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Purification of a Phosphatidylinositol 4-Phosphate Kinase from Bovine Brain Membranes

Albrecht Moritz, *Pierre N. E. De Graan, Peter F. Ekhart, *Willem H. Gispen, and Karel W. A. Wirtz

*Center for Biomembranes and Lipid Enzymology and *Division of Molecular Neurobiology, Rudolf Magnus Institute and Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht, The Netherlands*

Abstract: A phosphatidylinositol 4-phosphate (PIP) kinase (EC 2.7.1.68) was purified from bovine brain membranes in a six-step procedure involving solubilization of the enzyme with 170 m*M* NaCl followed by chromatography on diethylaminoethyl-cellulose, phosphocellulose, Ultrogel AcA44, hydroxylapatite, and ATP-agarose. The enzyme preparation was nearly homogeneous and was purified 5,600-fold with a final specific activity of 85 nmol/min/mg of protein and a yield of 20%. Its molecular mass was 110 kilodaltons, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was specific for PIP; phosphorylation of phosphatidylinositol and diacylglycerol was not observed. **Key Words:** Phosphatidylinositol 4-phosphate kinase–Enzyme purification. **Moritz A. et al.** Purification of a phosphatidylinositol 4-phosphate kinase from bovine brain membranes. *J. Neurochem.* **54,** 351–354 (1990).

Receptor-controlled activation of phosphoinositide-specific phospholipase C results in the enhanced breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2), thereby generating the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (Berridge and Irvine, 1984; Nishizuka, 1984; Berridge, 1987; Williamson and Hansen, 1987). In response to this breakdown, levels of PIP_2 in the plasma membrane are thought to be replenished by the sequential phosphorylation of phosphatidylinositol (PI) by PI kinase and phosphatidylinositol 4-phosphate (PIP) kinase (Berridge and Irvine, 1984; Williamson and Hansen, 1987). PI kinase activity is predominantly membrane bound and has been found to be associated with subcellular organelles throughout the cell, including the plasma membrane. PIP kinase is preferentially associated with plasma membrane-rich subcellular fractions (Lundberg et al., 1985), yet it also occurs in the membrane-free cytosol (Kai et al., 1968). Recently, the purification of PI kinase to apparent homogeneity was reported (Belunis et al., 1988; Hou et al., 1988; Porter et al., 1988; Walker et al., 1988; Yamakawa and Takenawa, 1988). The PI kinase purified from bovine brain myelin was shown also to have PIP kinase activity (Saltiel et al., 1987). To date, PIP kinase has been extensively purified from human erythrocyte membranes (Ling et al., 1989) and from rat brain cytosol (Van Dongen et al., 1984). In the latter purification, very low recoveries of enzyme activity were obtained. This was due to the extreme instability of brain PIP kinase, a major problem also encountered by others during purification of the brain enzyme (Kai et al., 1968; Lundberg et al., 1986). In an effort to stabilize PIP kinase from brain, we have found buffer conditions that effectively conserve the activity during purification. Here, we report the purification of PIP kinase from bovine brain membranes to apparent homogeneity at a high yield.

MATERIALS AND METHODS

Materials

Diethylaminoethyl (DEAE)-cellulose (DE52) and phosphocellulose (P11) were purchased from Whatman (Maidstone, U.K.). Ultrogel AcA44, ATP-agarose (AGATP type 3), and MonoQ HR 5/5 were from Pharmacia LKB (Uppsala, Sweden). Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad (Richmond, VA, U.S.A.). Silica gel plates were from Merck (Darmstadt, F.R.G.). ATP was purchased from Boehringer (Mannheim, F.R.G.). [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol) was purchased from Amersham (U.K.). PIP was purified from bovine brain using Folch extraction and DEAE–cellulose chromatography (Hendrickson and Ballou, 1964). Other reagents were of reagent grade.

Preparation of membrane fraction

Three fresh bovine brains were collected on ice at the local slaughterhouse. The brains (1,140 g wet weight) were freed of blood vessels and connective tissues, and a 25% (wt/vol) homogenate was prepared in 50 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 0.32 *M* sucrose, and 0.3% (vol/vol) 2-mercaptoethanol (buffer A) containing the protease inhibitors leu-

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Address correspondence and reprint requests to Dr. K. W. A. Wirtz at Center for Biomembranes and Lipid Enzymology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Abbreviations used: DEAE, diethylaminoethyl; kDa, kilodalton(s); PAGE, polyacrylamide gel electrophoresis; PEG 20,000, polyethylene glycol 20,000; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

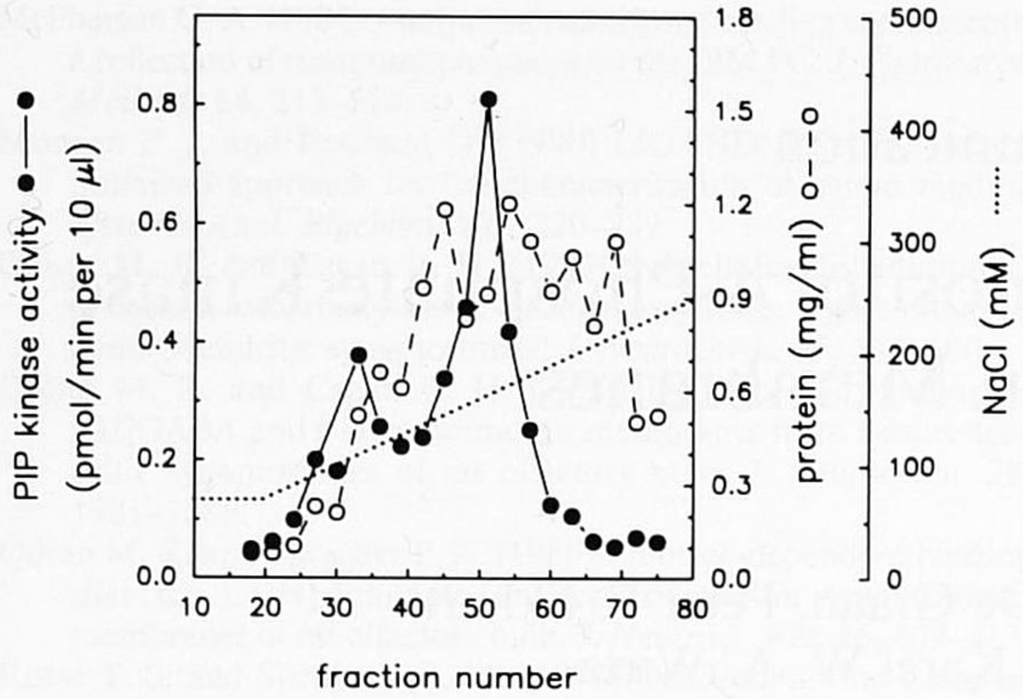

FIG. 1. DEAE–cellulose column chromatography: PIP kinase activity (●) and protein content (○). The membrane supernatant from bovine brain was loaded onto DEAE-cellulose and eluted with a linear NaCl gradient (•••) from 70 to 300 m*M*. Fractions of 21.6 ml were collected. The elution pattern is representative of four purifications.

peptin (0.5 mg/L), soybean and lima bean trypsin inhibitors (1 mg/L each), and phenylmethylsulfonyl fluoride (PMSF; 1 m*M*). Homogenization was carried out with an Ystral homogenizer for 1 min at high speed. The homogenate was centrifuged at 14,000 *g* for 1 h, and the membrane pellet was washed once by rehomogenization in 3,000 ml of buffer A followed by centrifugation at 14,000 *g* for 1 h. All procedures were carried out at 0–4°C.

PIP kinase assay

The assay procedure was, with some modifications, similar to the method described by Van Dongen et al. (1984). Protein solutions (10 μl) were assayed for PIP kinase (EC 2.7.1.68) activity in a final volume of 50 μl containing 50 m*M* Tris-HCl (pH 7.4), 80 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 80 μ*M* bovine brain PIP (Folch fraction), bovine serum albumin (0.4 mg/ml), 15 μ*M* [γ-^{32}P]ATP (0.75 μCi/assay), and 0.02% Triton X-100. The reaction was started by addition of the enzyme solution, continued for 10 min at 30°C, and then stopped by addition of 2 ml of chloroform/methanol/concentrated HCl (200:100:0.75 by volume). The extraction and further analysis of the ^{32}P incorporated into PIP_2 were performed as described by Jolles et al. (1981). Before estimation of the recovery of PIP kinase activity in the different steps of the purification procedure, the pools of active fractions were diluted fivefold with buffer C (see Purification of PIP kinase, step 4) to exclude the influence of differences in buffer composition and ionic strength on the PIP kinase activity.

Purification of PIP kinase

All procedures were carried out at 0–4°C. As a first step, the membranes were homogenized in 3,300 ml of Tris-HCl (pH 7.8), 1 m*M* EDTA, 170 m*M* NaCl, 1 m*M* PMSF, and 0.3% (vol/vol) 2-mercaptoethanol. After stirring for 30 min, the membrane suspension was centrifuged at 14,000 *g* for 1 h. PIP kinase activity was recovered from the supernatant and was stabilized by addition of 0.2 volumes of buffer containing sixfold-concentrated stabilizing agents, resulting in a final concentration of 50 m*M* Tris-HCl (pH 7.8), 1 m*M* EDTA, 0.25 *M* sucrose, 0.3% (vol/vol) 2-mercaptoethanol, 0.1% Triton X-100, 0.1% polyethylene glycol 20,000 (PEG 20,000), 50 μ*M* ATP, 0.8 m*M* PMSF, and ~105 m*M* NaCl. The final solution was centrifuged at 14,000 *g* overnight to sediment remaining membrane particles.

In step 2, the ensuing supernatant (3,950 ml) was diluted with 2,000 ml of 50 m*M* Tris-HCl (pH 7.8), 1 m*M* EDTA, 0.25 *M* sucrose, 0.3% (vol/vol) 2-mercaptoethanol, 0.1% Triton X-100, 0.1% PEG 20,000, and 50 μ*M* ATP to lower the ionic strength (equivalent to ~70 m*M* NaCl). The solution was applied to a DEAE–cellulose column (50 × 3.6 cm), which was equilibrated with 10 volumes of 50 m*M* Tris-HCl (pH 7.8), 1 m*M* EDTA, 0.25 *M* sucrose, 0.3% (vol/vol) 2-mercaptoethanol, 0.1% Triton X-100, 0.1% PEG 20,000, 50 μ*M* ATP, and 0.1 m*M* PMSF (buffer B) containing 70 m*M* NaCl. After the column was washed with 2 volumes of buffer B containing 70 m*M* NaCl, the enzyme was eluted with a 2,000-ml linear gradient from 70 to 300 m*M* NaCl in buffer B at a flow rate of 90 ml/h. The active fractions eluting at ~160 m*M* NaCl were pooled (yield, 37%).

In step 3, the pool of active fractions from step 2 (345 ml) was centrifuged at 35,000 *g* overnight to remove insoluble matter and then applied to a phosphocellulose column (10 × 1.5 cm), which was equilibrated with 10 volumes of buffer B containing 200 m*M* NaCl. After the column was washed with 5 volumes of the latter buffer, the enzyme was eluted with 1.2 *M* NaCl in buffer B at a flow rate of 18 ml/h (yield, 29%).

In step 4, the pool of active fractions from step 3 (14.2 ml) was applied to an Ultrogel AcA44 column (168 × 2.1 cm), which was equilibrated with 2 volumes of 10 m*M* potassium phosphate (pH 6.8), 1 m*M* EDTA, 0.25 *M* sucrose, 100 m*M* NaCl, 0.3% (vol/vol) 2-mercaptoethanol, 0.1% Triton X-100, 0.1% PEG 20,000, 50 μ*M* ATP, and 0.1 m*M* PMSF (buffer C). The enzyme was eluted with buffer C at a flow rate of 20 ml/h (yield, 36%).

In step 5, the pool of active fractions from step 4 (28 ml) was applied to a hydroxylapatite column (7.5 × 1.4 cm), which was equilibrated with 10 volumes of buffer C. After the column was washed with 2 volumes of buffer C, the enzyme was eluted with a 120-ml linear gradient from 10 to 350 m*M* potassium phosphate in buffer C at a flow rate of 2.6 ml/h. The activity eluted at ~250 m*M* potassium phosphate (yield, 22%).

In step 6, the pool of active fractions from step 5 (25.9 ml) was dialyzed overnight against 100 volumes of 50 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 0.25 *M* sucrose, 50 m*M* NaCl, 0.3% (vol/vol) 2-mercaptoethanol, 0.1% Triton X-100, 0.1% PEG 20,000, 50 μ*M* ATP, and 0.1 m*M* PMSF (buffer D).

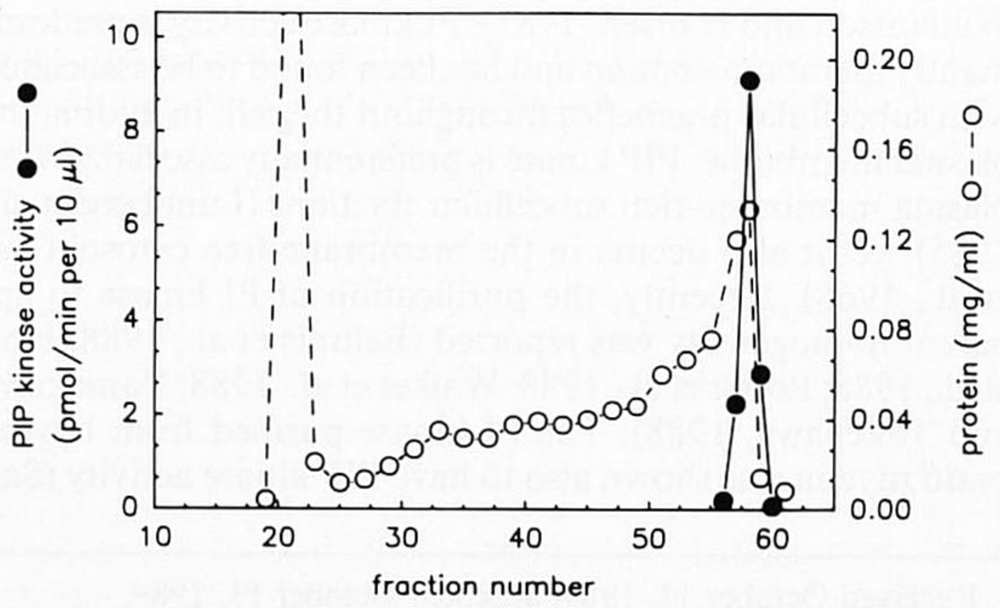

FIG. 2. Ultrogel AcA44 column chromatography: PIP kinase activity (●) and protein content (○). The enzyme solution from step 3 was loaded onto Ultrogel AcA44 and eluted as described in Materials and Methods. Fractions of 9.9 ml were collected. The elution pattern is representative of four purifications.

TABLE 1. *Purification of PIP kinase*

Purification step	Protein (mg)	Total units (nmol/min)[a]	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Membrane supernatant	2,449	36.7	0.015	1	100
DEAE-cellulose	362	13.8	0.038	2.5	37
Phosphocellulose	22	10.8	0.49	32.5	29
Ultrogel AcA44	2.8	13.4	4.8	315	36
Hydroxylapatite	0.86	8.2	9.6	626	22
ATP-agarose	0.088	7.5	85	5,566	20

[a] PIP kinase activity was assayed as described in Materials and Methods.

The dialysate was applied to an ATP–agarose column (4 × 1.1 cm), which was equilibrated with 10 volumes of buffer D. After the column was washed with 5 volumes of buffer D, the enzyme was eluted with a 80-ml linear gradient from 50 to 1,000 m*M* NaCl in buffer D at a flow rate of 2.5 ml/h. The activity eluted at ~400 m*M* NaCl (yield, 20%). The purified enzyme was stored at −20°C.

Other methods

Protein concentrations were determined using the BCA protein assay kit from Pierce (Rockford, IL, U.S.A.) with bovine serum albumin as the standard. Before content determination, proteins were precipitated twice with trichloroacetic acid in the presence of sodium deoxycholate (Bensadoun and Weinstein, 1976). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970) using 11% slab gels. The proteins were stained with Fast Green.

RESULTS AND DISCUSSION

PIP kinase activity in bovine brain was found to be present in both the cytosolic and membrane fractions, as was observed in rat brain (Van Dongen et al., 1984; Cochet and Chambaz, 1986). Under our assay conditions, we found ~40% of the PIP kinase activity in the cytosol and ~60% of the activity associated with the membranes. We have purified the membrane-bound kinase, which could be solubilized very effectively by treating the membranes with a buffer containing NaCl (170 m*M*). Using human erythrocytes, the PIP kinase was also released from the membranes by extraction with NaCl (1 *M*) in the absence of detergent (Ling et al., 1989). In this respect, PIP kinase behaves differently from the membrane-bound PI kinases, which require detergents for solubilization (Endemann et al., 1987).

Previous attempts to purify PIP kinase from rat brain were severely hampered by the instability of the enzyme in the various purification steps (Kai et al., 1968; Van Dongen et al., 1984; Lundberg et al., 1986). We have overcome this problem by using throughout the purification buffers containing EDTA, sucrose, NaCl, 2-mercaptoethanol, Triton X-100, PEG 20,000, PMSF, and ATP. This combination of stabilizing components was prompted by previous reports on the purification of diacylglycerol kinase (Kanoh et al., 1983; Kanoh and Ono, 1984) and PI kinase (Saltiel et al., 1987). Omission of EDTA, NaCl, and ATP in the course of the purification led to complete loss of PIP kinase activity within a few days. Under our buffer conditions, the enzymatic activity was extremely stable throughout the purification. At each stage of the purification, PIP kinase activity remained stable for several weeks at 4°C, and even with the purified enzyme, no detectable loss of activity was observed after storage at 4°C for 1 week.

Chromatography of the salt-extracted membrane proteins on DEAE-cellulose yielded at least three separable PIP kinase activities (Fig. 1). Approximately 50% of the total activity appeared in the flow-through of the column (data not indicated); the second activity peak (~15%) eluted at ~110 m*M* NaCl, whereas the third activity peak eluted at ~160 m*M* NaCl. The PIP kinase activity in the last peak, which represented 37% of the total activity, was further purified. In subsequent steps of the purification, it was found to behave as a single enzymatic activity. At this stage, we do not know the nature and properties of the two other PIP kinase activities.

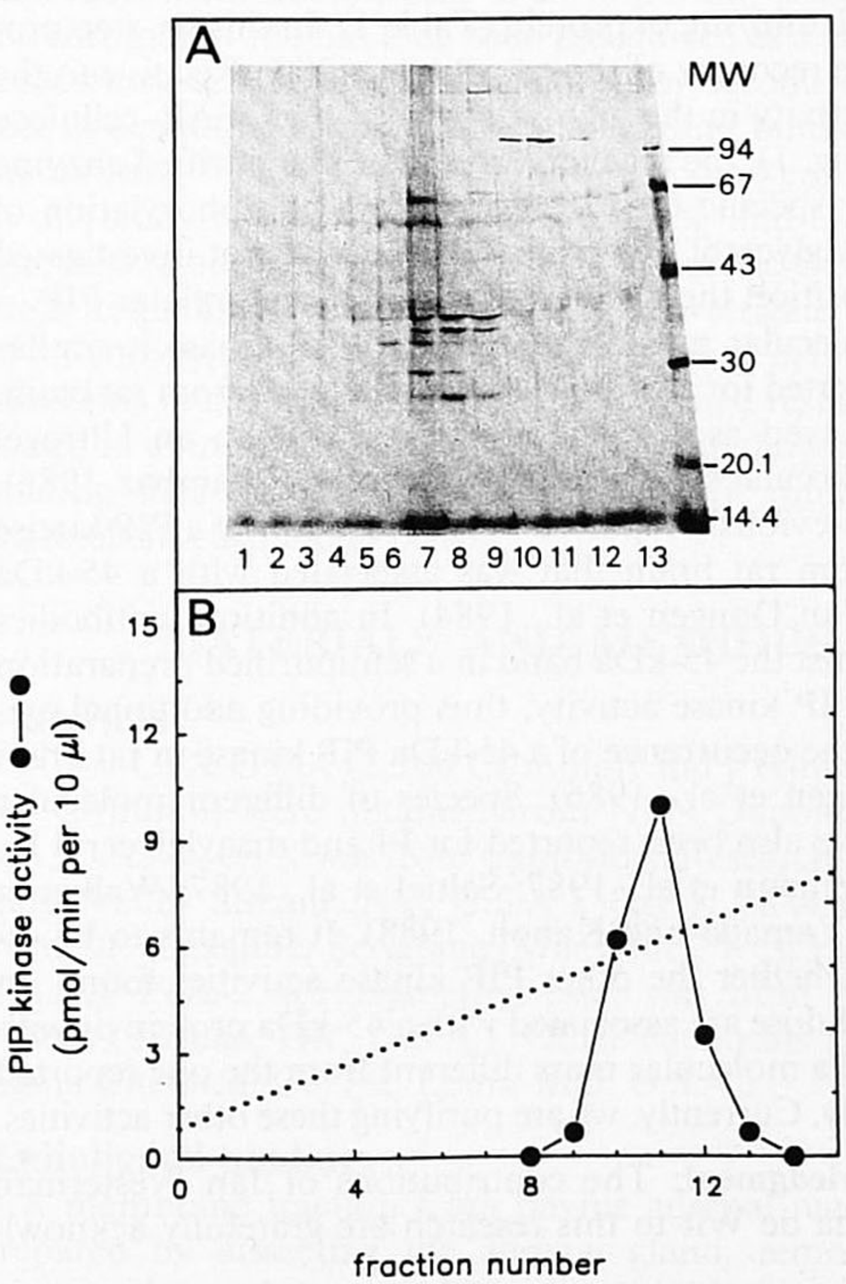

FIG. 3. ATP–agarose column chromatography. The active fractions from step 5 were loaded onto ATP-agarose and eluted with a linear NaCl gradient (•••) from 50 to 1,000 m*M*. **A:** Aliquots (25 μl) of fractions 1–13 were subjected to SDS-PAGE. **B:** The fractions were assayed for PIP kinase activity (●). The estimated amount of protein in fraction 11 (top fraction of PIP kinase activity) was 15 μg of protein/ml. Fraction numbers in A correspond to those in B. The faint bands seen throughout the gel are from 2-mercaptoethanol in the buffer. The elution pattern is representative of two purifications.

PIP kinase has a high affinity for phosphocellulose (Cochet and Chambaz, 1986), which makes this material an attractive step in the purification as well as an effective means to concentrate the enzyme activity before gel filtration chromatography on Ultrogel AcA44. On the latter material, PIP kinase showed an unusual, retarded elution (Fig. 2), which may be due to hydrophobic interactions of PIP kinase with the column material. This anomalous behavior led to an excellent separation of PIP kinase, yielding a 10-fold purification on this column (Table 1). It was repeatedly observed that the total PIP kinase activity was increased after this gel filtration step, possibly owing to the removal of an inhibitor or inhibitors.

The last two purification steps involved chromatography on hydroxylapatite and ATP-agarose. In the latter step, the enzyme was bound to the column in the presence of ATP (50 μM). Apparently, ATP in the buffer does not interfere with the binding of PIP kinase to the ATP affinity column, as was also observed for diacylglycerol kinase (Kanoh et al., 1983). The PIP kinase was eluted with a salt gradient, yielding one activity peak (Fig. 3B). This peak coincided with a protein band clearly separated from most of the contaminating proteins (Fig. 3A). This protein band has an estimated molecular mass of 110 kilodaltons (kDa). By removing the last impurities on a MonoQ column or by reeluting the activity peak on the ATP–agarose column, it was confirmed that the 110-kDa protein was PIP kinase. Up to the ATP–agarose step, the enzyme was purified 5,600-fold to yield a specific activity of 85 nmol/min/mg of protein (Table 1). In this six-step procedure, the recovery of the enzyme was 20%. Relative to the enzyme activity in the high-salt peak of the DEAE–cellulose column (Fig. 1), the recovery was 50%. The purified enzyme was highly specific for PIP, because no phosphorylation of PI or diacylglycerol was observed. We have not investigated at what position the purified enzyme phosphorylates PIP.

The molecular mass of the purified PIP kinase is similar to that reported for a partially purified enzyme from rat brain, which behaved as a 100–110-kDa protein on an Ultrogel AcA44 molecular sieve column (Cochet and Chambaz, 1986). We have previously reported the purification of a PIP kinase activity from rat brain that was associated with a 45-kDa protein (Van Dongen et al., 1984). In addition, antibodies raised against the 45-kDa band in a semipurified preparation inhibited PIP kinase activity, thus providing additional evidence for the occurrence of a 45-kDa PIP kinase in rat brain (Van Dongen et al., 1986). Species of different molecular masses have also been reported for PI and diacylglycerol kinase (Endemann et al., 1987; Saltiel et al., 1987; Walker et al., 1988; Yamada and Kanoh, 1988). It remains to be established whether the other PIP kinase activities found on DEAE-cellulose are associated with a 45-kDa protein or with proteins of a molecular mass different from the one reported in this study. Currently, we are purifying these other activities.

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