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THE SOLUBILIZATION OF PLATELET MEMBRANE-BOUND ACETYLCHOLINESTERASE AND ARYL ACYLAMIDASE BY EXOGENOUS OR ENDOGENOUS PHOSPHATIDYLINOSITOL SPECIFIC PHOSPHOLIPASE C

R. MAJUMDAR and A. S. BALASUBRAMANIAN*

Neurochemistry Laboratory, Department of Neurological Sciences, Christian Medical College and Hospital, Vellore—632 004, India

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Abstract—Phosphatidylinositol specific phospholipase *C* from *Staphylococcus aureus* could solubilize acetylcholinesterase up to 55% from sheep platelets in the presence of ethylenediaminetetra acetic acid (EDTA). The endogenous phosphatidylinositol specific phospholipase *C* of platelets activated by deoxycholate (at 3–5 mM) could also solubilize the enzyme to a similar extent. The solubilized enzyme could be further purified to apparent homogeneity by affinity chromatography without the use of any detergents. It is suggested that phosphatidylinositol specific phospholipase *C* will be a useful tool in the solubilization of acetylcholinesterase from mammalian sources and its purification free of detergents. The present study also demonstrates the parallel behaviour of acetylcholinesterase and aryl acylamidase in platelets confirming their identity.

Phosphatidylinositol specific phospholipase *C* (PIPL*C*) has been a subject of increasing interest for its involvement in the stimulus induced receptor mediated hydrolysis of phosphatidylinositol (PI) that has been implicated in the initiation of a signal cascade resulting in the mobilization of calcium, the release of arachidonic acid for the synthesis of prostaglandins, thromboxane and leukotriene and the activation of protein kinase *C* [1–3]. Recent evidence has also suggested that PIPL*C* causes a substantial release of membrane-bound exoenzymes indicating a specific interaction between PI and certain enzymes in the membrane [4].

Acetylcholinesterase (AChE) exists in a number of molecular forms usually bound to the cell membrane. Detergents are commonly used for its solubilization. Earlier studies have indicated that AChE is associated with a serotonin-sensitive aryl acylamidase (AAA) in different mammalian systems. This was evidenced by their identical behaviour on different affinity chromatographic purification procedures, gel electrophoresis, gel filtration, immunoprecipitation and response to inhibitors and lipid requirements [5–9].

It is postulated that platelets represent a useful model for neural cells in certain limited ways in terms of metabolism of biogenic amines and acetylcholine [10]. Most of the AChE and AAA present in platelet is membrane-bound [8]. Although the presence of acetylcholine and AChE in platelets is known, their function in platelets is unclear. During platelet activation a particular form of AChE is released [11]. Acetylcholine stimulates an increase in the metabolism of membrane phosphoinositides in different cells [1, 12] and also plays a role in the aggregation and release reaction of platelets [11]. Exploration of the nature of attachment between AChE and platelet membrane may therefore provide insight not only into the polymorphic nature of AChE that is observed in the excitable tissues [13] but also indicate how the enzyme is hooked to the membrane. Moreover the platelet is a rich source of PIPL*C* that plays a major role in platelet aggregation and release reaction [14]. We report here that AChE and AAA can be significantly released from platelet membranes by either *S. aureus* PIPL*C* or deoxycholate (DOC) activated platelet PIPL*C* and further purified to apparent homogeneity in a detergent-free form.

MATERIALS AND METHODS

Phosphatidyl choline, dipalmitoyl or dimyristoyl (PC), PI (soybean), phospholipase A_2 (*Naja naja*), PC specific phospholipase *C* (*Clostridium perfringens*), DOC, sodium cholate, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor and marker proteins were purchased from Sigma Chemical Co, MO, U.S.A. Amberlite CG-50 resin was from BDH, Poole, U.K. *Staphylococcus aureus* (Cowan I strain) was obtained from the Microbiology unit, Wellcome Research Laboratory of this institution.

Isolation of platelets. Platelets were isolated from sheep blood as described earlier [8]. The final sediment of platelets obtained by centrifugation at 2400 *g* for 15 min was washed repeatedly with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–NaOH, pH 7.4. The preparation contained less than 0.1% contamination by erythrocytes as revealed by microscope.

Preparation of S. aureus *PIPLC.* PIPL*C* from *S. aureus* was prepared by purification of the culture supernatant by passage through Amberlite CG-50

* Address reprint requests to Dr. A. S. Balasubramanian.

column as described earlier [15]. The active fractions that showed PIPLC activity were pooled and precipitated with ammonium sulfate at 65% saturation. The sediment obtained by centrifugation at 12,000 *g* for 30 min was dissolved in 20 mM Tris–HCl, pH 7.4, dialyzed against water to remove ammonium sulfate and redialyzed against 10 mM HEPES–NaOH, pH 7.4.

Preparation of platelet 105,000 g supernatant containing PIPLC activity. A cytosolic fraction enriched in PIPLC was prepared from platelets by the following procedure. Sheep platelets in 20 mM HEPES–NaOH, pH 7.4 were sonicated at 0–4° for 60 sec (every 20 sec burst followed by an interval of 30 sec) with a microprobe (MSE Mullard) at maximum frequency. The particulate and soluble fractions were separated by centrifugation at 105,000 *g* for 1 hr at 4°. The supernatant was dialyzed against 5 mM HEPES–NaOH, pH 7.4 and used in subsequent assays. The pellet that still retained 90% AChE and AAA activity was washed several times with 5 mM HEPES–NaOH, pH 7.4.

DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) treatment of platelet 105,000 g supernatant. To study the effect of DTNB on the solubilization of AChE mediated by PIPLC, the 105,000 *g* platelet supernatant (1 mg protein) was incubated in 50 mM Tris–HCl, pH 7.2 and 0.1–0.4 mM DTNB in dimethyl sulfoxide (final concentration of dimethyl sulfoxide was 1.5% v/v which had no effect on enzyme activity) in a total vol. of 0.8 ml at 37° for 5 min. The mixture was cooled and dialyzed at 4° against 50 mM Tris–acetate, pH 5.5 for PIPLC assay or 20 mM HEPES–NaOH, pH 7.4 for solubilization experiments.

Enzyme assays and protein estimation. Bacterial PIPLC was assayed in 1.0 ml of 20 mM HEPES–NaOH, pH 7.4 containing 2 mg PI (sonicated in water) and 0.2–0.5 ml Amberlite CG-50 purified enzyme. For the platelet 105,000 *g* supernatant PIPLC, the same assay mixture was used except that 1 mM $CaCl_2$ was included and the pH of the buffer was 5.5 instead of 7.4 [16]. The control reaction mixture contained the enzyme that had been heated for 10 min at 100° and cooled. Incubation was carried out for 30–60 min at 37° and was terminated by adding 5 ml $CH_3OH/CHCl_3/HCl$ (500/500/3 v/v). The upper aqueous phase was estimated for organic phosphorus by the method of Galliard *et al.* [17]. One unit of phospholipase *C* was defined as 0.01 μmoles water soluble organic phosphorus released in 60 min at 37°. For PC specific phospholipase *C* assay, the substrate used was PC (dimyristoyl or dipalmitoyl) and the procedure was similar to that described above. AChE according to Ellman *et al.* [18] and AAA were assayed as described before [5, 8]; for AAA the incubation mixture contained 6.2 μmoles *o*-nitroacetanilide instead of 4.6 μmoles. Protein was estimated according to Lowry *et al.* [19] using crystalline bovine serum albumin as the standard.

Protease activity was determined by the method of Kunitz [20] or Lundh [21] using α-casein or *N*-benzoyl arginine ethyl ester respectively as substrates.

Solubilization experiments. Solubilization of AChE and AAA was done either with platelet homogenate (protein 15–19 mg/ml) or the washed 105,000 *g* pellet (protein 12–15 mg/ml) in 20 mM HEPES/NaOH, pH 7.4. The solubilization mixture (0.9 ml) consisted of the above homogenate or pellet and the following additions in different experiments. (a) 0.3–0.5 ml *S. aureus* PIPLC (10–30 units); (b) DOC, 1–10 mM; (c) 0.2–0.6 ml of platelet 105,000 *g* supernatant (75 units/ml, PIPLC) and 1 mM $CaCl_2$ in the presence or absence of 3–5 mM DOC. The incubation was for 30–60 min at 37° with constant shaking. After cooling the mixture in ice, it was centrifuged at 105,000 *g* for 1 hr at 4° and the supernatant was assayed for AChE and AAA activities.

Affinity chromatography. Meta-aminophenyltrimethylammoniumchloride hydrochloride, an inhibitor of AChE linked through a 30 carbon spacer arm to Sepharose 4B was prepared as described earlier [6]. The affinity column (4 × 1 cm) was equilibrated with 20 mM Tris–HCl, pH 7.4. AChE and AAA solubilized from platelets with *S. aureus* PIPLC was dialyzed against the equilibrating buffer and 7.3 ml of the enzyme was passed through the column at a flow rate of 2–3 ml/hr. The column was washed with 60 ml of 20 mM Tris–HCl, pH 7.4/0.1% Triton X-100/0.5 M NaCl and subsequently with 50 ml equilibrating buffer containing 0.5 M NaCl. Elution was carried out with 20 mM Tris–HCl, pH 7.4/0.3 M tetraethylammonium bromide and fractions of 1.5 ml were collected. The active fractions were pooled and dialyzed against 5 mM Tris–HCl, pH 7.4 and used for gel electrophoresis.

Gel electrophoresis. Disc gel electrophoresis was performed in 5% gel at pH 8.3 according to Davis [22]. After electrophoresis the gels were either stained by Coomassie blue G for protein or assayed for activity after slicing the gel (1.5 mm thickness) and extraction with 20 mM potassium phosphate buffer, pH 7.0/0.5% Triton X-100 as described earlier [8]. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out in 10% gel, under reducing conditions according to Laemmli [23]. Gels were stained with 0.1% Coomassie Blue G solution in water/methanol/acetic acid mixture (65/25/10) for 2 hr and destained with the same mixture without dye.

RESULTS AND DISCUSSION

Solubilization of AChE and AAA by S. aureus *PIPLC*

Staphylaroccus aureus PIPLC (20 units) purified by Amberlite CG-50 chromatography, solubilized about 55% of both AChE and AAA from platelet membrane in the presence of 1 mM EDTA (Table 1). There was no loss of enzyme activity as the insolubilized activity was fully recovered in the 105,000 *g* pellet. The solubilization was not further enhanced either by increasing the amount of PIPLC up to 30 units or by prolonging the incubation time up to 90 min. Several lines of evidence indicated that solubilization was mediated by PIPLC (Table 1). The solubilization was partially or completely suppressed by 0.25 M KCl, 5 and 10 mM $CaCl_2$, the known inhibitors of *S. aureus* PIPLC [24]. Neither PMSF (60 μM) nor trypsin inhibitor (2 mg) had any effect on the process, indicating that the release was

Table 1. Release of AChE and AAA from the platelet homogenate by *S. aureus* PIPLC

Additions	% activity released into 105,000 *g* supernatant AChE	AAA
No addition	5	6
1 mM EDTA	10	10
PIPLC (20 units)	15	12
PIPLC + 1 mM EDTA	55	54
PIPLC + 1 mM EDTA + 60 μM PMSF	55	53
PIPLC + 1 mM EDTA + 2 mg trypsin inhibitor	52	53
PIPLC + 1 mM EDTA + 5 mM Ca^{2+}	29	30
PIPLC + 1 mM EDTA + 10 mM Ca^{2+}	9	9
PIPLC + 1 mM EDTA + 0.25 M KCl	10	12
PIPLC + 1 mM EDTA + 3 mg PI	30	31
PIPLC + 1 mM EDTA + 6 mg PI	16	15
PIPLC + 1 mM EDTA + 3 mg PC	54	55
PIPLC + 1 mM EDTA + 6 mg PC	45	42
PC specific PLC (*C.perfringens*, 2 mg)	6	6
PC specific PLC (*C.perfringens*, 2 mg) + 1 mM EDTA	6	6
Phospholipase A_2 (*Naja naja*) 100 units	6	5

Platelet homogenate (0.4 ml) in 20 mM HEPES–NaOH, pH 7.4 was incubated for 1 hr at 37° with the above additions as described in the text.For phospholipase A_2 treatment homogenate was in 20 mM Tris–HCl, pH 9.2. The 105,000 *g* supernatant was assayed for AChE and AAA activities. Values are expressed as % activity of control homogenate incubated at 37° for 1 hr. EDTA, $CaCl_2$, PMSF, PI or PC alone at the concentration used did not affect AChE or AAA activity or their solubilization as observed in appropriate control tubes.

not mediated by protease action. Moreover, the Amberlite CG-50 preparation did not exhibit either protease activity or PC specific PLC activity. While exogenous PI (as a competitive inhibitor of PIPLC) prevented the solubilization in a dose dependent manner, PC was unable to do so. The solubilization was effected only by PIPLC as evidenced by the fact that phospholipiase A_2 (*Naja naja*) or PC specific PLC (*Clostridium perfringens*) did not solubilize the AChE or AAA from platelet membrane (Table 1).

Effect of EDTA

EDTA was essential for bacterial PIPLC mediated solubilization although EDTA alone had no effect on solubilization (Table 1). Figure 1 shows the EDTA dependency of solubilization. The maximum solubilization of AChE and AAA occurred at 1 mM EDTA. It was, however, noteworthy that PIPLC activity by itself was independent of EDTA under the conditions of assay described using exogenous PI as substrate (data not shown). It is possible that the chelation of cationic molecule(s) present in the platelet membrane and/or modulation of the membrane surface by EDTA could be responsible for the increased susceptibility to hydrolysis of the endogenous PI molecules by the bacterial PIPLC in the presence of EDTA resulting in greater solubilization of the enzyme. An earlier study has indicated that rat brain and liver lysosomes contain an EDTA insensitive PIPLC which however exhibits a 40–110% stimulation of its activity in the presence of EDTA. This specific stimulation was attributed to the chelation of Ca^{2+} [25].

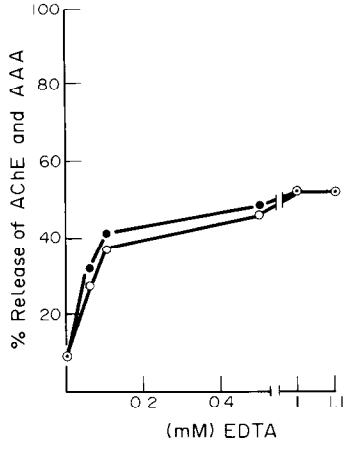

Fig. 1. The effect of EDTA on the solubilization of AChE (●) and AAA (○) by *S. aureus* PIPLC. The incubation mixture contained platelet homogenate (0.4 ml) in 20 mM HEPES–NaOH, pH 7.4, bacterial PIPLC (20 units) and increasing concentrations of EDTA.

Effect of DOC on solubilization

DOC has been shown to activate PIPLC in platelets particularly when membrane phospholipid is the substrate [26, 27]. The results on solubilization of AChE and AAA from the platelet homogenate by DOC are presented in Fig. 2. The maximum release (52%) of AChE and AAA into the 105,000 *g* supernatant occurred at 5 mM DOC. At lower (1–2 mM) concentrations of DOC there was only insignificant solubilization. The decrease in solubilized activity at >5 mM DOC was found to be due to the marked inactivation of the enzyme activities by DOC at >5 mM when added to the platelet homogenate as shown in the inset of Fig. 2. The following findings suggested that the solubilization of AChE and AAA by DOC at 5 mM from the platelet homogenate was mediated through an endogenous PIPLC and not by simple detergent action alone.

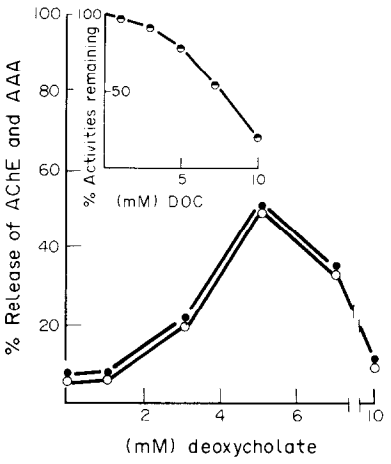

Fig. 2. Percentage activity of AChE (●) and AAA (○) released by DOC into the 105,000 *g* supernatant (main figure) and inactivation of AChE and AAA in the platelet homogenate by DOC treatment (inset). The homogenate (0.4 ml) was incubated with DOC at various concentrations. Activity is expressed as percentage of incubated control sample of untreated homogenate.

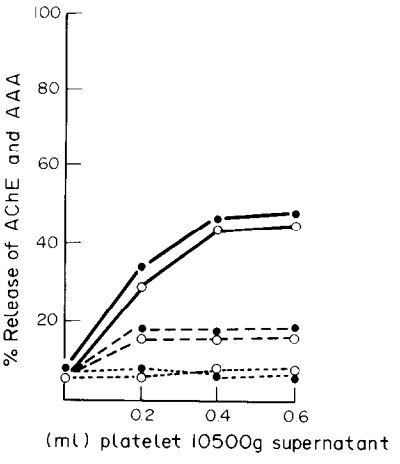

Fig. 3. The effect of platelet 105,000 *g* supernatant on the solubilization of AChE (●) and AAA (○) from the washed 105,000 *g* pellet in the presence of Ca^{2+}. The 105,000 *g* pellet in 20 mM HEPES–NaOH, pH 7.4 (0.3 ml) was incubated for 45 min with: (a) 105,000 *g* supernatant in the absence of DOC (· · · ·); (b) boiled supernatant in the presence of 4 mM DOC (– – –) and (c) supernatant in the presence of 4 mM DOC (———).

(a) Sodium cholate, another detergent at 5 mM did not release the enzyme activities (Table 2). (b) When the homogenate was first centrifuged at 105,000 *g* and the residue (that contained almost 90% of original activity) after washing with 5 mM HEPES–NaOH, pH 7.4, subjected to 5 mM DOC treatment, there was only very little (18–19%) solubilization of AChE and AAA from the residue (Table 2). (c) On the other hand, the 105,000 *g* supernatant that had detectable PIPLC activity and no PC specific phospholipase *C* activity, upon addition to the 105,000 *g* residue in the presence of 4 mM DOC and 1 mM Ca^{2+} resulted in about 48% solubilization of both AChE and AAA activities (Fig. 3). Omission of DOC nearly abolished solubilization and use of denatured cytosolic fraction (by previous heating at 100° for 10 min) gave only 18–19% solubilization of enzyme activities (Fig. 3). It is evident from Fig. 3 that endogenous PIPLC alone is not sufficient to release AChE and AAA without inclusion of DOC which probably facilitates the accessibility of the membrane PI to the endogenous PIPLC [26]. (d) The maximum solubilization of the enzyme from the 105,000 *g* pellet by the supernatant in the presence of DOC was effective at pH 6.8–7.4, whereas pH 5.5 and 9.2 were not favourable for solubilization (data not shown). It is known that endogenous PIPLC of seminal vesicles, brain and platelets in the presence of DOC shows optimum activity at pH 7.0 [28–30]. (e) More convincing evi-

Table 2. The solubilization of AChE and AAA from platelet homogenate by DOC

Source of enzyme	Additions	% Activity released into 105,000 *g* supernatant	
		AChE	AAA
Platelet homogenate			
	No addition	6	6
	Sodium cholate 5 mM	6	7
	DOC 5 mM	55	54
	DOC 5 mM + 1 mM $CaCl_2$	55	55
	DOC 5 mM + 2 mM EDTA	40	37
	DOC 5 mM + 2 mg PI	34	36
	DOC 5 mM + 4 mg PI	19	17
	DOC 5 mM + 2 mg PC	50	46
	DOC 5 mM + 4 mg PC	42	39
105,000 *g* pellet			
	DOC 5 mM	18	19
	Triton X-100, 1%	55	55

The conditions of the reaction in the presence of the above agents and subsequent procedures are similar to that described under Table 1, except that the incubation period is for 30 min.

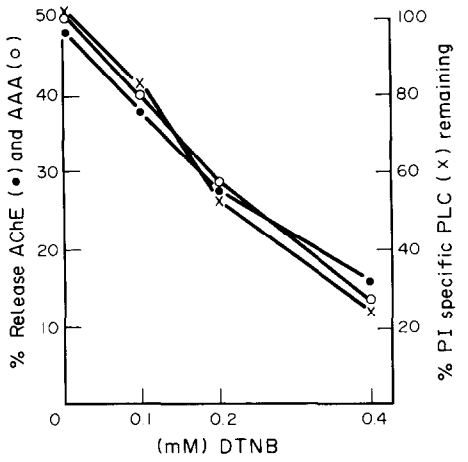

Fig. 4. The effect of DTNB treatment of the platelet cytosolic fraction on the solubilization of AChE and AAA from the 105,000 *g* pellet in 20 mM HEPES–NaOH, pH 7.4 (0.3 ml). The 105,000 *g* supernatant (0.8 ml) was treated with different concentrations of DTNB and dialyzed. The supernatant was incubated with the 105,000 *g* pellet under the conditions described in the text. AChE (●) and AAA (○) activity released are given as a percentage of the total activity in the pellet (100%). PIPL*C* activity (X) is expressed as a percentage of the DTNB untreated control.

dence came from the finding that exogenously added PI (used as competitive inhibitor of endogenous PIPL*C*) at different concentrations was able to prevent the solubilization in the platelet homogenate (Table 2). The minor inhibition of solubilization caused by PC (Table 2) might be due to either its ability to alter the PC to endogenous PI ratio [31] or to its masking effect on the membrane PI [32] which prevented the PIPL*C* interacting with the PI molecules of the membrane.

To further corroborate that the solubilization was mediated by the endogenous PIPL*C* present in the 105,000 *g* supernatant of platelets, the supernatant was pre-treated with DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), a selective cystein-SH group modifying agent. Figure 4 shows that DTNB treatment inhibited the cytosolic PIPL*C* in a dose-dependent fashion as reported by Kyger and Franson [33]. The figure also shows that the inhibition was correlated with a corresponding decrease in the solubilization of AChE and AAA from the platelet 105,000 *g* pellet.

DOC has also been shown to activate endogenous phospholipase A_2 of platelets [27]. However, the involvement of a phospholipase A_2 in the solubilization process was ruled out because phospholipase A_2 of platelets is not inhibited by DTNB [33] and *Naja naja* phospholipase A_2 did not solubilize the enzymes (Table 1).

Affinity chromatographic purification of the bacterial PIPLC solubilized AChE and AAA

The *S. aureus* PIPL*C* solubilized platelet enzyme was subjected to affinity chromatography and the results of purification are summarized in Table 3. The enzyme completely bound to the affinity column and the recovery on elution was always more than 80%. Triton X-100 was not needed for elution from the affinity column. It is noteworthy that almost all the membrane-bound detergent soluble forms of AChE known so far need a detergent for solubilization and elution from the affinity columns [6, 8, 34–37]. Table 3 also shows that AAA and AChE were purified to a similar extent with approx. constant ratio of specific activities and percentage recoveries, thereby supporting the fact that both the activities were associated with the same protein [5, 6, 8].

Gel electrophoresis

Figure 5 shows the gel electrophoretic pattern and the profile of enzyme activities on the gel slices. AAA and AChE were both detected in two different slices, one corresponding to the observed protein band and the other a few millimeters away towards the anode as shown in Fig. 5. There was no visible protein band corresponding to the second peak of activities. The aggregation properties of AChE resulting in multiple bands of activity during gel electrophoresis is known [6, 8, 35] and it is likely that the two peaks of activities arose from an aggregated polymeric form and a non-aggregated form of AChE. SDS–gel electrophoresis of the enzyme under reducing conditions showed a single protein band of mol. wt. 72,000 (Fig. 6).

The treatment of tissues or cells with PIPL*C* from

Table 3. Purification of AChE and AAA from platelet homogenate

Step	Total protein (mg)	AChE Total activity (units)	AChE Specific activity (units/mg)	AChE Recovery (%)	AAA Total activity (units)	AAA Specific activity (units/mg)	AAA Recovery (%)	Ratio of specific activities
Homogenate	60	7.19	0.12	–	0.275	0.0045	–	27
105,000 *g* supernatant after *S. Aureus* PIPL*C* treatment	16.4	3.5	0.21	48	0.12	0.007	44	30
Enzyme after affinity chromatography	0.081	3.0	37.03	43	0.11	1.35	40	27

The methods of solubilization and affinity chromatography are described under Materials and Methods. One unit AAA is defined as 1 μmole of *o*-nitroaniline formed per hour; one unit AChE is defined as 1 μmole of thiocholine formed per min under the standard assay conditions.

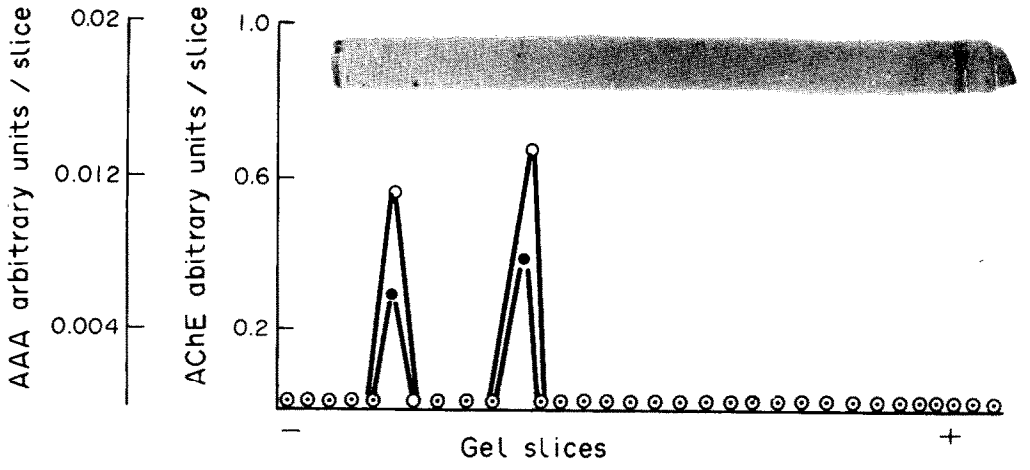

Fig. 5. Polyacrylamide gel electrophoresis under non-denaturing conditions and activity profiles of AChE (●) and AAA (○) of bacterial PIPLC solubilized enzyme (26 μg) after affinity chromatography.

bacterial culture supernatants has demonstrated a selective release of enzymes in a soluble form from the plasma membrane [4, 9, 24]. Their selective release has provided a new concept that these enzymes are anchored to PI in the membranes. The present studies indicate that PIPLC, either endogenous or from *S. aureus*, can be utilized for the solubilization and purification of AChE in a detergent free form from platelet membranes. With the possibility of a wider occurrence in mammalian tissues of PIPLC [38], there are better chances of obtaining in true solution enzymes that are membrane-bound through PI residues. The conditions for solubilization may, however, differ from source to source (such as the need for EDTA when *S. aureus* PIPLC is used and a critical concentration of DOC for activation of the endogenous PIPLC in platelets as shown in the present studies).

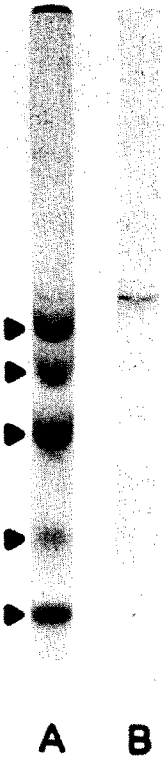

Fig. 6. SDS–polyacrylamide gel electrophoresis of 15 μg of the purified reduced enzyme (gel B). Molecular wt was determined by using the following market proteins (indicated by arrows in gel (A) from top to bottom, bovine serum albumin (68,000), ν-globulin heavy chain (50,000), ovalbumin (43,000), ν-globulin light chain (23,500) and myoglobin (17,800).

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