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ENZYMOLOGICAL CHARACTERISTICS OF PLASMA MEMBRANE PHOSPHATIDATE PHOSPHOHYDROLASE (PAP_2) FROM RAT LIVER*

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Abstract – Phosphatidate phosphohydrolase (PAP_{2b}, fraction b) was purified from the plasma membrane of rat liver cells. The K_m for the surface concentration of phosphatidic acid was 0.43 mol%. The subunit of the enzyme had an M.W. of 33.8 kDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The native enzyme shows a molecular weight of 182 kDa in a gel filtration column packed with Sephacryl S_{300} in the presence of Triton X-100. The pH optima obtained for PAP_{2b} were 5.5 and 7 in imidazole and Tris- HCl buffers, respectively. The membrane homogenate enzyme (PAP_2) consumed the lamellar (L_a) phase of phosphatidate and was activated (approximately 3-fold) by Lubrol PX, CTAB and Tween 80 and inhibited by Zn^{2+} and Mn^{2+}. The inhibition was concentration dependent. These cations affected PAP_{2b} activity through the phase transition of phosphatidate from lamellar (L_a) to inverted hexagonal (H_{II}) form. Guanidine hydrochloride and urea increased PAP_2 activity (2-fold) up to 20mM concentrations by stabilizing the L_a phase. Optimum activity of purified PAP_{2b} was obtained at 3% trehalose and 7% sucrose. The data suggested that the stability of the L_a form of phosphatidate by detergent micelles may take place through surface dilution processes.

Keywords – Phosphatidate phosphohydrolase, phosphatidic acid

1. INTRODUCTION

Phosphatidate phosphohydrolase (PAP, EC 3.1.3.4) catalyzes the dephosphorylation of phosphatidate to yield diacylglycerol and inorganic phosphate (P_i) [1]. So far, two isoforms of PAP have been distinguished in rat liver [2]. The first isoform, designated as $PAP_{1,}$ is associated with the cytosol and microsomes. PAP_1 is responsible for the synthesis of triacylglycerols and phospholipids, is sensitive to N-ethylmaleimide (NEM) and requires Mg^{2+} for its activity [1, 2]. The second isoform (PAP_2) is bound to the plasma membranes and is insensitive to Mg^{2+} and N-ethylmaleimide. This form has been considered to be involved in lipid signaling pathways [1, 2].

Gel filtration chromatography of PAP_2 yields two separate peaks designated as PAP_{2a} and PAP_{2b}. Both forms can consume different diacylglycerol phosphates as substrate, inactivated by the removal of detergents from their media, and are inhibited by bivalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} [3]. PAP_{2a} and PAP_{2b} differ in sialic acid content [4]. Most studies have used PAP_{2b} because PAP_{2a} consists of only 35% of total PAP_2, and has not yet been purified [3]. Amphiphilic amines have been described as potent inhibitors of PAP_2. The polyamins spermine, spermidine and puterscine have no effect on the activity of purified PAP_{2a} and $PAP_{2b.}$ Neither form is activated by fatty acids or phospholipids [3]. PAP_1

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has been extensively studied, and it has recently been reported that the phase transition of substrate plays an important role in its activity [5]. For PAP_2, however, the suitable substrate configuration has not yet been clarified.

In this study, we have investigated some enzymological characteristics of PAP_2 and PAP_{2b} including the effects of cations, detergents, urea and guanidine HCl (structure breaking compounds) on their activities and on phase transition form L_a to H_{II} configurations of phosphatidate.

2. MATERIAL AND METHODS

a) Materials

Phosphatidic acid (sodium salt), dithiothreitol (DTT), N-ethylmaleimide (NEM), leupeptin, hydroxylapatite, pepstatin, soya bean trypsin inhibitor, Affi Gel Blue, heparin Sepharose, molecular weight markers, n-octyl glucoside, CTAB (Cetyltrimethylammonium bromide), Lubrol PX and guanidine HCl were obtained from Sigma Chemical Co. (U.S.A). All other chemicals were reagent grade.

b) Animals

The source of rats and their maintenance were the same as reported by Haghighi et al [6].

c) Enzyme purification

PAP_{2b} was purified from rat liver plasma membrane as described by Fleming et al [3] except that hydroxylapatite chromatography was done by the batchwise method.

d) Enzyme assay

PAP_2 and PAP_{2b} activities were measured in the assay buffer (250µl) containing 50mM Tris- HCl buffer pH 7.4, 1 mM DTT, 1mg/ml bovine serum albumin, 3.2mM Triton X-100, 4 mM NEM, 1 mM EGTA, 1 mM EDTA, 0.35 mM phosphatidate and an appropriate amount of the enzyme solution [7]. The assay mixture was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml trichloroacetic acid (10%). Hence, the P_i released was measured [6]. One unit (U) of PAP_2 activity was defined as the amount of enzyme that catalyzed the release of 1µ mole of P_i per minute under the standard assay conditions.

e) Kinetic studies

Kinetic constants were determined based on the surface dilution kinetic model [8]. The mole percent of phosphatidate in the mixed micelles of the Triton X-100/ phosphatidate was calculated using the formula: mol %=([PA] / [PA]+ [Triton X-100])×100 [8]. The line of double reciprocal plots were drawn using linear regression analysis.

f) Separation of L_a and H_{II} forms of phosphatidic acid

This separation was performed as previously reported [5]. In brief, phosphatidate (0.35 mM) was incubated in the assay buffer and after phase equilibration (10 min) the L_a and H_{II} phase were separated by centrifugation at 1,500 ×g for 20 min at 4°C. The supernatant containing L_a phase was removed and H_{II} phase in the pellet was confirmed by sudan black [9].

g) Determination of suitable configuration of phosphatidate required for PAP_2 activity

PAP_2 activity was measured in the presence of the L_a or H_{II} form of PA at various concentrations of Triton X-100. The H_{II} form of PA was prepared by the addition of 100mM Ca^{2+} to the aqueous emulsion of PA and centrifugation at 1,500 ×g for 20min at 4ºC [5]. The platelet was then washed with distillated water and centrifuged again. The washing step was repeated five times, and finally the H_{II} form obtained was dissolved in the assay buffer.

h) The effects of cations, urea and guanidine HCl on the L_a and H_{II} formation

In order to determine the L_a to H_{II} phase transition, phosphatidate (0.35 mM) was incubated in the assay buffer in the absence and presence of cations, urea and guanidine HCl at different concentrations for 10 min and the change in absorbance at 356 nm was recorded [9]. The L_a to H_{II} transition was determined by acidic digestion of phosphatidic acid in L_a phase as described by Haghighi et al [5].

i) The effects of Mg^{2+} and Triton X-100 on the L_a to H_{II} transition phase of phosphatidate in the assay buffer

The effects of various concentrations of Mg^{2+} in the presence and absence of Triton X-100 were examined on L_a to H_{II} phase transition in an assay buffer by 10 min incubation and then separation and measurement of the L_a form by acidic digestion of phosphatidic acid in the L_a phase

j) pH profile

PAP_{2b} activity was examined in imidazole buffer (pH 5-6.5) and Tris- HCl buffer (pH 6-9.5) containing the other components as in the assay buffer.

k) The effects of trehalose and sucrose on the purified PAP_{2b}

Various concentrations of trehalose and sucrose were added in the assay mixture and enzyme stability was examined at 24, 48 and 72 h at 4 ºC.

l) Other methods

Protein concentration was determined by the Bradford method [10], with bovine serum albumin as the standard.

SDS poly acrylamide gel electrophoresis was performed using 10% slab gel as described by Thompson [11]. Prior to electrophoresis, protein samples were precipitated in acetone/ammonia (30 / 1.7, V/V) to remove Triton X-100 [12]. Protein bands were detected by silver staining [13]. The native molecular weight of PAP_{2b} was estimated by gel filtration on a Sephacryl S_{300} column (2.5×43 cm) equilibrated with buffer A (25 mM Tris HCl, pH 7.4, 10%glycerol, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% W/V Triton X-100, 1 mM benzamidine and 5µg/ml each of lupeptin, pepstatin or soya bean trypsin inhibitor). The column was calibrated with thyroglobulin (669 kDa), catalase (232 kDa), albumin (67kDa) and chymotrypsinogenA (25 kDa).

3. RESULTS

The specific activity of purified PAP_{2b} was 7350mU/mg protein and the enzyme showed a major band (over 90%) on SDS-PAGE with an M.W. of about 33.8 kDa (Fig. 1). PAP_{2b} exhibits an apparent molecular mass of 272 kDa on gel filtration in the presence of Triton X-100 (Fig. 2).

PAP_{2b} activity was measured as a function of the surface concentration of PA, expressed as mol % of PA in Triton X-100 mixed micelles. A hyperbolic curve was produced, suggesting that rat liver PAP_{2b}

obeys surface dilution kinetic. At a bulk concentration of 0.3 mM PA, a K_m value of 0.43 mol % was obtained using Lineweaver-Burk plot (Fig. 3).

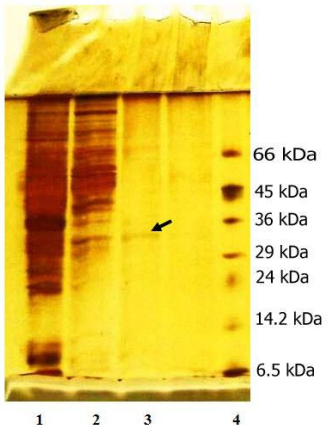
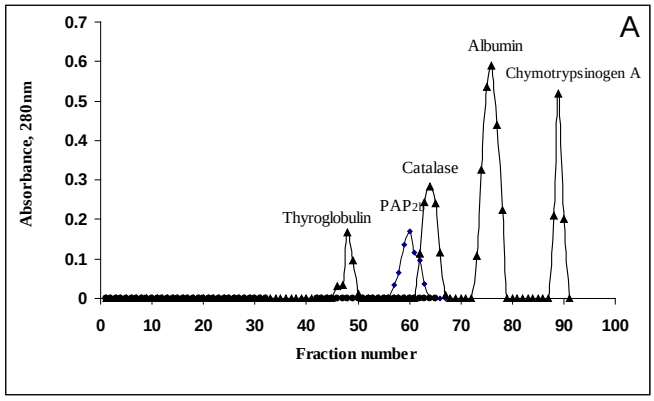


Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified PAP_{2b}. Lanes 1 to 3 are homogenate (180µg), membrane fraction(40µg) and purified enzyme(10µg), respectively. Line 4 is molecular mass standards (each of 5µg), from the top, bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldhyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), lactalbumin (14.2 kDa) and aprotinin (6.5 kDa). For details see text.



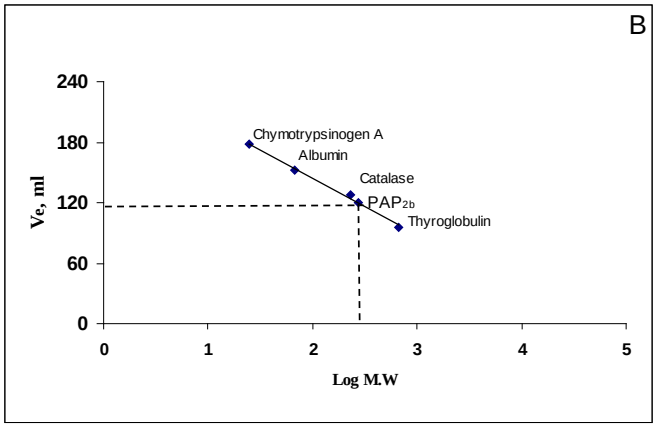


Fig. 2. Gel filtration was done on a Sephacryl S_{300} column (43 × 2.5 cm) equilibrated with buffer A. The column was calibrated with 10 mg / ml each of thyroglobulin (669 kDa), catalase (232 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa). From the purified PAP_{2b} 100 µg was applied. Fractions were collected in 2ml volumes. A; the elution profile of standard proteins and PAP_{2b}. B; the data from A were plotted against M.W's. The dotted lines show the enzyme elution volume and M.W. of the enzyme. For details see text.

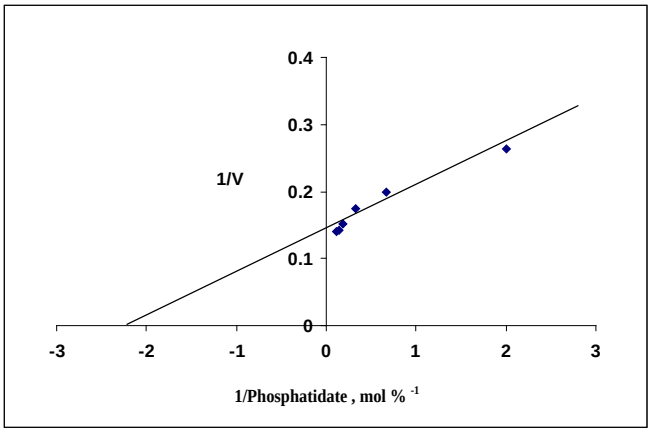


Fig. 3. Double reciprocal plot of PAP_{2b} activity against surface concentration of PA. The activity of PAP_{2b} was measured at different surface concentrations (mol %) of PA. The molar concentration of PA was held constant at 0.3 mM while the Triton X-100 concentration was varied. Each point represents the mean of two independent experiments.

Fig. 4 demonstrated that the membrane homogenate of PAP_2 consume the L_a phase of PA rather than the H_{II} phase as substrate. The activity of PAP_{2b} was measured in the presence of different concentrations of Zn^{2+} and Mn^{2+} (Fig. 5A). Both cations inactivated PAP_{2b}, but Zn^{2+} was a more effective inhibitor than Mn^{2+} at the concentration of 1 mM. These cations stimulate the L_a to H_{II} phase transition of PA at different rates, so that Zn^{2+} has high potential for the L_a to H_{II} phase transition (Fig. 5B). The effect of different concentrations of guanidine HCl and urea (structure breaking compounds) on the activity of membrane homogenate of PAP_2 (native enzyme) was determined (Fig. 6A). Both urea and guanidine HCl decrease the L_a to H_{II} phase transition of PA and increased enzyme activity (Fig. 6B). At 10 mM of guanidine- HCl or urea, membrane homogenate activity (PAP_2) was increased by approximately 2-fold (Fig. 6A). Other detergents such as CTAB (1mM) and Lubrol PX or Tween 80 (4 mM) also stimulated about a 3-fold membrane homogenate of PAP_2 activity (Fig. 7). The presence of Triton X-100 increased the L_a fraction of PA by 20 % (Fig. 8). Addition of Mg^{2+} in the absence of Triton X-100 decreased the L_a phase to about 40 %, whereas in the presence of Triton X-100 only up to a 20% decrease was observed. Thus, the stimulatory effect of Mg^{2+} on L_a to H_{II} phase transition was decreased by the presence of Triton X-100 (Fig. 8). Maximum PAP_{2b} activity was observed at pH 5.5 and 7 for imidazole and Tris- HCl buffers, respectively (Fig. 9A). Fig. 9B shows that the maximum remaining L_a form level in imidazole and Tris-HCl buffers is consistent with the pH optimum obtained for PAP_{2b} activity. Fig. 10 shows the effects of various concentrations of trehalose and sucrose on the activity of PAP_{2b} at different times.

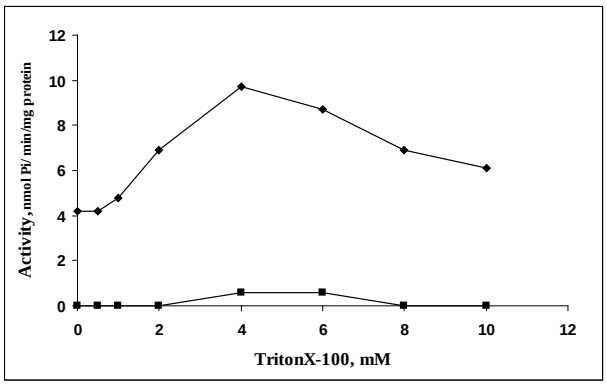


Fig. 4. The effects of L_a and H_{II} form of PA on PAP_2 activity. PAP_2 activity was measured in the assay buffer using L_a (♦) and H_{II} (■) phases of PA in the presence of indicated Triton X-100 as described in the method. Each point represents the average of two independent experiments.

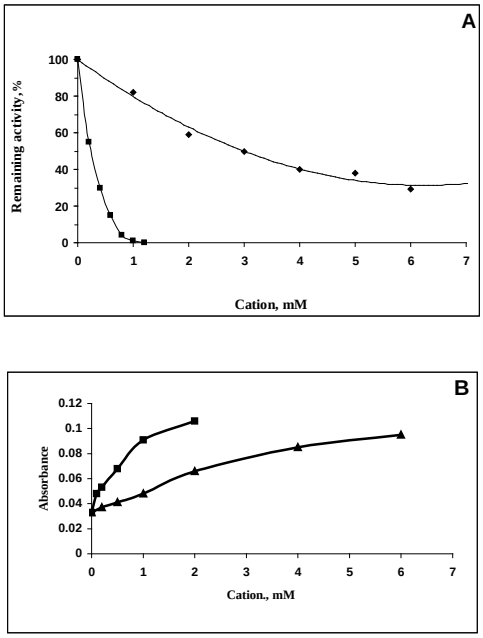


Fig. 5. The effects of Mn^{2+} and Zn^{2+} on PAP_{2b} activity and phosphatidic acid configuration. (A) The enzyme activity was measured in the presence of indicated concentrations of Mn^{2+} (♦) and Zn^{2+} (■) as described in the methods. (B) The absorbance of phosphatidic acid (0.35mM) in the assay buffer at 356 nm was measured in the indicated concentrations of Mn^{2+} (▲) and Zn^{2+} (■). Each point represents the mean of two independent experiments.

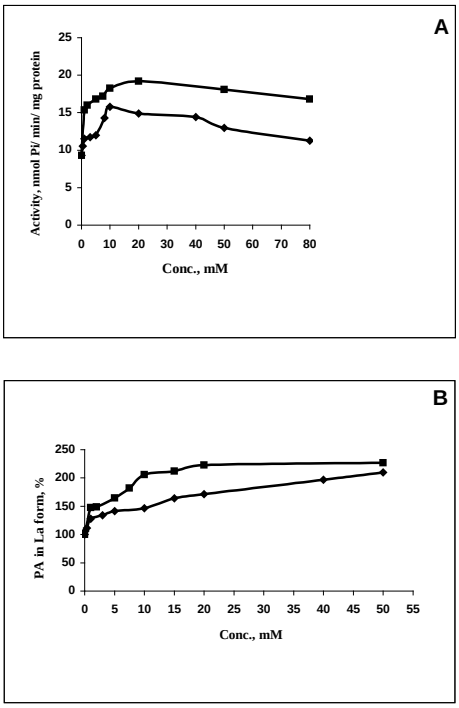


Fig. 6. The effects of urea and guanidine- HCl on PAP_2 activity and phosphatidic acid configuration. (A) The enzyme activity was measured in the presence of indicated concentrations of urea (♦) and guanidine HCl (■) as described in the methods. (B) phosphatidic acid (0.35mM) was incubated in the assay buffer in the presence of indicated concentrations of urea (♦) and guanidine HCl (■), and after equilibrium L_a phase was separated, digested with perchloric acid and released phosphate was measured as described in the methods. Each point represents the average of two independent experiments.

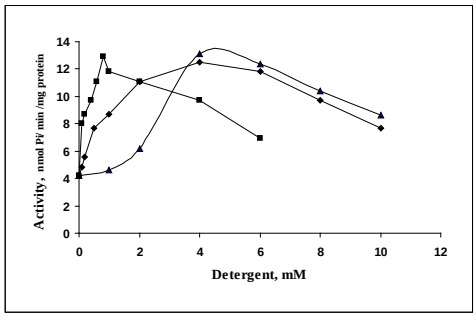


Fig. 7. The effect of detergents on PAP_2 activity. The enzyme activity was measured in the presence of indicated concentrations of CTAB (■), Tween 80 (♦) and Lubrol PX (▲) as described in the method. Each point represents the mean of two independent experiments.

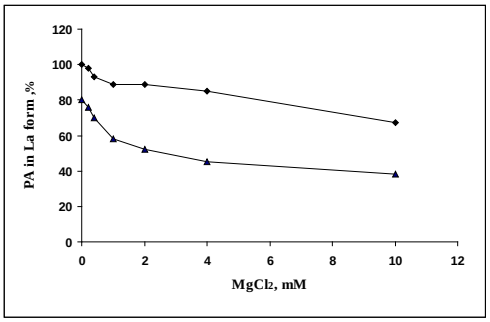


Fig. 8. The effects of Mg^{2+} on L_a phase formation of PA. Phosphatidic acid (0.35mM) was incubated at different concentrations of Mg^{2+} in the presence (♦) and absence (▲) of Triton X-100 in assay buffer, and after equilibrium L_a phase was separated and digested with perchloric acid and the phosphate released was measured as described in the methods. Each point represents the average of two independent experiments.

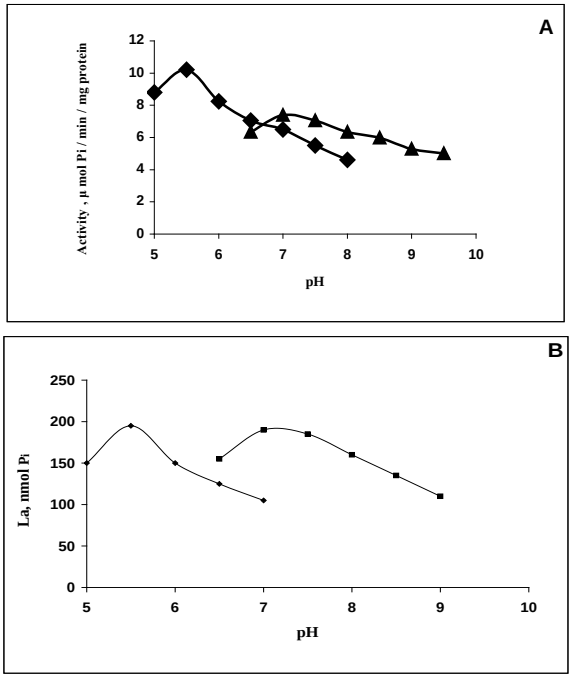


Fig. 9. The effect of pH on the activity of PAP_{2b} and L_a levels of PA. The activity of PAP_{2b} measured in imidazole (♦) and Tris HCl (■) buffers at the indicated pH (A). Phosphatidic acid (0.35mM) was incubated in imidazole (♦) and Tris HCl (■) buffers at the indicated pH and after equilibrium L_a phase was separated and digested with perchloric acid and phosphate released was measured as described in the methods (B). Each point represents the average of two independent experiments.

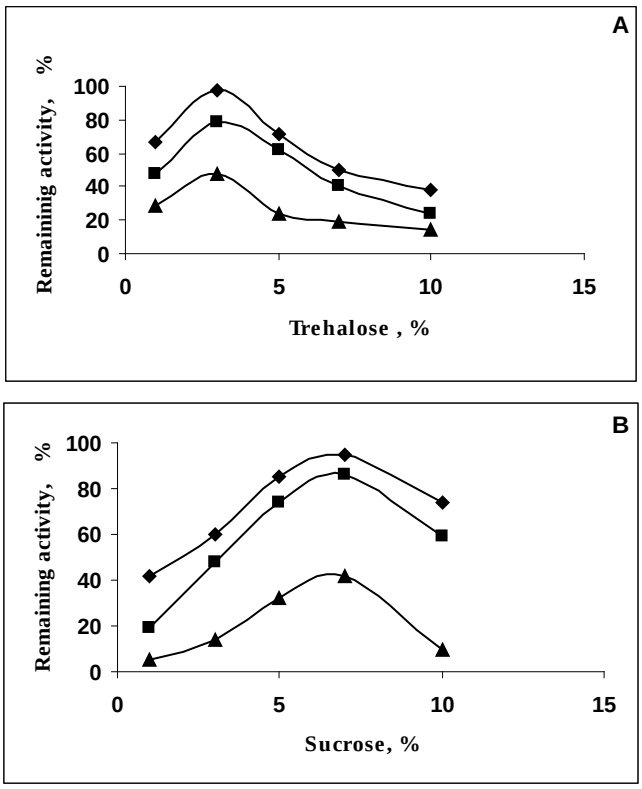


Fig. 10. The effects of trehalose and sucrose on the activity of PAP_{2b}. The enzyme activity was measured in the presence of indicated concentrations of trehalose (A) and sucrose (B) at 24(♦), 48 (■) and 72 (▲) h. Each point represents the mean of two independent experiments.

4. DISCUSSION

SDS gel electrophoresis of the purified PAP_{2b} enzyme showed a major distinct band having an M.W. of 33.8 kDa. Other workers have reported a subunit M.W. of 35 [14] and 31 [15] kDa. Gel filtration of PAP_{2b} in the presence of Triton X-100 micelles shows a molecular mass of 272 kDa. Assuming that each Triton X-100 micelle contains one protein molecule and that each micelle is 90 kDa in size [16], then the native PAP_{2b} would have a molecular mass of 182 kDa and is likely to be a hexamer.

PAP_{2b} activity when using the L_a phase of PA as a substrate was much higher than that of the H_{II} phase (Fig. 4). Increase in PAP_2 activity observed by urea and guanidine HCl results in the stabilization of the L_a phase of PA (Fig. 6A). This stabilization may be due to the hydrophobic interactions of these reagents with lipids [9]. Cations such as Zn^{2+} and Mn^{2+} both inhibit PAP_{2b} activity and stimulate H_{II} phase formation. This is consistent with the report that the bivalent cations tend to stoichiometrically bind to lipid headgroups [9]. Farren et al [15] have demonstrated that calcium and other bivalent ions tend to induced the H_{II} phase possibly through a decrease in dehydration of PA, which has two negative phosphate groups per molecule, inducing H_{II} core particles. On the other hand, Papahadjopoulos et al [17] have demonstrated that the potential of various cations to bind PA are different and directly related to the H_{II} phase. The difference in the PAP_2 inhibition by various cations may be related to the potential ability of the cations for the L_a to H_{II} phase conversion. Haghighi et al [5] have previously reported that PAP_1 utilizes an intermediate configuration of PA when the L_a to H_{II} phase transition takes place. The report demonstrated that cations (bivalent and trivalent) at low concentrations (0.25–0.3 mM), where the equilibrium between the L_a and H_{II} phases took a longer time, stimulated PAP_1 activity, but at higher concentrations the L_a to H_{II} transition was rapid, enzyme activity decreased. PAP_{2b}, however, was inhibited by these cations, even at their low concentrations because only the L_a form is the real substrate.

Detergents are known to provide a surface of catalysis for the enzymes using lipid substrate [7, 18]. Kanoh et al [7] have demonstrated that PAP_2 exhibit a clear preference to Triton X-100 among other detergents such as 3-[(3-Cholamidopropyl) dimethylammonio]-1-propansulfonate (CHAPS), deoxycholate and cholate, however, they did not analyze in detail the mechanism(s) of the enzyme activation by detergents. They proposed that these detergents not only provide a catalytic surface, but also mimic a microenvironment favorable to the action of enzyme in the absence of phospholipids [7]. In this study, the effects of Lubrol PX, CTAB and Tween 80 on PAP_2 activity were tested. These detergents stimulate enzyme activity up to 3-fold (Fig. 7). Studies on the activation of phospholipase C (PLC) by detergents showed that the rate limiting step in the reaction is the release of the hydrophobic product, diacylglycerol (DG), from the active site and DG inhibits PLC via product inhibition [19]. The presence of detergents, however, stimulates the release of DG and causes a stimulatory effect on the PLC activity [19].The DG resulted from dephosphorylation of PA is also a product inhibitor to PAP_2 [3]. Hence, we propose that the stimulatory effect of detergents could be exerted; 1) by providing a catalytic surface and microenvironment, 2) by inhibiting the L_a to H_{II} phase transition through substrate surface dilution (see below and 3) by preventing the inhibitory effect of DG on the enzyme activity.

The yeast membrane PAP and PAP_1 of rat liver cytosol require Mg^{2+} for their activity, whereas PAP_2 appears to be unaffected by this factor [3]. PAP_1 is stimulated by Mg^{2+} up to 2mM by inducing favorable substrate configurational, but Mg^{2+} up to 4 mM has no effect on PAP_2 activity [7, 20]. The activity of PAP_1 can be independent of Mg^{2+} in the presence of Triton X-100 [21, 22]. These differences may be explained by the result that, up to 4 mM Mg^{2+} concentration and in the presence of Triton X-100, about 85 % of the PA remains in the L_a form which is a favorable substrate for PAP_2. At higher Mg^{2+} levels, however, because L_a form is declined, the activity of PAP_2 will also decrease (Fig. 8). The phase transition from L_a to H_{II} of PA by Mg^{2+} in the absence of Triton X-100 was more rapid, suggesting that Triton X-100 prevents the phase transition from L_a to H_{II}.

The pH optima obtained in this study and those reported by other investigators [3, 7, 23] have been between 5.5 to 7.4 (Fig. 9A). Having different pH for PAP_2 in different buffer systems has not been interpreted so far. Thus the different pH optima can be explained by the L_a to H_{II} phase transition of PA in the media (Fig. 9B). The hydration of PA is affected by various pH values and the low hydration of PA results in H_{II} phase formation [24, 25]. Farren et al [15] have demonstrated that dioleoyl-PA increasingly prefers the H_{II} phase below pH 5. Thus, the maximum remaining L_a form level in different buffer systems is consistent with pH optimum.

Polyhydric alcohols and sugars have been used for many years as stabilizing agents for the maintenance of the biological activities of macromolecules [26]. The dominant mechanism by which sugars and polyols stabilized proteins against heat denaturation is through their effect on the structure of water, which in turn determines the strength of hydrophobic interactions [27]. Optimum activity of PAP_{2b} was obtained in the presence of 3% trehalose or 7% sucrose (Fig. 10). Aurell et al [28] have suggested that the trehalose promotes direct transition of dioleoylphosphatidylethanolamine from L_a to H_{II} phase. Hence, the declined activity of enzyme above 3% trehalose and 7% sucrose in this study must be due to the promotion of the L_a to H_{II} transition phase of PA, and not through the inactivation of enzyme. These sugars decrease the hydration of PA, and subsequently induce the H_{II} phase [27]. On the other hand, Mauro et al [29] have demonstrated that trehalose occupies at least a 2.5 times larger volume than sucrose, maltose, glucose, and fructose, which correlates this property with the ability to protect the structure and function of enzymes against thermal inactivation. They also demonstrated that when the concentrations of all sugars were corrected by the percentage of the occupied volume, the sugars will present the same effectiveness. These data explain why 7% sucrose has the same effect as 3% trehalose.

NOMENCLATURE

PAP Phosphatidate phosphohydrolase
PA Phosphatidic acid
L_a Lamellar phase
H_{II} Inverted hexagonal phase
CTAB Cetyltrimethylammonium bromide

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