters appears to involve a negative allosteric interaction (oligomeric structure), suggesting that PAP-1 could be regulated *in vivo* by the intracellular ration of PA/fatty acids [11]. Therefore, this work would provide a valuable basis for further purification and characterisation of PAP-1 in order to elucidate the regulatory mechanisms operating in the conversion of PA to diacylglycerol at the molecular level.

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Résumé

La phosphatidique acide phosphohydrolase-1 (EC 3.1.3.4), enzyme clé dans la biosynthèse des glycérolipides chez les mammifères, est purifiée à partir de la fraction soluble (cytosol) du foie de rat. Après précipitation par le poly (éthylène glycol) 8000, l'enzyme est solubilisée à l'aide du Triton X-100 et stabilisée par l'acide phosphatidique et le diacylglycérol. Les extraits au Triton X-100 sont alors fractionnés par

chromatographie sur colonne Hydroxyapatite, Mono Q, Héparine et CDP-diacylglycérol-Sépharose. L'analyse sur gel de polyacrylamide de la fraction hautement purifiée, montre deux bandes principales à 110 kDa et 55 kDa. La détection directe de l'activité enzymatique dans le gel de polyacrylamide a permis d'identifier clairement que la bande 110 kDa coïncide avec l'activité phosphatidique acide phosphohydrolase-1. L'enzyme purifiée reste sensible au N-ethylmaleimide (thioréactif) mais faiblement stimulée par Mg2+. L'activité enzymatique spécifique est de 2850 ± 100 nmol/min/mg de protéine et le Km pour l'acide phosphatidique est de 0.14 ±. 0.02 mM. Le présent travail nous a permis de purifier pour la première fois la phosphatidique acide phosphohydrolase-1 (forme soluble) à partir d'un tissu animal avec un enrichissement de l'activité spécifique supérieur à 10000 fois. Ce travail constitue une base fondamentale pour l'étude au niveau moléculaire des propriétés de cette activité phosphatidique acide phosphohydrolase-1, longtemps restée inconnue.

Mots-clés: *Phosphatidique acide phosphohydrolase – purification – foie de rat*

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