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RAPID PURIFICATION OF INOSITOL MONOPHOSPHATE PHOSPHATASE FROM BEEF BRAIN

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A procedure is described for preparation of homogeneous inositol monophosphate phosphatase (EC 3.1.3.25) from beef brain in less than 2 days with an overall recovery of 15-25%. This enzyme, an essential part of the inositol phospholipid cycle in brain, is a proposed site of action of lithium ions in manic-depressive disorders. The major purification steps are:
a) removal of most interfering protein by heat denaturation at 75° C for 1 h,
b) separation by anion exchange at a pH (6.0) near the enzyme's pI (4.9), and
c) adsorption of most remaining impurities on a Procion Red affinity column.

Many hormones, growth factors and neurotransmitters act on cells via stimulation of hydrolysis of inositol phospholipids, causing the formation of the "second messengers" diacylglycerol and inositol-1,4,5 trisphosphate (IP_3) (1). Resynthesis of inositol phospholipids in brain (which lacks an inositol uptake mechanism) requires that released inositol phosphates be hydrolyzed to form free inositol. Lithium uncompetitively inhibits the monophosphate phosphatase that catalyses the hydrolysis of D-inositol-1-phosphate (which arises from phosphatidylinositol), D-inositol-3-phosphate (from glucose-6-phosphate) and D-inositol-4-phosphate (from breakdown of IP_3) (2-4). This phophatase also catalyses the hydrolysis of a few other compounds including 2'-AMP, and β-glycerophosphate. The latter compound is a convenient inexpensive substrate for routine studies. Although it has recently been suggested that therapeutic concentrations of Li salts also inhibit an inositol polyphosphate phosphatase (5) and a "G protein" (6), the inositol monophosphate phosphatase remains an enzyme of considerable interest. A convenient source of this enzyme would be useful for sequence analysis, kinetic and mechanistic studies, preparation of antibodies, etc. This enzyme has been purified from rat brain (3), and during the course of this work, from beef brain (4). We report here a much simpler isolation procedure.

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Materials and Methods

Frozen beef brains (250-400 g) were obtained from Pel-Freez, Rogers, AR. Triton X-100, D,L-inositol-1-phosphate and β-glycerophosphate were purchased from Sigma (St. Louis, MO). Triton X-100 was freed of inorganic phosphate by stirring a 10 % solution in water with Dowex 1. For kinetic analyses, the β-glycerophosphate was freed of traces of inorganic phosphate by conversion to the cyclohexylamine form and crystallization from ethanol. Elemental analysis of the resulting needles showed it to be β-glycerophosphate bis-(cyclohexylamine)·1/2 H_2O. Sephacryl S-200 HR, Q Sepharose Fast Flow, and precast "Phastgels" (10-15% gels for SDS-polyacrylamide gel electrophoresis, 8-25% gels for non-denaturing electrophoresis, and pH 4-6.5 isoelectric focusing gels) and Coomassie stain were obtained from Pharmacia (Uppsala). Matrex Red A (Procion Red) agarose and a kit of other dye affinity columns were purchased from Amicon (Danvers, MA).

Inorganic phosphate was assayed with malachite green as described by Lanzetta et al (7) except that instead of 1 ml of Sterox/50 ml of color reagent, we used 100 µl of 10 % Triton X-100/50 ml. The absorbance was linear with phosphate from 0.5-20 nmol. Protein was assayed using Coomassie Blue reagent and γ-globulin standard from Bio Rad (Richmond, CA). Gel filtration of crude preparations was perfomed on a 90 x 1.6 cm Sephacryl S-200 HR column with a flow rate of 1 ml/min and a mobile phase of 0.2 M KCl, 50 mM tris (pH 7.4) and 5 % glycerol. Reverse phase HPLC was performed on a Vydac 5 cm X 4.6 mm "C4" column with a gradient from 10% acetonitrile, 0.1% TFA to 70% acetonitrile, 0.1% TFA in 30 min at 1 ml/min.

INOSITOL MONOPHOSPHATE PHOSPHATASE ASSAY To 50 µl solution of enzyme containing solution (diluted as needed with tris HCl, pH 7.4 at 25°) in 1.5 ml Eppendorf tubes was added 5 µl of 5 mg/ml bovine serum albumin and 5 µl of a solution of 50 mM β-glycerophosphate (cyclohexylamine salt), 20 mM $MgCl_2$ and 2.5 M NaCl in 20 mM tris pH 7.4. After incubation at 37° for 15 min, the reaction was stopped with 5 µl 4 M $HClO_4$. After centrifugation for 2 min, 50 µl of the supernatant solution was assayed for inorganic phosphate.

In initial experiments with gel-filtration and ion exchange columns, a microtiter-plate assay was used to examine large numbers of fractions. Falcon 96-well plates were rinsed with water to remove phosphate, then 50 µl enzyme solution was incubated at 37° with 10 µl substrate/cofactor solution. After 15 min, 12 µl of the mixture was transferred with an 8 channel pipette to wells containing 0.2 ml malachite green reagent. The plates were examined visually or absorbance measured at 660 nm in a Titertek plate reader.

PURIFICATION OF INOSITOL MONOPHOSPHATE PHOSPHATASE

Step 1) 15,000 g Supernatant A frozen brain (300 - 400 g) was thawed for 30 min to allow cutting into pieces. The tissue was homogenized in a Waring blendor in 2 volumes of a solution containing 150 mM KCl, 50 mM tris HCl (pH 7.4) and 0.1 mM EGTA, then centrifuged at 15,000 g for 30 min.

Step 2) 40-60% Ammonium Sulfate Precipitation Ammonium sulfate (242 g/L) was added to the supernatant solution. After 30 min at 4°, the suspension was centrifuged at 15,000 x g for 30 min. Ammonium sulfate (130 g/L) was added to the supernatant solution. The suspension was centrifuged as before.

Step 3) 75° Heat Treatment The resulting pellet was dissolved in homogenization buffer (28 ml/300 g brain weight), and placed in screw capped 25 ml centrifuge tubes. DTT and EDTA were added to a final concentration of 1mM each. The tightly sealed tubes were placed in a preheated 75° C waterbath for 1 hour, then centrifuged at 15,000 x g for 30 min in a Sorvall HB-4 swinging bucket rotor. The clear supernatant solution (with fresh DTT added to 1 mM final concentration) was dialysed first for 1 h, then overnight at 4° against 2 liters of 50 mM tris HCl (pH 7.4 at 25°).

Step 4) Anion Exchange The dialysed enzyme solution was passed at room temperature at 1.0 ml/min over a 1 cm dia. X 25 cm column of Q Sepharose Fast Flow anion exchanger which had been equilibrated with 20 mM tris HCl pH 7.4. The column was eluted with a 55 ml linear gradient of NaCl (0-0.3 M) in 20 mM bis-tris HCl pH 6.0 containing 1 mM DTT, followed by 30 ml 1 M NaCl.

Step 5) Dye Affinity Fractions of eluate containing enzyme activity were combined and concentrated by ultrafiltration to approximately 2 ml on an Amicon YM10 membrane to reduce the amount of salt present. The concentrate was diluted to 8 ml with 20 mM bis-tris acetate pH 6, containing 2 mM $MgCl_2$ and 1 mM DTT and passed at approximately 0.5 ml/min, 4°, over a 0.5 cm dia. x 3 cm Procion Red A column which had been equilibrated with the same buffer.

Step 6) FPLC The column effluent plus a 2 ml wash with the same buffer was adjusted to pH 8 with tris base and loaded via a 10 ml "Superloop" sample injector onto a 5 mm dia. x 5 cm MONO-Q anion exchange column. The enzyme was eluted with a NaCl gradient (0-0.2 M) in 20 min, followed by 0.2 M-.5 M in 10 min) in 20 mM histidine HCl buffer, pH 5.5. The fractions containing enzyme were adjusted to pH 7.4 with tris base and stored frozen at -70°.

Results and Discussion

The remarkable heat resistance of inositol monophosphate phosphatase (2,3,8-10) provided the basis for a most convenient means of precipitating about 99% of interfering proteins while losing only moderate amounts of enzyme activity (Table 1). The heating step eliminates the inositol polyphosphate phosphatase which was a major difficulty in an earlier enzyme preparation (4). Enzyme heated in the presence of DTT and EDTA eluted as a single symmetrical peak on a Sephacryl S-200 HR size exclusion column with an apparent molecular weight of 46,000. Enzyme heated in the absence of DTT gave a broader peak with a higher apparent molecular weight, possibly due to aggregation via disulfide interchange.

Previous preparations of the enzyme (2-4) employed anion exchange separation at pH 8.0. We found that the separation was much better when carried out at a pH closer to the pI of the enzyme (4.9). We examined DEAE Sephacel and Q Sepharose columns. With both columns at pH 6.0, a large degree of purification of protein, and removal of DNA could be rapidly achieved (Fig. 1). The sample was loaded at pH 7.4 in order to maximize the column capacity. SDS-PAGE analysis of the active fractions showed a band with the apparent molecular weight of the enzyme monomer (29,000) contaminated with 1 major and several minor protein bands (Fig. 2).

Table 1. Purification of inositol monophosphate phosphatase

Step	Volume (ml)	Protein (mg)	Total Activity*	Specific Activity*	Purif. (fold)	Yield (%)
15,000 g supernatant	610	8,140	31.6	0.004	1.0	100
40-60% Ammonium SO_4	60	3,330	28.9	0.009	2.2	91
Heat, 75° C, 1 h	40	24.4	17.8	0.73	188	56
Q Sepharose Fast Flow	9	4.9	15.3	3.14	810	48
Procion Red Effluent	10	1.9	8.5	4.49	1150	27
MONO Q	1.8	1.0	6.7	6.66	1710	21

* Total activity = μmol Pi formed per min from β-glycerophosphate.
Specific activity = μmol Pi min^{-1} mg $protein^{-1}$.

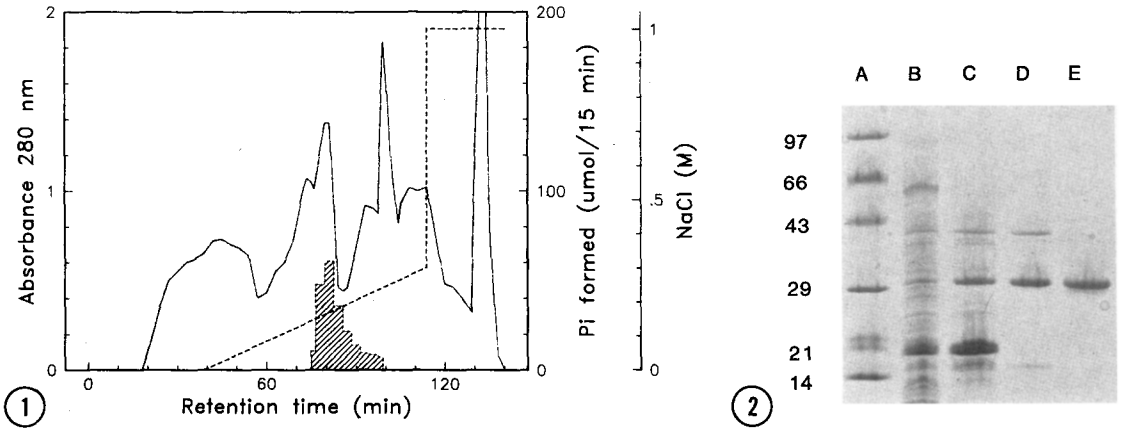

Fig. 1 Anion exchange purification of inositol monophosphate phosphatase. A heat-treated beef brain extract (40 ml, pH 7.4) was loaded onto a Q Sepharose Fast Flow column, then eluted with a NaCl gradient at pH 6.0 The hatched area represents enzyme activity.

Fig. 2. SDS-PAGE of molecular mass markers (lane A), and of proteins present after various purification steps: Lane B, 75° heat treated; Lane C, Q Sepharose eluate; Lane D, Procion Red effluent; Lane E, Mono Q eluate. Samples were heated 15 min at 95° (5 min was insufficient to completely denature the phosphatase) with SDS and mercaptoethanol, and separated on 10-15% "Phastgels". Mass markers (kD): lysozyme (14), soybean trypsin inhibitor (21), carbonic anhydrase (29), ovalbumin (43), bovine serum albumin (66), rabbit muscle phosphorylase B(97). The gel was stained with Coomassie Blue.

Removal of most of the remaining impurities was achieved with a red dye affinity column. The separation was carried out at 4°, pH 6.0, and in the presence of magnesium ions in order to maximize retention of proteins (11). Under these conditions, the monophosphatase was not retained either by the Matrex Red (Procion Red) column, or by any of the other dye affinity columns in the Amicon test kit. The Procion Red column was chosen as the "negative" column, since it adsorbs the most protein from crude tissue extracts (11).

The dye affinity column effluent contained the enzyme contaminated with small amounts of a few other proteins (Fig. 2). These minor contaminants could be effectively removed by chromatography at pH 5.5-6.25 on a MONO Q column. Chromatography at pH 7.4 was much less effective. The enzyme was not retained at pH 5.0

The purified protein gave a single band on SDS-PAGE (with a migration similar to that of carbonic anhydrase, molecular weight 29,000), a single band on non-denaturing gradient gels (with a migration slightly slower than bovine serum albumin), a single band on isoelectric focusing (pI=4.9), and a single peak on reverse phase HPLC, t_r= 23.2 min (ovalbumin= 21.4 min). The strong retention on the reverse phase column indicates that the enzyme is relatively lipophilic. The data obtained by SDS-PAGE and isoelectric focusing are similar

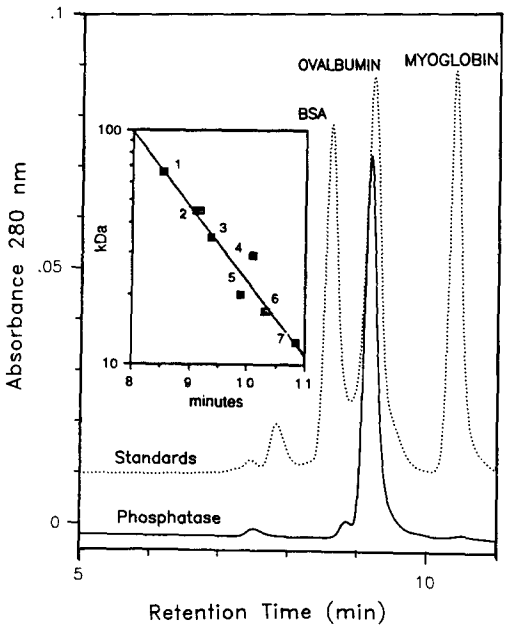

Fig. 3. HPLC-Gel Filtration analysis of the molecular weight of inositol monophosphate phosphatase. The enzyme and standards were chromatographed on a Dupont Bio-series GF250 column (25 x 0.94 cm) with a mobile phase of 0.2 M sodium sulfate, 20 mM tris (pH 7.4) and a flow rate of 1 ml/min. The sample was 20 ul of the MONO Q effluent concentrated approximately 6 fold with an Amicon Centricon 10 centrifugal concentrator. The inset shows the calibration curve: 1, bovine serum albumin; 2, ovalbumin; 3, β-lactoglobulin A; 4, carbonic anhydrase; 5, soybean trypsin inhibitor; 6, myoglobin; 7, cytochrome C.

to that reported previously (4). In non-denaturing and isoelectric focusing gels, monophosphatase activity co-migrated with the protein band as shown by assay of enzyme activity in slices of a lane of the gel adjacent to one subsequently stained with Coomassie blue.

High performance gel filtration chromatography of the enzyme (Fig. 3) indicated a molecular mass of the native enzyme of 46 kDa, a lower value than the 58 and 50 kDa reported previously for the beef brain enzyme (2,4). The value obtained by gel filtration is considerably smaller than twice the apparent 29 kDa mass of monomer given by SDS-PAGE. A better understanding of the correct size of the protein awaits sequence analysis.

The apparent K_m for D,L inositol-1-phosphate was 0.12 mM and 1.0 mM for D,L-β-glycerophosphate. LiCl (20 mM) caused a 90% inhibition of hydrolysis of β-glycerophosphate. In 8 separate preparations of enzyme, the final specific activity for β-glycerophosphate ranged from 2.9 to 6.7 μmol/min/mg protein.

Purified enzyme lost more than 50% of its activity in less than a week at 4°, with or without addition of DTT, β-glycerophosphate, glycerol or $MgCl_2$. The enzyme lost about 10 % activity when stored at -70° for 2 months.

The enzyme was reported to be activated 1.5-2 fold by high concentrations of NaCl, KCl, or NH_4Cl (2). Since the enzyme is inhibited by Li ions, we wondered if there was a requirement for a monovalent metal. We found less than 20% stimulation by addition of 0.25 M NaCl with inositol monophosphate cyclohexylammonium salt as substrate in tris buffer, and no stimulation with β-glycerophosphate as substrate. There was no stimulation by NaCl of β-glycerophosphate hydrolysis even when the cyclohexylammonium ion of the substrate was replaced by the more hindered tetraethylammonium ion, and the incubation buffer was HEPES adjusted to pH 7.4 with tetraethylammonium hydroxide. These data suggest that the effect of NaCl is unlikely to be on a specific mono-cation site.

Purified enzyme was found to be stimulated about 50% by the presence of 0.25-2 mg/ml bovine serum albumin. The stimulation was not due to prevention of enzyme inactivation during incubation since activity was linear with time both with and without albumin addition.

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