

Purification of PDE6 Isozymes From Mammalian Retina

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Summary

The photoreceptor phosphodiesterase (PDE6) is the central effector of visual transduction in vertebrate retinal photoreceptors. Distinct isozymes of PDE6 exist in rods and cones. Mammalian retina serves as an abundant source of tissue for PDE6 purification. Methods are described for the isolation and purification of membrane-associated PDE6 from rod outer segment membranes. Purification of cone PDE6 from the soluble fraction of retinal extracts is also described. Several procedures that can purify the rod and cone isozymes to homogeneity, including anion exchange, hydrophobic interaction, gel filtration, hydroxyapatite, and immunoaffinity chromatography, are presented. A method to activate PDE6 by limited proteolysis of its inhibitory γ -subunit is also provided.

Key Words

Photoreceptor; phosphodiesterase; retina; phototransduction; anion-exchange chromatography; cone and rod; hydrophobic interaction chromatography.

1. Introduction

The cyclic nucleotide phosphodiesterase (PDE) that is abundantly expressed in retinal photoreceptor cells constitutes the PDE6 family of PDEs. Features of PDE6 that distinguish it from other PDE families include primary localization in photoreceptive cells, a catalytic mechanism operating at the diffusion-controlled rate, association with a low molecular weight protein inhibitor of catalysis (γ -subunit), and attachment to cellular membranes via isoprenylated C-termini.

Rod and cone photoreceptors express distinct isoforms of PDE6. The rod PDE6 enzyme (PDE6R) is the only PDE that is a catalytic dimer of nonidentical catalytic subunits, α and β ($P\alpha\beta$). PDE6R forms a holoenzyme on binding of two identical γ -subunits ($P\gamma$) to the catalytic $\alpha\beta$ dimer: $\alpha\beta\gamma_2$. Cone PDE6

(PDE6C) is a catalytic dimer of the cone-specific α' -subunit, to which two cone-specific γ' -subunits bind to form $\alpha'_2\gamma'_2$. PDE6C, as well as a fraction of the total PDE6R, is found in a soluble form in mammalian retinal homogenates in association with a prenyl-binding protein, formerly defined as the δ -subunit of PDE6), which is believed to be responsible for solubilizing PDE6 from photoreceptor membranes (1).

This chapter presents a set of methods for purification of each of the isoforms of PDE6 from mammalian retina.

2. Materials

All solutions used to isolate and purify PDE6 are supplemented just before use with 1 mM dithiothreitol (DTT) and 0.3 mM phenylmethylsulfonyl fluoride (PMSF) or protease inhibitor cocktail (following the manufacturer's recommendations). All chromatography buffers are filtered with a 0.45- μ m membrane under vacuum immediately before use to remove particulates and degas the solvent.

1. Bovine retinas (W. Lawson, Lincoln, NE).
2. Chromatography columns and media: Mono Q and Superdex prepacked columns, butyl-Sepharose, and Q-Sepharose (Amersham Biosciences), ceramic hydroxyapatite (Type I, 40- μ m particle size) (Bio-Rad, Hercules, CA), Sulfolink coupling gel and Immunopure (G) IgG Purification Kit (Pierce, Rockford, IL).
3. Membrane filtration devices (Millipore, Bedford, MA).
4. ROS1 monoclonal antibody (MAb) cell line: This was a kind gift from Dr. R. L. Hurwitz (2).
5. Miscellaneous stock solutions: 1 M DTT in water, 100 mM PMSF in 95% ethanol, Mammalian Protease Inhibitor Cocktail (P8340; Sigma Chemical Corporation, St. Louis, MO).
6. Solution A: 20 mM 3-morpholinopropane-1-sulfonic acid (MOPS), pH 7.2; 2.0 mM MgCl_2 , 60 mM KCl; 30 mM NaCl.
7. Solution B: 50% (w/v) sucrose in solution A. It is used to prepare the following sucrose density gradient solutions, which are checked for the correct density with a hydrometer at 4°C:
 - a. Solution B1: 51 mL of B diluted to 100 mL with A ($\rho = 1.105$ g/mL).
 - b. Solution B2: 54.25 mL of B diluted to 100 mL with A ($\rho = 1.115$ g/mL).
 - c. Solution B3: 64.5 mL of B diluted to 100 mL with A ($\rho = 1.135$ g/mL).
8. Solution C: 45% (w/v) sucrose in solution A.
9. ROS membrane solutions:
 - a. High Mg^{2+} hypotonic buffer: 5 mM Tris-HCl (pH 7.5 at 4°C), 10 mM MgCl_2 , 10 mM DTT.
 - b. PDE6R extraction buffer: 5 mM Tris-HCl (pH 7.5 at 4°C), 5 mM DTT.
10. Mono Q chromatography solutions:
 - a. MQ-A buffer: 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl_2 .
 - b. MQ-B buffer: MQ-A containing 1.0 M NaCl.

11. Hydrophobic interaction chromatography (HIC) solutions:
 - a. HIC-A: 400 mM $(\text{NH}_4)_2\text{SO}_4$, 5 mM Tris-HCl, pH 7.5.
 - b. HIC-B: 5 mM Tris-HCl, pH 7.5.
12. Gel filtration chromatography (GFC) buffer: 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM MgCl_2 .
13. Q-Sepharose chromatography solutions:
 - a. Q-A buffer: 10 mM Tris-HCl, pH 7.5, 1 mM MgCl_2 , 100 mM NaCl.
 - b. Q-B buffer: 10 mM Tris-HCl, pH 7.5, 1 mM MgCl_2 , 350 mM NaCl.
14. Hydroxyapatite (HAP) chromatography solutions:
 - a. 0.5 M sodium phosphate solution, pH 7.2.
 - b. HAP-A buffer: 75 mM sodium phosphate, pH 7.2, 50 mM NaCl.
 - c. HAP-B buffer: 150 mM NaH_2PO_4 , 150 mM K_2HPO_4 , pH 7.2.
15. Immunoaffinity purification solutions:
 - a. TMN buffer: 50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.5 mM MgCl_2 .
 - b. pH 9.0 wash buffer: 25 mM 3-cyclohexylaminopropane-1-sulfonic acid (CAPS), 200 mM NaCl, 2 mM MgCl_2 , pH 9.0.
 - c. pH 10.8 elution buffer: 25 mM CAPS, 200 mM NaCl, 2 mM MgCl_2 , 10% glycerol, pH 10.8.
 - d. Neutralizing buffer: 1.0 M Tris-HCl, pH 6.7.
16. PDE storage buffer: 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM MgCl_2 , 2 mM DTT, sterile filtered.
17. Solutions for trypsin activation of PDE6:
 - a. 2X Proteolysis buffer: 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM MgCl_2 , 40% glycerol.
 - b. 2X Proteolysis stop solution: 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM MgCl_2 , 0.5 mg/mL of soybean trypsin inhibitor (T9128; Sigma Chemical Corporation), 0.4 mg/mL of bovine serum albumin, 0.4 mM Pefabloc, 4 mM DTT.

3. Methods

An overview of the purification scheme for rod and cone PDE6 is shown in **Fig. 1**. Following the general procedure of McDowell (3), photoreceptors are detached from bovine retina by mechanical disruption, and the rod outer segments (ROSs) are separated from soluble proteins by centrifugation. Following removal of soluble ROS proteins, the membrane-associated PDE6R is extracted from the membrane with a hypotonic buffer lacking magnesium (4) and then chromatographically purified. PDE6C and a soluble form of PDE6R found in the soluble retinal extract are separated by strong anion-exchange chromatography (5) and then further purified by several chromatographic techniques. Purified PDE6R and PDE6C can be stored at -20°C for several months with minimal loss of activity. The final method in this chapter is a procedure to proteolytically activate the PDE6 holoenzyme by digesting the inhibitory $\text{P}\gamma$ -subunits (6), leaving the PDE6 catalytic dimer fully activated.

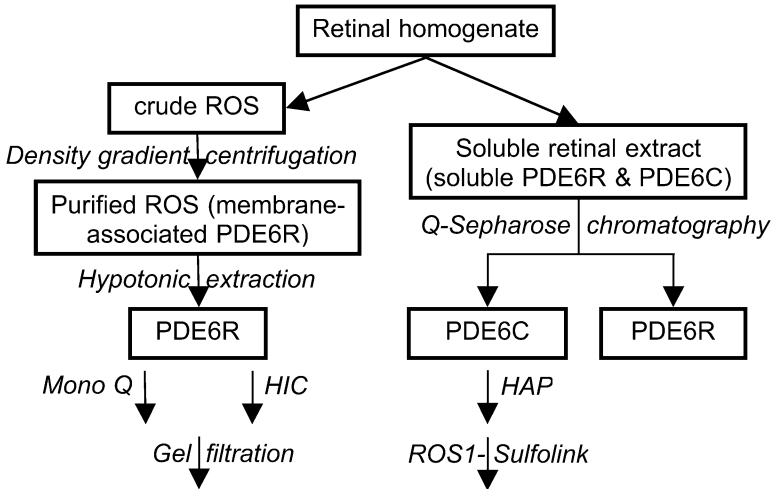


Fig. 1. Flow chart showing major strategies used to purify PDE6R and PDE6C from bovine retina. Once the initial PDE6 isozyme has been isolated, various combinations of chromatographic techniques can be used to purify the enzyme to homogeneity. Mono Q, anion-exchange chromatography using Mono Q resin; HIC, hydrophobic interaction chromatography on butyl-Sepharose resin; HAP, hydroxyapatite chromatography; ROS1-Sulfolink, immunoaffinity purification using the ROS1 antibody coupled to Sulfolink resin.

3.1. Initial Isolation of PDE6 Isoforms From Bovine Retina and Purification of ROS

In this section, mechanical disruption of photoreceptor cells from the neural retina is the starting point for isolating both the rod photoreceptor PDE6 associated with the outer segment membranes, and the soluble rod and cone PDE6 that are recovered in the soluble portion of the retinal extract. For the case of the membrane-associated PDE6R, sucrose density gradient centrifugation results in purified ROS in which rhodopsin (~70% of total protein), transducin (~10% of total protein), and PDE6R (1 to 2% of total protein) are membrane bound. Because of the relative purity of the membrane-associated PDE6R, we use only the soluble retinal extract for purification of PDE6C and discard the soluble PDE6R, which is difficult to purify to homogeneity.

To prevent activation of the components of the phototransduction pathway, the following procedures should be performed in a darkroom with infrared (IR) illumination and IR viewers. All solutions should be ice cold throughout the ROS purification process.

3.1.1. Preparation of Retinal Homogenate From Previously Frozen Bovine Retinas

1. Quickly thaw 50 frozen bovine retinas and keep on ice once thawed.
2. Add 45 mL of solution C (supplemented with 200 μ L of Mammalian Protease Inhibitor Cocktail) to a beaker containing the 50 retinas.
3. Use a magnetic stir bar in the bottom of the beaker at low speed to mechanically disrupt photoreceptors from the retinas for 1 h in the dark (*see Note 1*).
4. Transfer the solution containing the disrupted retinas to 50-mL centrifuge tubes and centrifuge at 3000 g_{\max} for 3 min to pellet the retinal debris. (Save the retinal debris that has pelleted if purification of PDE6C is desired [*see Subheading 3.1.3.*]).

3.1.2. Fractionation of Retinal Extract to Separate ROS From Soluble Retinal Proteins

1. Pour off the retinal extract (containing ROS) through a nylon sock into a cold beaker.
2. Add 1.5 vol of solution A to the supernatant to dilute the sucrose concentration. Mix well.
3. Centrifuge the retinal extract in 50-mL tubes for 30 min at 23,000 g_{\max} in a fixed-angle rotor.
4. Pour off the supernatant (containing soluble retinal proteins, including PDE6C and soluble PDE6R, to be used for PDE6C purification; *see Subheading 3.6.*). Further purify the pellet (containing ROS and its membrane-associated PDE6R) (*see Subheading 3.1.4.*).

3.1.3. Re-Extraction of Retinal Debris Pellet to Recover Additional Soluble PDE6

If the primary purpose is to purify PDE6C, then the pelleted retinal debris should be resuspended to extract additional soluble PDE6.

1. Recover debris trapped by filtering the retinal extract (*see Subheading 3.1.2., step 1*) by rinsing with 100 mL of solution A, and combine this with the retinal debris pellets (*see Subheading 3.1.2., step 4*).
2. Vortex vigorously to release additional soluble PDE6.
3. Centrifuge the solution for 20 min at 30,000 g_{\max} . Combine this supernatant with the soluble retinal proteins obtained previously in **Subheading 3.1.2., step 4**. This is the starting material for purification of PDE6C in **Subheading 3.6**.

3.1.4. Purification of ROS

1. Resuspend the ROS-containing pellets from **Subheading 3.1.2.** in 15 mL of solution B1 ($\rho = 1.105$ g/mL).
2. Prepare discontinuous sucrose gradients just before use in 18-mL centrifuge tubes (Beckman) by layering 5 mL of solution B3, then 5 mL of solution B2. Layer the resuspended ROS pellets (in solution B1) to within 1 cm of the top of the tubes.

3. Centrifuge the sucrose gradients for 60 min at $116,000g_{\max}$ in a swinging-bucket rotor at 4°C .
4. Remove the interface of solutions B2 and B3 containing the purified ROS with a 15-gage needle attached to a 5-mL syringe.
5. Dilute the ROS with 2 vol of solution A, and then centrifuge for 60 min at $30,000g_{\max}$ to pellet the ROS (*see Note 2*).

3.2. Extraction of PDE6R From ROS Membranes

In this section, the soluble proteins present in the ROS are removed by disrupting the plasma membrane of the ROS in a moderate ionic strength buffer in the dark, and separating ROS membranes from the soluble proteins by centrifugation (7). The resulting ROS membranes contain integral membrane proteins (predominantly rhodopsin), along with peripheral membrane proteins (notably PDE6R and a reduced amount of transducin). Because transducin undergoes a light-dependent binding to photoactivated rhodopsin (4), the ROS membranes are exposed to light just before releasing PDE6R with a low ionic strength buffer.

3.2.1. Preparation of ROS Membranes Enriched in PDE6R

1. Resuspend the ROS pellets in 15 mL of solution A, and then homogenize 10–12 times with a tight-fitting Teflon pestle homogenizer driven by a Talboy Model 134-1 overhead stirrer.
2. Spin the ROS homogenate for 45 min at $110,000g_{\max}$, and discard the soluble ROS proteins in the supernatant.
3. Resuspend the pellets in 15 mL of solution A and recentrifuge to remove residual soluble proteins.
4. Resuspend the ROS membranes in 15 mL of high Mg^{2+} hypotonic buffer to deplete the ROS membranes of some peripheral membrane proteins, and centrifuge as in **step 2**. Discard the supernatant following centrifugation (*see Note 3*).

3.2.2. Extraction of PDE6R From Washed ROS Membranes With Hypotonic Buffer

1. Expose the ROS membrane pellets to room light for 1 min at 4°C to photoactivate rhodopsin, thereby inducing tight binding of transducin to the ROS membranes.
2. Resuspend the light-exposed ROS membranes in a hypotonic PDE6R extraction buffer.
3. Homogenize the ROS membranes in a Dounce tissue grinder at 4°C using a pestle with a tight clearance (~ 0.025 mm).
4. Centrifuge the hypotonic extract for 60 min at $30,000g_{\max}$, and recover the hypotonic supernatant, which contains solubilized PDE6R.
5. Repeat the hypotonic extraction procedure (without homogenization) two additional times.
6. Pool the hypotonic extracts, and clarify the solution by ultracentrifugation at more than $100,000g_{\max}$ for 30 min (*see Note 4*).

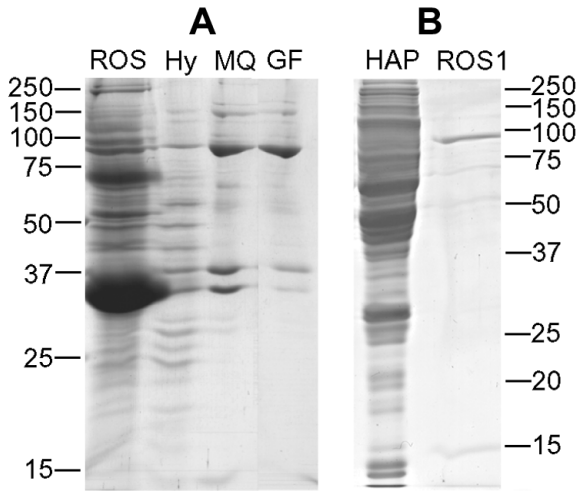


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of PDE6 at various stages of purification. Samples were applied to 12% acrylamide gels and electrophoresed and the gel was stained with Coomassie Blue. **(A)** Membrane-associated PDE6R purification. Purified ROS contain high concentrations of rhodopsin, which are removed by hypotonic extraction of PDE6R from ROS membranes (Hy). Mono Q chromatography (MQ) removes most impurities except for major bands at 35–37 kDA (transducin subunits). Gel filtration chromatography (GF) yields a PDE6R preparation that is more than 95% pure. **(B)** Soluble PDE6C purification. PDE6C that has been resolved from soluble PDE6R by Q-Sepharose chromatography (not shown) is further purified and concentrated by hydroxyapatite chromatography (HAP). A greater than 100-fold purification of the PDE6C to more than 95% purity is achieved by immunoaffinity purification on the ROS1-Sulfolink column (ROS1).

3.3. Purification of PDE6R by Mono Q Anion-Exchange Chromatography

Anion-exchange chromatography of the PDE6 family has traditionally utilized the weak anion exchanger, diethylaminoethyl (DEAE), as the functional group (5,7). We find that greater reproducibility and better resolution are achieved for PDE6 isozymes when a strong anion exchanger (quaternary ammonium [Q]) is used. The purification of hypotonically extracted PDE6R on a Mono Q column eliminates most other proteins, with transducin subunits remaining the predominant impurity following chromatography (**Fig. 2A**).

1. Equilibrate a Mono Q column with 10 column vol of MQ-A buffer prior to loading the sample.

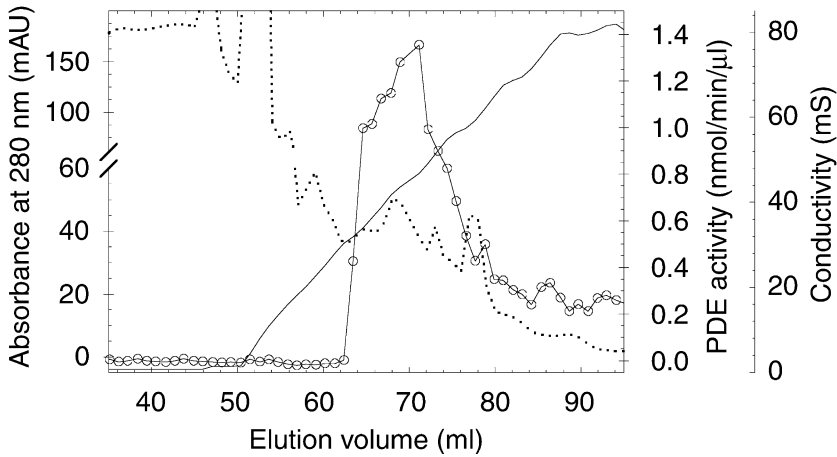


Fig. 3. Mono Q chromatography of hypotonically extracted PDE6R. Forty-five milliliters of hypotonically extracted PDE6R was loaded onto a Mono Q column followed by 5 mL of MQ-A. PDE6R was eluted with a linear gradient from 0 to 100% MQ-B. For this particular experiment, the buffers were different from the standard procedure: MQ-A lacked NaCl, and MQ-B was 800 mM NaCl. Absorbance (dotted line) and conductivity (continuous line) were recorded, and PDE activity (—○—) was assayed for each fraction collected.

2. Adjust the hypotonic PDE6R sample to the approximate ionic strength of MQ-A buffer by adding 0.10 vol of MQ-B buffer. Filter the sample with a low-protein-binding 0.22- μ m filter to remove particulates.
3. Load the PDE6R sample onto the Mono Q column at a flow rate of 0.5 mL/min and wash the column with 5 column vol of MQ-A buffer.
4. Perform a linear salt gradient from 0% MQ-B (100 mM NaCl) to 100% B (1.0 M NaCl) in a total volume of 40 mL, and collect 1-mL fractions.
5. After the elution is completed, wash the Mono Q column with 5 column vol of MQ-B, and then store as directed by the manufacturer.
6. Identify fractions containing PDE6R by a colorimetric PDE activity assay (8). See Fig. 3 for a typical result.

3.4. Removal of the PDE6R-Binding Protein, Glutamic Acid-Rich Protein-2, by Hydrophobic Interaction Chromatography

(HIC) separates proteins based on their relative hydrophobicity. This method is useful for the purification of PDE6R free of a high-affinity PDE6R-binding protein, glutamic acid-rich protein-2 (GARP2) (9), that is found associated with Mono Q-purified PDE6R.

1. Adjust hypotonically extracted PDE6R (see **Subheading 3.3.**) or Mono Q-purified PDE6R (see **Subheading 3.3.**) to 400 mM $(\text{NH}_4)_2\text{SO}_4$ by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to the solution (see **Note 5**).
2. Wash a 30-mL butyl-Sepharose column with 5 column vol of HIC-A prior to loading the PDE6R sample at a flow rate of 0.5 mL/min.
3. Remove unbound proteins (including PDE6R) with 4 column vol of HIC-A.
4. Perform concentration and buffer exchange of the recovered PDE6R by ultrafiltration (see **Subheading 3.9.**).
5. To elute GARP2 from the butyl-Sepharose column, perform stepwise elution with 2 column vol of 65% HIC-B and then 2 vol of 100% HIC-B.
6. Regenerate the HIC column following the manufacturer's instructions.

3.5. Gel Filtration Chromatography of PDE6

Gel filtration (size exclusion) chromatography is the most suitable final step for PDE6 purification. It not only purifies PDE6 from other proteins based on size, but it also equilibrates the enzyme in a buffer more suitable for long-term storage.

1. Reduce the PDE6 sample to be purified in volume by ultrafiltration (see **Subheading 3.9.**) to $\leq 2\%$ of the total volume of the gel filtration column in order to obtain maximum resolution of the PDE6R peak (see **Note 6**).
2. Equilibrate the gel filtration column with 2 column vol of GFC buffer prior to injecting the PDE6 sample on the column.
3. Operate the column at a flow rate of 0.4 mL/min and collect 0.4-mL fractions.
4. Analyze the absorbance at 280 nm and the activity of each fraction (see **Fig. 4**).

3.6. Separation of PDE6C From Soluble PDE6R by Q-Sepharose Anion-Exchange Chromatography

The purification of PDE6C is hampered by the low abundance of cone photoreceptors in most retinas, the lack of procedures for isolating intact cone photoreceptor cells, and the occurrence of PDE6C in the soluble retinal extract in conjunction with a more than 10-fold excess of soluble PDE6R. Gillespie and Beavo (5) exploited differences in the surface charges of PDE6C and PDE6R to effect a separation of the rod and cone enzyme using DEAE-cellulose anion-exchange chromatography. Using a strong anion exchanger attached to a rigid bead (Q-Sepharose) and a batch adsorption method, we have enhanced the resolution and reduced the time required to separate PDE6C from PDE6R.

1. Equilibrate 45 mL of Q-Sepharose resin with 3 column vol of Q-A buffer at a flow rate of 3 mL/min.
2. Before mixing the Q-Sepharose with the soluble retinal proteins, check the conductivity of the protein solution to ensure that it is less than 16 mS.

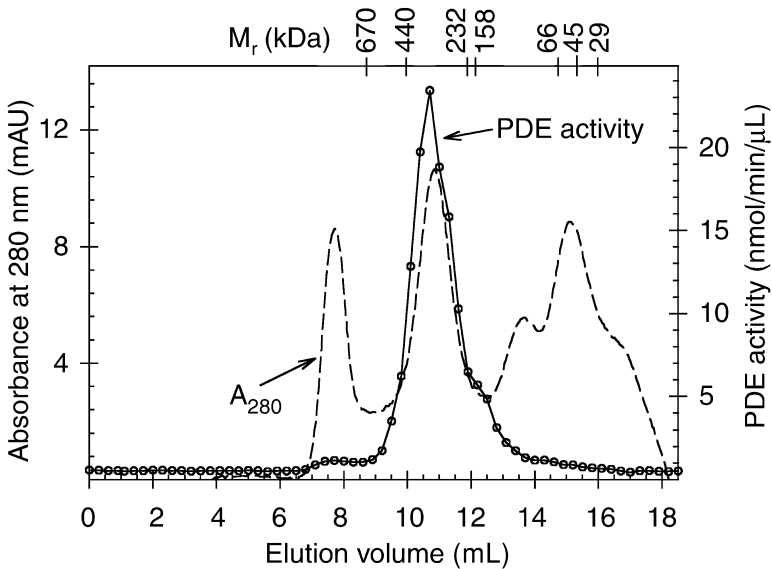


Fig. 4. Gel filtration chromatography of PDE6R on Superdex 200. A 0.5-mL sample of Mono Q-purified PDE6R was injected onto a Superdex 200 HR 10/30 column at 0.4 mL/min, and 0.3-mL fractions were collected. The 280-nm peak at 7.5 mL represents material in the void volume, with other impurities eluting at less than 100 kDa. The protein and PDE activity peak at 11.0 mL has an apparent mol mass of 300 kDa.

3. Mix the Q-Sepharose and the soluble retinal proteins (*see Subheadings 3.1.2. and 3.1.3.*) by gently stirring with a cross-shaped magnetic stir bar. Incubate for 45 min.
4. Pour the suspension into 250-mL centrifuge bottles, and centrifuge for 1 min at $1000g_{\max}$. Decant the supernatant and discard.
5. Add the Q-Sepharose slurry to a wide-diameter chromatography column (e.g., Bio-Rad 2.5-cm Econo column or Amersham XK 26 column), attach the flow adapter, and hook up to the chromatography system.
6. Wash the packed column with 3 column vol of Q-A buffer at 3 mL/min, and then perform a linear gradient from 0% Q-B to 100% Q-B over 5 column vol, collecting 5-mL fractions.
7. Continuously monitor the absorbance at 280 nm and the conductivity, and perform a colorimetric PDE activity assay on every other fraction to identify the PDE6C (~24 mS) and PDE6R (~32 mS) (*see Note 7*).
8. Regenerate the Q-Sepharose resin by washing with 2 vol of 1 M NaCl, 3 vol of 0.5 M NaOH, and then equilibrate and store in 10 mM NaOH containing 20% ethanol.

3.7. Purification of PDE6C by HAP Chromatography

HAP is a crystalline form of calcium phosphate suitable for chromatographic separations based on the interaction of the Ca^{2+} and PO_4^{3-} ions of the column matrix with oppositely charged ionic groups on the surface of proteins. Because of its unique adsorptive properties, HAP can remove contaminants from PDE6 samples that copurify by other methods. HAP has been previously used to purify hypotonically extracted PDE6R (10), and it is utilized here as a prelude to immunoaffinity purification of PDE6C (see **Subheading 3.8.**).

1. Pack a 1×10 cm column with 1.5 mL of swollen HAP, wash with 10 column vol of HAP-B buffer, and then equilibrate with 10 vol of HAP-A.
2. Prepare a pooled Q-Sepharose-purified PDE6C sample by adding 0.10 vol of 0.5 M sodium phosphate.
3. Load the PDE6C onto the column at a flow rate of 0.2 mL/min, and then wash the column with 10 column vol of HAP-A.
4. Elute the PDE6C with a linear gradient of 0–100% HAP-B over 20 column vol, and monitor the absorbance at 280 nm and the conductivity.
5. Collect 1-mL fractions, assay for PDE activity, and pool the peak fractions. HAP-purified PDE6C has severalfold higher specific activity than Q-Sepharose PDE6C but remains highly impure (**Fig. 2B**) (see **Note 8**).
6. Regenerate the HAP column following the manufacturer's recommendations.

3.8. Immunoaffinity Chromatography of PDE6

Immunoaffinity purification is the single most effective chromatographic technique used in protein purification. Because antibodies are raised against unique epitopes of a particular protein, they afford the capability of enriching for the protein of interest more than 1000-fold in a single step.

The murine MAb ROS1 was originally raised against the PDE6R holoenzyme (2). The epitope recognized by the ROS1 antibody has not been defined, but the affinity of the antibody for PDE6R and PDE6C is very high (2). Elution of active PDE6 from ROS1 has required extensive manipulation of buffer conditions, with high pH and the inclusion of glycerol serving to elute the antibody with the least loss of biological activity. Inclusion of a pH 9.0 wash step just prior to elution serves to eliminate proteins that would otherwise contaminate PDE6 preparation.

3.8.1. Coupling of ROS1 Antibody to Sulfolink Beads

1. Purify the ROS1 antibody from ascites fluid on a Pierce Immunopure Protein G-agarose column using the manufacturer's protocols.
2. Concentrate the ROS1 antibody eluted from the Protein G column to less than 1 mL using a Centricon Plus-20 (see **Subheading 3.9.**), dilute 10-fold with the coupling buffer, and reconcentrate to ≥ 10 mg/mL in a volume of ≤ 2.5 mL.

3. Couple the ROS1 antibody to the Sulfolink beads at a coupling density of 5 mg of antibody/mL of resin following the manufacturer's instructions.

3.8.2. Purification of PDE6C by Adsorption to ROS1-Sulfolink Column and Elution of Active Enzyme

1. Wash the ROS1-Sulfolink column with 10 column vol of pH 10.8 elution buffer at 1 mL/min, and then equilibrate with 10 column vol of TMN buffer.
2. Dilute the PDE6C sample in TMN buffer (*see Note 9*).
3. Load PDE6C onto the column at a flow rate of 0.5 mL/min.
4. Recover the eluate and reapply it to the ROS1 column to ensure that all PDE6C has bound.
5. Wash the beads with 5 column vol of TMN at 1 mL/min.
6. Wash with 5 column vol of pH 9.0 wash buffer to remove contaminating proteins that nonspecifically bind to the ROS1-Sulfolink beads.
7. Elute the PDE6C with 10 column vol of pH 10.8 elution buffer.
8. Collect 1.0-mL fractions in tubes containing 0.1 mL of neutralization buffer.
9. Assess fractions for PDE activity using standard activity assays (*8*).
10. Pool fractions containing PDE activity and concentrate prior to long-term storage (*see Subheading 3.9*).
11. Regenerate the ROS1-Sulfolink resin by washing with 5 column vol of pH 10.8 elution buffer, then 10 column vol of TMN buffer containing 0.05% NaN_3 (*see Note 10*).

3.9. Concentration of PDE6 by Ultrafiltration

Centrifugal ultrafiltration of dilute PDE6 samples serves three purposes: concentration of the PDE6 prior to storage, removal of low molecular weight impurities, and exchange of the purification buffer with the PDE storage buffer. In our experience, the Amicon/Millipore devices with the Ultracel PL membrane (Centricon and Centricon Plus-20) offer the best rate of concentration and highest recoveries for PDE6 isozymes.

1. Before adding a PDE6 sample to the centrifugal filtration unit, add PDE storage buffer, and centrifuge briefly to prewet the membrane. Discard the buffer in the filtrate and retentate compartments.
2. For PDE6 samples with volumes <2 mL, load the Centricon YM-100 with the PDE6, and centrifuge at $1000g_{\text{max}}$ until the desired volume is achieved (*see Note 11*).
3. For PDE6 samples with volumes of 2–20 mL, load the Centricon Plus-20 with PL-30 membrane (30,000 mol wt cutoff) with the PDE6 sample and then centrifuge at $4000g_{\text{max}}$ until the volume is reduced sufficiently.
4. To carry out buffer exchange, resuspend the concentrated PDE6 sample to the full volume capacity of the device with PDE storage buffer and reconcentrate the sample. Repeat the process until the contaminating solutes are reduced to an acceptable level (typically $<5\%$ of the original concentration).

5. Recover the concentrated PDE6 from the retentate chamber following the manufacturer's directions.
6. For long-term storage of PDE6, mix the concentrated enzyme 1:1 (v/w) with molecular biology-grade glycerol, and keep at -20°C .

3.10. Preparation of Activated PDE6 Lacking Inhibitory P γ -Subunit

Because of the very high affinity with which P γ binds to the catalytic dimer of PDE6 (**11**), the most effective way to prepare fully activated PDE6 is by limited proteolysis. Under controlled conditions, trypsin can effectively degrade the P γ -subunits (and relieve their inhibition of the active site) without harming the properties of the catalytic dimer of PDE6 (**6,12,13**).

3.10.1. Determining Optimal Conditions for Trypsin Activation of PDE6

In this procedure, PDE6 is proteolyzed with trypsin at 4°C for various times to determine the minimum time necessary to activate the enzyme fully. After quenching the reaction with soybean trypsin inhibitor, samples are assayed for PDE activity.

1. Dilute PDE6 to a concentration of 200 nM in 2X proteolysis buffer, and add an equal volume of 100 $\mu\text{g}/\text{mL}$ of TPCK-treated trypsin (T1426; Sigma Chemical Corporation) in 10 mM Tris-HCl (pH 7.5) at 4°C .
2. At various intervals, remove portions and quench with an equal volume of 2X proteolysis stop solution (final PDE6 concentration is 50 nM).
3. Assay the quenched samples for PDE6 catalytic activity using a colorimetric PDE activity assay (**8**).

3.10.2. Removal of Proteolytic Fragments of P γ From Trypsin-Activated PDE6

Although limited proteolysis with trypsin effectively activates PDE6 without adversely affecting the catalytic subunits, a large proteolytic fragment of P γ consisting of the C-terminal half of the protein is generated (**Fig. 5**). This peptide has low affinity for binding to the active site of the enzyme (**14,15**), but at high concentrations P γ C-terminal peptides can act as competitive inhibitors of catalysis (**15**). This section provides a procedure to remove this P γ fragment to prepare purified P $\alpha\beta$ dimer lacking P γ or P γ fragments (**Fig. 5**).

1. Concentrate trypsin-activated PDE6 (*see Subheading 3.10.1.*) by ultrafiltration (*see Subheading 3.9.*) using a 30-kDa mol mass cutoff filter, and resuspend in MQ-A buffer and reconcentrate to remove trypsin, trypsin inhibitor, and some P γ fragments from the PDE6 sample.
2. Load the concentrated PDE6 sample onto a Mono Q column as described in **Subheading 3.3**.

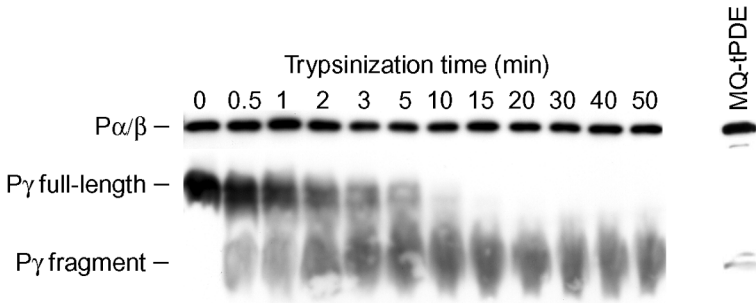


Fig. 5. Proteolytic digestion of PDE6R holoenzyme preferentially destroys the P γ -subunit. PDE6R (50 nM) purified by Mono Q and gel filtration chromatography was incubated with 50 μ g/mL of trypsin at 4°C as described in **Subheading 3.10.1**. At the indicated times, samples were mixed with soybean trypsin inhibitor and run on SDS-PAGE. After transfer to a nitrocellulose membrane at 60 V for 1 h, the membrane was probed with a mixture of catalytic and inhibitory subunit antibodies. The time course of disappearance of the 11-kDa P γ -subunit correlates with the appearance of an approx 5 kDa P γ fragment (amino acids 45–87; see refs. 13 and 14). The α and β catalytic subunits are not degraded by this treatment. Following purification of the trypsin-activated PDE6R on Mono Q (MQ-EPDE), most of the P γ fragment is removed.

3. Concentrate PDE6-containing fractions from the Mono Q column by ultrafiltration (see **Subheading 3.9.**) to a volume of less than 500 μ L and store at -20°C with 50% glycerol (see **Note 12**).

4. Notes

1. To optimize recovery of soluble rod and cone PDE6 instead of the membrane-associated PDE6R, the magnetic stir bar is replaced with a four-blade propeller paddle attached to an overhead electric stirrer. The use of the propeller blade substantially reduces the yield of ROS (and hence membrane-associated PDE6R) from the sucrose gradient centrifugation step.
2. At this stage, the pelleted ROS can be frozen at -70°C (wrapped in aluminum foil to maintain a dark-adapted state) and PDE6R extracted at a later time. The yield of purified ROS can be estimated by spectrophotometric determination of the rhodopsin concentration (16).
3. The ROS and ROS membrane preparations should be kept in a dark-adapted state until this step is completed.
4. At this stage of purification, a typical yield of hypotonically extracted PDE6R from 50 retinas is 3 nmol when the ROS are mechanically disrupted with the magnetic stir bar. PDE6R constitutes approx 10% of the total protein in the hypotonic extract.
5. The high concentration of ammonium sulfate disrupts the high-affinity interaction of GARP2 with PDE6R, thus allowing their separation on the HIC column.

6. A Superdex 200 HR 10/30 column with a total volume of 24 mL allows 0.5 mL of PDE6 to be loaded. We use the Superdex 200 beads for good separation of PDE6 elution from the void volume of the column as well as high resolution over the mol mass range of 10,000–600,000 Daltons.
7. The typical yield of PDE6C from 50 bovine retinas is 40 μ g at this stage. The PDE6C is quite impure at this step and should be further purified as soon as possible.
8. The phosphate buffer used for HAP chromatography interferes with the colorimetric PDE activity assay unless precautions are taken. Assaying 1- μ L portions of 1/20 diluted fractions will reduce the phosphate concentration sufficiently to avoid interference with the colorimetric PDE assay. Alternatively, a radiotracer PDE activity assay (8) can be used.
9. The PDE6C buffer conditions are not critical to the success of ROS1 immunopurification. However, we routinely dilute the PDE6C 1:1 with TMN buffer to adjust the pH and ionic strength to be similar to the equilibration conditions. It is important to determine the effective binding capacity of the ROS1-Sulfolink column by testing each batch of ROS1 beads for the maximum amount of PDE6 that will bind per milliliter of resin.
10. ROS1-Sulfolink columns can be reused for many years, but a slow decline in the maximum PDE6-binding capacity is observed. To avoid cross-contamination, separate ROS1 columns are reserved for PDE6R and for PDE6C purifications.
11. The Centricon YM-100 membrane (100,000 molecular mass cutoff) retains all of the PDE6 activity in the retentate chamber when the holoenzyme is being concentrated. However, for trypsin-activated PDE6 (see **Subheading 3.10.**), significant amounts of PDE6 activity pass through into the filtrate, and the YM-50 membrane is recommended in this case.
12. The use of ultrafiltration and diafiltration of the trypsin-treated PDE6 prior to Mono Q chromatography causes the P γ fragments to dissociate from the catalytic dimer, and the P γ fragments are then able to pass through the membrane in the subsequent concentration step. An additional gel filtration step can be added to this procedure if residual P γ fragments must be removed.

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