

Purification of G Protein Subunits from Sf9 Insect Cells Using Hexahistidine-Tagged α and $\beta\gamma$ Subunits

Tohru Kozasa

Summary

G protein-mediated pathways are the most fundamental mechanisms of cell signaling. In order to analyze these pathways, the availability of purified recombinant G proteins are critically important. Using Sf9-Baculovirus expression system, a general and simplified method to purify various G protein subunits is described in this chapter. This method is useful for purification of most of G protein subunits.

Key Words: G protein; recombinant protein; Sf9-Baculovirus expression system.

1. Introduction

G protein-mediated signal transduction is a fundamental mechanism of cell communication, being involved in various cellular functions (1–3). G proteins receive signals from a large number of heptahelical cell surface receptors, and they transmit these signals to various intracellular effectors. Each heterotrimeric G protein is composed of a guanine nucleotide-binding α subunit and a high-affinity dimer of β and γ subunits. Agonist-bound receptor activates G protein to facilitate guanosine diphosphate–guanosine triphosphate (GDP–GTP) exchange on $G\alpha$ subunit, which induces subunit dissociation to generate GTP-bound α and free $\beta\gamma$ subunits. Both of these molecules are able to regulate the activity of downstream effectors. GTP on $G\alpha$ is hydrolyzed to GDP by its own GTPase activity as well as GTPase-activating proteins, such as regulator of G protein signaling (RGS) proteins. GDP-bound $G\alpha$ reassociates with $\beta\gamma$ subunit to form an inactive heterotrimer to complete the G protein cycle.

G α subunits are commonly classified into four subfamilies based on their amino-acid sequence homology and function: G $_s$ family (G $_s\alpha$ and G $_{olf}\alpha$; activate adenylyl cyclase); G $_i$ family (G $_{i1}\alpha$, G $_{i2}\alpha$, G $_{i3}\alpha$, G $_o\alpha$, G $_t\alpha$, G $_z\alpha$, and G $_g\alpha$; substrate for pertussis toxin-catalyzed adenosine-5'-diphosphate (ADP) ribosylation except for G $_z\alpha$, inhibit adenylyl cyclase, or stimulate guanosine-2',3'-cyclic phosphate (cGMP) phosphodiesterase, and so forth); G $_q$ family (G $_q\alpha$, G $_{11}\alpha$, G $_{14}\alpha$, G $_{15}\alpha$, and G $_{16}\alpha$; stimulate phospholipase C- β isozymes); and G $_{12}$ subfamily (G $_{12}\alpha$ and G $_{13}\alpha$; regulate Rho guanine nucleotide exchange factor [GEF] activity) (4–6). Five β subunits and 13 γ subunits have been identified in mammals. $\beta\gamma$ subunits directly regulate several effectors, such as adenylyl cyclase, phospholipase C β , K $^+$ channels, Ca $^{2+}$ channels, or PI3 kinase (7). Functional specificity of different combinations of $\beta\gamma$ subunit have been shown for several cases, particularly for the combination with β_5 subunits.

Purification of G proteins from natural tissue requires lengthy procedures, and quantity is often limiting. It is also difficult to resolve closely related members of G α subunits or practically impossible to purify specific combinations of $\beta\gamma$ subunit. Expression of G $_s\alpha$, G $_i\alpha$, and G $_o\alpha$ in *Escherichia coli* (*E. coli*) yields large amounts of protein that can be myristoylated where appropriate (G $_i\alpha$ and G $_o\alpha$) (8), but the proteins are not palmitoylated and may be missing some other unknown modifications. Alpha subunits of G $_q$ and G $_{12}$ subfamilies and the $\beta\gamma$ complex have not been successfully expressed in *E. coli* as active proteins.

The Sf9-Baculovirus expression system has advantages to overcome these problems. First, a variety of posttranslational modification mechanisms, especially lipid modifications, such as palmitoylation, myristoylation, and prenylation, are present in Sf9 cells. These lipid modifications are critically important for the interactions of G protein subunits with receptors, RGS proteins, or effectors. With these modifications present, the recombinant G protein subunits from Sf9 expression system are almost as active as native proteins (9,10). Second, we can coinfect multiple viruses encoding α , β , and γ subunits to express desired G protein heterotrimer or $\beta\gamma$ complex on Sf9 cells. The inactive heterotrimer is the stable structure for G α subunits. Coexpression of $\beta\gamma$ was particularly required to purify properly folded α subunits of G $_q$ subfamily (10,11). Without $\beta\gamma$ subunit, these α subunits aggregated in Sf9 cells and could not be purified. It was also shown that the amount of membrane-bound G α increases by coexpressing of $\beta\gamma$ subunit. In spite of these advantages, the yield of recombinant G protein subunit from Sf9 cells was often low, and the purification procedure was laborious with conventional purification methods.

A general and simple method for purification of G proteins from Sf9 cells is described in this chapter. The G protein subunit to be purified is coexpressed with an associated hexahistidine-tagged subunit. The oligomer is adsorbed to a

Ni²⁺-containing resin and the desired untagged protein is eluted with aluminum tetrafluoride (AlF₄)⁻, which reversibly activates the α subunits of G proteins and causes dissociation of α from $\beta\gamma$. This method takes advantage of the high affinity and large capacity of Ni-NTA resin for the hexahistidine tag, as well as the extremely specific elution of the untagged subunit with AlF₄⁻. It is especially useful for purification of G α subunits that can not be purified using *E. coli* expression system (G_z α , G_q α , G₁₁ α , G₁₂ α , and G₁₃ α) and for the purification of defined combinations of $\beta\gamma$ subunits. The detailed procedures to purify each of these subunits are described in the following sections.

2. Materials

2.1. Sf9 Cells, Baculoviruses, and Culture Supplies

1. Baculoviruses for expression of the appropriate G protein combination: For all α subunit purifications, 6-His- γ_2 or with hexahistidine tag at the N-terminus, a wild-type β_1 subunit and the appropriate wild-type α subunit are needed. For $\beta\gamma$ subunit purification, 6-His-G₁₁ α (hexahistidine tag is inserted at position 121 of G₁₁ α) and the appropriate wild-type β and γ subunit combinations are necessary. Recombinant viruses encoding each G protein subunit have already been described (9,10,12–14). General methods for construction, isolation, and amplification of recombinant viruses are described in refs. 15 and 16 (see Note 1).
2. Frozen stock of Sf9 cells (Invitrogen/LifeTechnologies, American Type Culture Collection, or Pharmingen).
3. IPL-41 medium (see Note 2).
4. 10% Heat-inactivated fetal bovine serum (FBS): heat-inactivated at 55°C for 30 min.
5. 10 mL to 2 L Glass culture flasks with steel closure (BELLCO).
6. Chemically defined lipid concentrate (Invitrogen/LifeTechnologies).
7. 0.1% Pluronic F-68 (Invitrogen/LifeTechnologies).

2.2. Chromatography Supplies and Solutions

Different supplies are required for different G protein subunit preparations. Check the specific G protein purification protocol outlined in Subheading 3.4. for the columns and solutions that will be required.

1. Ni²⁺ containing resin (Ni-NTA agarose; Qiagen, cat. no. 30230).
2. Ceramic hydroxyapatite (macroprep; Bio-Rad, cat. no. 158-4000).
3. 2.5 × 5-cm Econo chromatography column (Bio-Rad).
4. Mono Q HR5/5 anion-exchange column (Amersham/Pharmacia).
5. Mono S HR5/5 cation-exchange column (Amersham/Pharmacia).
6. Fast Protein Liquid Chromatography (FPLC) system (Amersham/Pharmacia).
7. Centricon YM30 centrifugal concentration devices (Millipore/Amicon).
8. Cholic acid (Sigma). Make a 20% stock solution in water and store at 4°C. Sodium cholate is purified from cholic acid by diethylaminoethyl (DEAE) Sepharose column as described (17).

9. Polyoxyethylene-10-lauryl ether ($C_{12}E_{10}$; Sigma). Make a 10% stock solution in water and store at 4°C.
10. CHAPS (Calbiochem or Sigma). Make a 0.1 M stock solution in water and store at 4°C.
11. *N*-octyl- β -D-glucopyranoside (octylglucoside). Prepare a fresh 10% stock solution in water.
12. *N*-dodecyl- β -D-maltoside (dodecylmaltoside; Calbiochem). Prepare a fresh 10% stock solution in water.
13. The stock solutions of proteinase inhibitors (Sigma) are prepared as follows; 800 mg each of phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), and *N* α -p-tosyl-L-lysine chloromethyl ketone (TLCK) are dissolved in 50 mL of 50% dimethylsulfoxide (DMSO)/50% isopropanol. 160 mg each leupeptin and lima bean trypsin inhibitor are dissolved in 50 mL H_2O . The stock solutions of proteinase inhibitors are stored at -20°C and used as 1000X stock.
14. The following solutions are used to prepare the buffers for purification; 1 M HEPES-NaOH, pH 8.0; 1 M HEPES-NaOH, pH 7.4; 0.1 M EDTA, pH 8.0; 4 M NaCl; 1 M $MgCl_2$; 1 M KPi, pH 8.0; 14 M 2-mercaptoethanol; 1 M DTT (store frozen); 2 M imidazole-HCl, pH 8.0; 1 M NaF, 10 mM $AlCl_3$; 50 mM GDP (store frozen); and 10 mM GTP γ S (purified over Mono Q column, store frozen). The compositions of the purification solutions are shown in **Tables 1 and 2**.

3. Methods

The methods outlined in **Subheadings 3.1–3.3** are general methods required for purification of all G protein subunits. **Subheading 3.4** gives specific protocols required for the individual G protein subunit desired.

3.1. Sf9 Cell Culture (see Note 3)

1. Sf9 cells are grown and maintained in IPL-41 medium supplemented with 10% heat-inactivated FBS heat-inactivated at 55°C for 30 min and 0.1% pluronic F-68.
2. Freshly thawed Sf9 cells are cultured in a 25-mL tissue culture flask at 27°C for about 1 wk to recover.
3. Then, they are transferred to suspension culture at 27°C with constant shaking at 125 rpm.
4. Stock culture, usually 50 mL in 100-mL flask, are passaged every 3 d and maintained at a density between 0.5 and 3×10^6 cells/mL.

3.2. Infection of Sf9 Cells and Membrane Preparation

The membrane preparation procedure from 4 L of Sf9 cell culture is described.

1. Sf9 cells are expanded from 50-mL stock culture to 250 mL ($0.5\text{--}1 \times 10^6$ cells/mL) by IPL-41 medium with 10% FBS and 0.1% pluronic F-68 in a 500-mL flask.
2. After 2–3 d, they are further expanded to 1 L ($\sim 1 \times 10^6$ cells/mL) with IPL-41 containing 10% FBS and are divided into four 500-mL flasks.

Table 1
Solutions for Sf9 Membrane Preparation

Stocks	Lysis buffer	Wash buffer
1 M HEPES-NaOH, pH 8.0	20	20
4 M NaCl	25	25
100 mM EDTA	1	0
1 M MgCl ₂	2	1
14 M 2-mercaptoethanol	0.7	0.7
50 mM GDP	0.2	0.2
Total volume (mL)	1000	1000

Addition in milliliters. Adjust volume of solutions to the indicated final volume. Stock solution of 1000X proteinase inhibitors are added before use.

- After 2 d, cells are transferred to four 2-L flasks and diluted with 750 mL of IPL-41 medium containing 1% FBS and 1% lipid concentrate and 0.1% pluronic F-68.
- The following day, cells (usual density of $1.5\text{--}2 \times 10^6$ cells/mL) are infected with amplified recombinant Baculoviruses encoding the desired combination of G protein subunits. For purification of G α subunit, viruses encoding G α , β_1 , and 6-His- γ_2 are infected. For purification of $\beta\gamma$ subunit, 6-His-G α is coinfecting with the desired combination of β and γ viruses. The typical infection is 15 mL of α , 10 mL of β , and 7.5 mL of γ amplified viruses to 1 L Sf9 culture (see **Note 4**).
- After 48 h of infection, cells are harvested by centrifugation at 500g for 15 min in a JLA-10 rotor (Beckman Coulter). Cell pellets can be frozen in liquid nitrogen and stored at -80°C , or they can be further processed for membrane preparation.
- Cell pellets from 4 L of Sf9 cells are resuspended in 600 mL ice-cold lysis buffer: 20 mM HEPES, pH 8.0, 0.1 mM EDTA, 2 mM MgCl₂, 10 mM 2-mercaptoethanol, 100 mM NaCl, 10 μM GDP; with fresh proteinase inhibitors.
- Cells are lysed by nitrogen cavitation (Parr bomb) at 500 psi for 30 min at 4°C .
- The lysates are collected and centrifuged at 500g for 10 min in a JLA-10 rotor to remove intact cells and nuclei.
- The supernatants are collected and centrifuged at 35,000 rpm for 30 min in a Ti-45 rotor (Beckman Coulter).
- The pellets are resuspended in 300 mL wash buffer: 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM 2-mercaptoethanol, 10 μM GDP, and fresh proteinase inhibitors. Then, the pellets are centrifuged again as above.
- The pellets (cell membranes) are resuspended in 200 mL wash buffer and the protein concentration is determined using a Bradford protein assay (Coomassie Protein Assay Reagent, Pierce).
- The membranes are frozen by slowly pouring into a container of liquid nitrogen to form small chunks that are similar to popcorn and stored at -80°C . The amount of membrane protein from 4 L of Sf9 cells is 1.2–2 g.

Table 2
Solutions for G Protein Subunit Purification

Stocks	A	B	C	D	E	F	G	H	I
1 M HEPES-NaOH, pH 8.0	30	2	0.6	0.6	0.6	0.6	4	1	0.6
4 M NaCl	37.5	7.5	0.375	0.375	0.375	0.375	5	0.625	0.75
1 M MgCl ₂	1.5	0.3	0.09	1.5	0.09	0.006	0.2	2.5	0.03
14 M 2-mercaptoethanol	1.05	0.07	0.021	0.021	0.021	0.021	0.14	0.035	0.021
50 mM GDP	0.3	0.02	0.006	0.006	0.006		0.04	0.02	0.012
1 mM GTP γ S						0.15			
2 M Imidazol-HCl, pH 8.0		0.5	0.15	0.15	2.25	0.15	1.5	0.25	2.25
1 M NaF				0.3				0.5	
10 mM AlCl ₃				0.09			10	0.15	
10% C ₁₂ E ₁₀	75	5						2.5	1.5
20% Sodium cholate			0.3	1.5	1.5	1.5			
Total volume (mL)	1500	100	30	30	30	30	200	50	30
Stocks	J	K	L	M	O	P	Q		
1 M HEPES-NaOH, pH 8.0	0.6	0.6	0.6	0.6	0.4	0.4	0.4		
4 M NaCl	0.75	0.75	0.375	0.375	0.5	0.5	0.5		
1 M MgCl ₂	0.03	0.03	1.5	0.03	0.02	1	0.02		
14 M 2-mercaptoethanol	0.021	0.021	0.021	0.021	0.014	0.014	0.014		
50 mM GDP	0.012	0.012	0.012	0.012	0.004	0.004	0.004		
2 M Imidazol-HCl, pH 8.0	0.15	0.15	0.15	2.25	0.1	0.1	1.5		
1 M NaF			0.3			0.2			
10 mM AlCl ₃			0.09			0.06			
10% Dodecylmaltoide	0.18	0.6	0.6	0.6					
10% Octylglucoside					0.6	2	2		
Total volume (mL)	30	30	30	30	20	20	20		

Table 2 (Continued)
Solutions for G Protein Subunit Purification

Stocks	R	R1000	S	S1000	T	T150	T300
1 M HEPES-NaOH, pH 8.0			8	2	0.8	0.4	0.4
1 M HEPES-NaOH, pH 7.4	8	2					
4 M NaCl		25		25	0.5	0.25	0.25
0.1 M EDTA	2	0.5	2	0.5			
1 M MgCl ₂	0.8	0.2	0.8	0.2	0.04	0.02	0.02
1 M DTT	0.4	0.1	0.4	0.1	0.04	0.02	0.02
0.1 M CHAPS	44	11	44	11			
10% Dodecylmaltoside					0.8	0.4	0.4
1 M KPi, pH 8.0						3	6
Total volume (mL)	400	100	400	100	40	20	20

Additions are in milliliters. Adjust volume of solutions to the indicated total volume.

3.3. Detergent Extraction of SF9 Cell Membranes and Loading onto Ni-NTA Agarose

1. 1500-mg Frozen cell membranes are thawed and diluted to 5 mg/mL with wash buffer containing fresh proteinase inhibitors.
2. 20% Sodium cholate stock solution is added to a final concentration of 1% (w/v), and the mixture is stirred on ice for 1 h prior to centrifugation at 9600g for 30 min in a Ti-45 rotor.
3. The supernatants (membrane extracts) are collected, diluted threefold with buffer A: 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM 2-mercaptoethanol, 10 μ M GDP, 0.5% C₁₂E₁₀, and loaded onto a 2.5 \times 5-cm Econo Column packed with 4 mL Ni-NTA agarose and equilibrated with 20 mL buffer A. The loading of approx 1 L of diluted membrane extract onto 4 mL Ni-NTA resin usually takes 6–7 h. After loading, the Ni-NTA column is processed differently according to the subunit to be purified as described in **Subheading 3.4**.

3.4. Purification Procedures for Individual G Protein Subunits

3.4.1. G_z α

1. Ni-NTA column is washed with 100 mL buffer B: 20 mM HEPES, pH 8.0, 300 mM NaCl, 3 mM MgCl₂, 10 mM 2-mercaptoethanol, 10 μ M GDP, 10 mM imidazole, 0.5% C₁₂E₁₀. This is followed by 12 mL buffer C: 20 mM HEPES, pH 8.0, 50 mM NaCl, 3 mM MgCl₂, 10 mM 2-mercaptoethanol, 10 μ M GDP, 10 mM imidazole, 0.2% sodium cholate, at 4°C.
2. The column is incubated at room temperature for 15 min, then washed with 12 mL buffer C at 30°C.
3. The G_z α protein is eluted with 32 mL buffer D: 20 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10 μ M GDP, 30 μ M AlCl₃, 50 mM MgCl₂, 10 mM NaF, 10 mM imidazole, 1% sodium cholate, at 30°C and 4-mL fractions are collected (**Fig. 1A**; *see Note 5*).
4. Finally, β_1 6-His- γ_2 is eluted with 12 mL buffer E: buffer D without AlCl₃, MgCl₂, NaF (AMF) and containing 150 mM imidazole. 4-mL Fractions are collected (**Fig. 1A**).
5. The peak fractions containing G_z α from the Ni-NTA column are diluted threefold with buffer R: 20 mM HEPES, pH 7.4, 0.5 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.7% CHAPS. The fractions are loaded onto Mono S HR5/5 column, which was equilibrated with buffer R at a flow rate of 0.5 mL/min using an FPLC system (Amersham Pharmacia).
6. G_z α is eluted with a 25-mL gradient of 0–550 mM NaCl. Fractions of 0.5 mL are collected and assayed for protein staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and GTP γ S-binding activity.
7. G_z α elutes as a broad peak in fractions approx 400–450 mM NaCl (**Fig. 1B**). The peak fractions are concentrated and the buffer is exchanged into buffer R with 100 mM NaCl and 5 μ M GDP by repeated concentration and dilution in Centricon 30 concentration device (*see Note 6*).

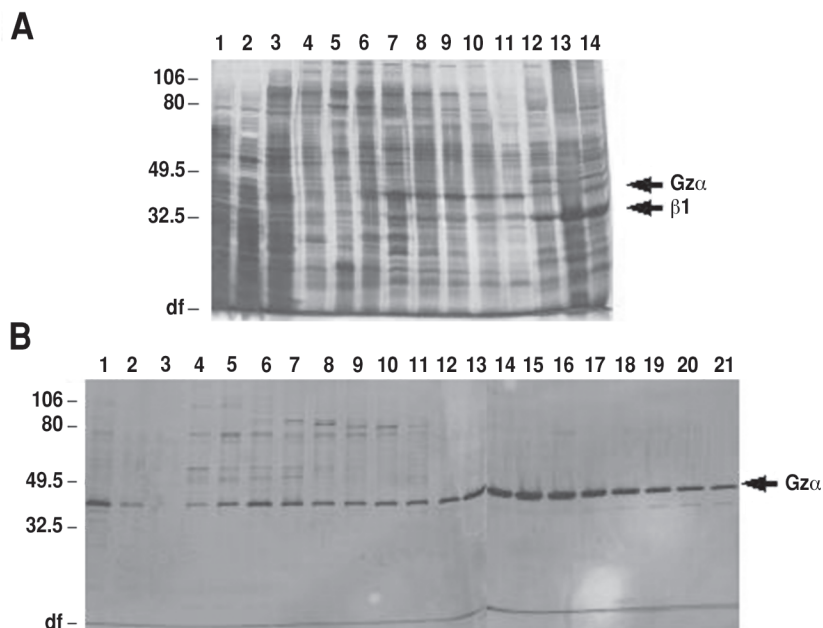


Fig. 1. Purification of G_zα. (A) Ni-NTA column for purification of G_zα. Fractions of 4 μL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with high salt and low imidazole; lanes 6–11, elution with AMF; lanes 12–14, elution with 150 mM imidazole. (B) Mono S chromatography of G_zα. The peak fractions from the Ni-NTA column were loaded onto a Mono S column and chromatographed as described. Fractions of 4 μL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through, lanes 3–21, NaCl gradient 0–500 mM.

3.4.2. G_qα

1. After loading the membrane extract from Sf9 cells expressing G_qα, β₁ and 6-His-γ₂, the Ni-NTA column is washed with 100 mL buffer B and 12 mL buffer C.
2. The column is incubated at room temperature for 15 min and is further washed with 12 mL buffer C at room temperature.
3. The column is then incubated with buffer F: 20 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM 2-mercaptoethanol, 0.2 mM MgCl₂, 5 μM GTPγS, 10 mM imidazole, and 0.2% sodium cholate, for 15 min at room temperature and washed with 32 mL the same buffer. Endogenous Sf9G_iα-like protein is removed from the column at this step (see **Note 7**).
4. Recombinant G_qα is eluted from Ni-NTA column by washing with 24 mL buffer D at room temperature and collecting 4-mL fractions (**Fig. 2**).

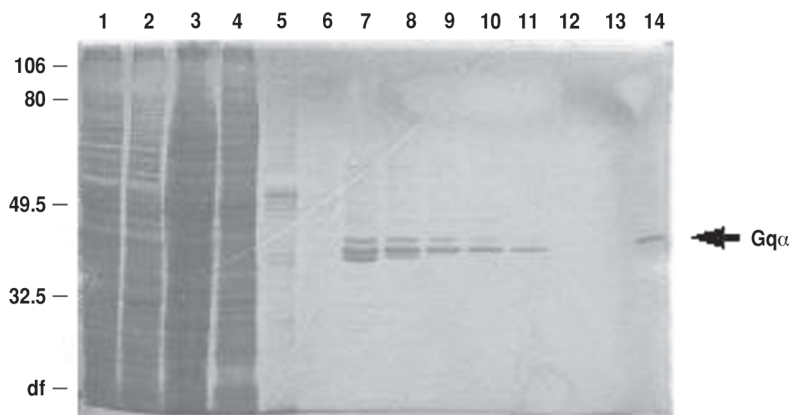


Fig. 2. Purification of $G_q\alpha$. Ni-NTA column for purification of $G_q\alpha$. Fractions of 4 μ L were subjected to SDS-PAGE and stained by silver nitrate. In this purification, $G_q\alpha$ was eluted with two steps; first with 1% cholate containing buffer, then with AMF containing buffer. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with high salt and low imidazole; lanes 6–12, elution with 1% cholate; lanes 13 and 14, elution with AMF.

5. The peak fractions containing $G_q\alpha$ from the Ni-NTA column are diluted three-fold with buffer S and loaded onto a Mono Q HR5/5 column that was equilibrated with buffer S. CHAPS in buffer S can be replaced with 1% octylglucoside.
6. $G_q\alpha$ is eluted with a linear gradient of 0–400 mM NaCl 20-mL gradient with a flow rate of 0.5 mL/min: collecting 0.5-mL fractions. Fractions are assayed by immunoblotting with $G_q\alpha/G_{11}\alpha$ antiserum Z811 and $G_{11}\alpha$ antiserum B825 (18).
7. Recombinant $G_q\alpha$ is recognized by both Z811 and B825 and eluted in fractions containing approx 220 mM NaCl. An endogenous Sf9 $G_q\alpha$ -like protein is recognized by Z811, but not by B825 (10), and eluted later in the gradient (\sim 280 mM NaCl).
8. The peak fractions that mainly contain recombinant $G_q\alpha$ are pooled. The sample is concentrated and the buffer is changed into buffer S containing 100 mM NaCl and 5 μ M GDP by repeated dilution and concentration using Centricon YM30 (see Notes 8 and 9).

3.4.3. $G_{12}\alpha$

$G_{12}\alpha$ has several different biochemical properties from other $G\alpha$ subunits, and the purification method for $G_z\alpha$ or $G_q\alpha$ cannot be used for $G_{12}\alpha$. The high-salt wash on Ni-NTA column disrupts the interaction between $G_{12}\alpha$ and $\beta_1\gamma_2$. Also, $G_{12}\alpha$ cannot be eluted from Ni-NTA column with buffers containing sodium cholate.

1. After loading the extract expressing $G_{12}\alpha$, β_1 , and 6-His- γ_2 , the Ni-NTA column is washed with 100 mL buffer G: buffer A containing 15 mM imidazole and 100 mM NaCl.

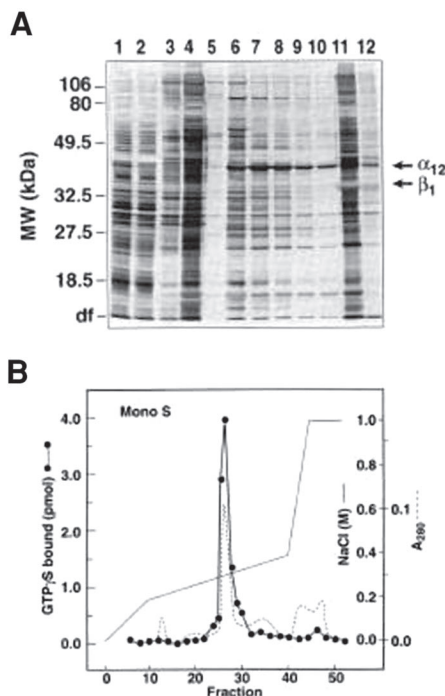


Fig. 3. Purification of $G_{12}\alpha$. (A) Ni-NTA column for purification of $G_{12}\alpha$. Fractions of 4 μ L were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with low imidazole; lanes 6–10, elution with AMF; lanes 11 and 12, elution with 150 mM imidazole. (B) Mono S chromatography of $G_{12}\alpha$. The peak fractions from the Ni-NTA column were loaded onto a Mono S column and chromatographed with NaCl gradient of 200–400 mM. Fractions of 3 μ L were assayed for GTP γ S binding activity with 5 μ M GTP γ S and 10 mM $MgSO_4$ for 90 min at 30°C. (Reproduced from ref. 12 with permission.)

2. The column is incubated at room temperature for 15 min. Then the column is washed with 12 mL buffer G and 32 mL buffer H: 20 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM 2-mercaptoethanol, 20 μ M GDP, 50 mM $MgCl_2$, 10 mM NaF, 30 mM $AlCl_3$, 10 mM imidazole, and 0.5% $C_{12}E_{10}$, at 33°C (Fig. 3A) collecting 4-mL fractions.
3. The dissociation of $G_{12}\alpha$ from $\beta\gamma$ on Ni-NTA column is usually incomplete. Approximately 50% of $G_{12}\alpha$ remains on the column after AMF elution based on Western blot. The temperature of the AMF elution buffer can be raised to 37°C to further facilitate the dissociation.
4. The peak fractions of $G_{12}\alpha$ are diluted threefold with buffer S and loaded onto Mono S HR5/5 column, which was equilibrated with buffer S and chromatographed with the gradient of 200–400 mM NaCl at 0.5 mL/min, and 0.5-mL frac-

tions are collected. 0.7% CHAPS in buffer S can be replaced by 1% octylglucoside. $G_{12}\alpha$ elutes from Mono S column as a sharp peak near 250 mM NaCl (**Fig. 3B**).

5. The peak fractions containing $G_{12}\alpha$ are concentrated and the buffer is exchanged into buffer S containing 100 mM NaCl, 1 μ M GDP, and 10% glycerol using a Centricon YM30 and repeated dilution and concentration (*see Note 10*).

3.4.4. $G_{13}\alpha$

1. After loading the membrane extract from Sf9 cells expressing $G_{13}\alpha$, β_1 and 6-His- γ_2 , the Ni-NTA column is washed with 100 mL buffer B, then is warmed up to room temperature for 15 min.
2. Then the column is washed with 12 mL buffer J: 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 μ M GDP, 10 mM imidazole, 0.06% dodecylmaltoside.
3. Wash with 12 mL buffer K: buffer J containing 0.2% dodecylmaltoside.
4. 24 mL buffer L: buffer K containing 50 mM NaCl, 30 μ M $AlCl_3$, 50 mM $MgCl_2$, and 10 mM NaF. 4-mL Fractions are collected. As shown in **Fig. 4A**, the peak fractions are almost pure on SDS-PAGE.
5. The column is then eluted with 12 mL buffer M: buffer K containing 50 mM NaCl and 150 mM imidazole, at 30°C to elute $\beta\gamma$ and to check for any remaining $G_{13}\alpha$.
6. The fractions from the elution containing relatively pure $G_{13}\alpha$ are combined and applied onto 1 mL of ceramic hydroxyapatite column equilibrated with buffer U: 20 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.2% dodecylmaltoside, 10% glycerol.
7. The flow-through is collected and applied again onto the column.
8. The column is then washed with 4 mL buffer T, 4 mL T150 (buffer T containing 150 mM KPi, pH 8.0), and 4 mL T300 (buffer T containing 300 mM KPi, pH 8.0). 1-mL fractions are collected. $G_{13}\alpha$ elutes in fractions of T150 (**Fig. 4B**).
9. The peak fractions are combined and the buffer is exchanged to buffer T containing 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μ M GDP and 10% glycerol by repeated dilution and concentration with a Centricon YM30 (*see Note 11*).

3.4.5. Wild-Type $\beta\gamma$ Subunit

Because $\beta\gamma$ subunit expresses at much higher level than the α subunits described previously, the purification from 1 L of Sf9 cells is usually enough for most of the experimental purposes.

1. To purify wild-type $\beta_1\gamma_2$ subunit, 1 L of Sf9 cells are infected with recombinant baculoviruses encoding β_1 , γ_2 , and 6-His- $G_{i1}\alpha$, the membrane extracts are prepared and loaded onto 1 mL Ni-NTA agarose column following the same procedure as described in **Subheadings 3.2.** and **3.3.**
2. Then the column is washed with 25 mL buffer B and 10 mL buffer O: 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 μ M GDP, 10 mM imidazole, 0.3% octylglucoside.

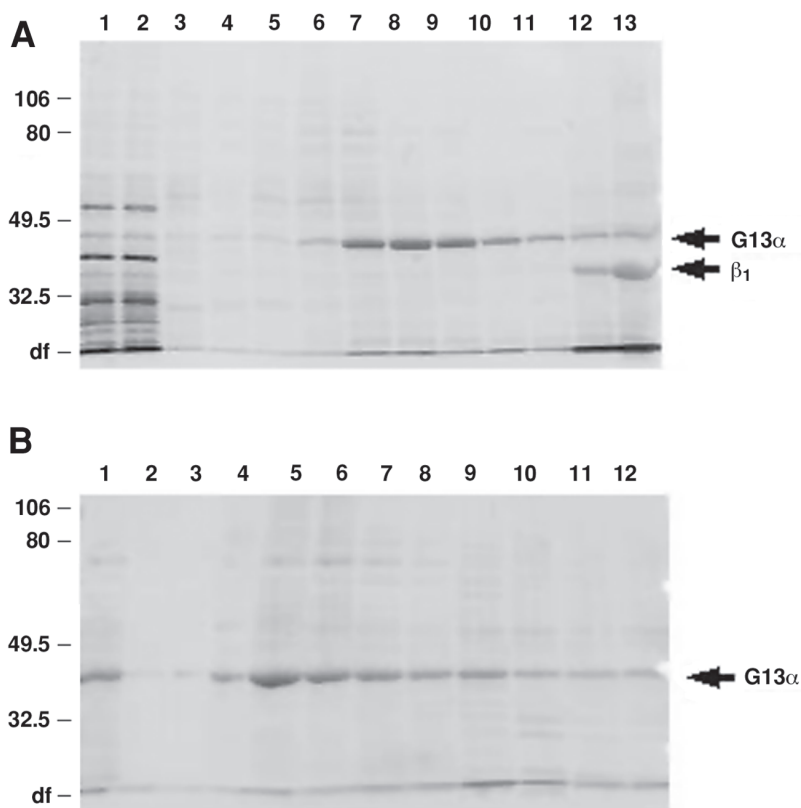


Fig. 4. Purification of G₁₃α. **(A)** Ni-NTA column for purification of G₁₃α. Fractions of 4 μL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with low imidazole; lanes 6–11, elution with AMF; lanes 12 and 13, elution with 150 mM imidazole. **(B)**. Hydroxyapatite chromatography of G₁₃α. The peak fractions from the Ni-NTA column were loaded onto a hydroxyapatite column and chromatographed as described. Fractions of 5 μL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lane 3, wash ; lanes 4–8, elution with 150 mM KPi; lanes 9–11, elution with 300 mM KPi.

3. The column is incubated at room temperature for 15 min and washed with 4 mL buffer P (buffer O containing AMF and 1% octylglucoside) and 4 mL buffer Q (buffer O containing 150 mM imidazole and 1% octylglucoside).
4. 1-mL Fractions are collected. β₁γ₂ or 6-His-G₁₁α elutes in fractions with buffer P or buffer Q, respectively (**Fig. 5A**).
5. The elution fractions from Ni-NTA column can be further purified by Mono Q chromatography. The peak fractions are diluted threefold with buffer S: 20 mM

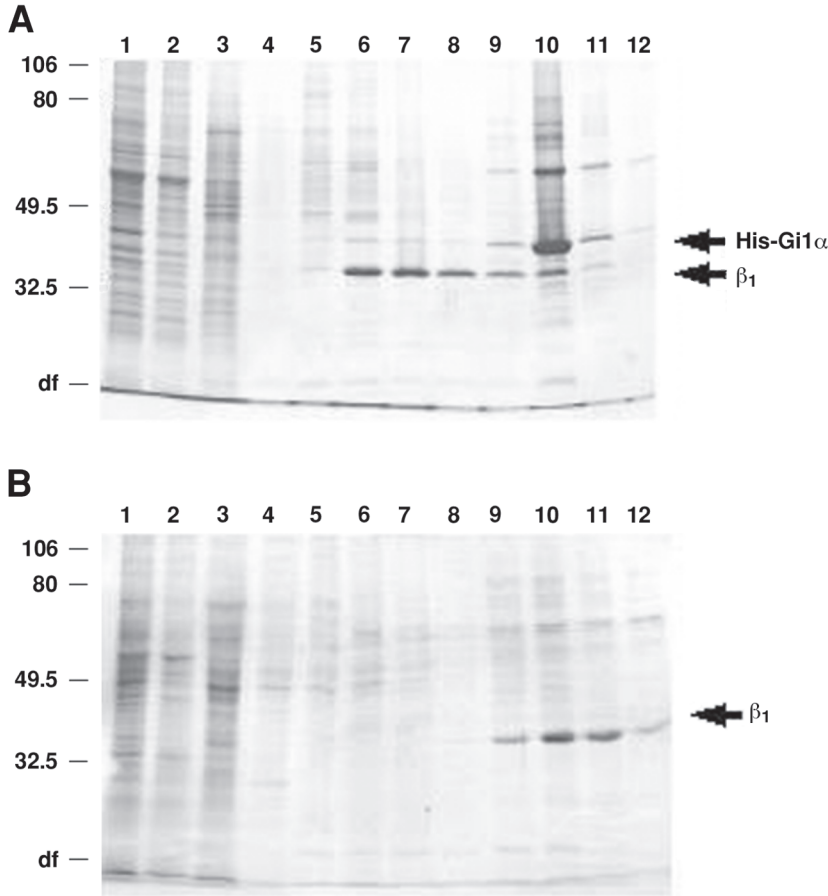


Fig. 5. Purification of $\beta\gamma$ subunit. Ni-NTA column for purification of wild-type $\beta_1\gamma_2$ (A) or 6-His- $\beta_1\gamma_2$ (B) Fractions of 4 μ L were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flowthrough; lanes 3–5, wash with high salt and low imidazole; lanes 6–11, elution with AMF; lanes 12 and 13, elution with 150 mM imidazole.

HEPES, pH 8.0, 0.5 mM EDTA, 2 mM MgCl_2 , 1 mM DTT, 0.7% CHAPS. The fractions are loaded onto Mono Q HR5/5 column that was equilibrated with buffer S. 0.7% CHAPS in buffer S can be replaced with 1% octylglucoside.

6. $\beta_1\gamma_2$ elutes from the column with a 20-mL gradient of 0–400 mM NaCl. Fractions of 0.5 mL are collected. Peak fractions at approx 200 mM NaCl are concentrated and the buffer is exchanged into buffer S containing 100 mM NaCl by Centricon 30 (see **Note 12**).

3.4.6. Purification of 6-His-Tagged $\beta\gamma$ Dimers

Functionally active $\beta\gamma$ subunit can also be purified without $G\alpha$ coexpression using either 6-His-tagged β or γ subunits (**19,20**).

1. Sf9 cells are infected with Baculoviruses encoding β_1 and 6-His- γ_2 .
2. Membrane extracts are loaded on Ni-NTA column as described previously.
3. The column is processed with the same procedure for wild-type $\beta\gamma$. Washing with AMF-containing buffer removes endogenous Sf9 $G\alpha$ subunits. However, this step can be omitted, and the entire procedure can be performed at 4°C. **Figure 5B** shows the elution profile.
4. The peak fractions are concentrated and the buffer is exchanged into buffer S containing 100 mM NaCl by Centricon-30 (see **Note 13**).

4. Notes

1. We keep low passage viruses as the progenitor stocks at 4°C and at -80°C. The operating virus stocks (usually 200–500 mL/batch) are amplified from these stocks and are stored at 4°C. These viral stocks are stable for at least several months.
2. Preparation of IPL-41 medium from powder (JRH) is more cost effective for large-scale cultures.
3. Early passage cells are frozen in medium containing 10% FBS and 10% DMSO and can also be used as cell stocks. Cells from one vial of frozen stock can be maintained for approx 4–6 mo. We change to a new frozen stock when we start to see cells with irregular shapes, a decrease of growth rate, or a reduction in the expression level of recombinant proteins. For large-scale culture >1 L, IPL-41 medium containing 1% FBS, 1% chemically defined lipid concentrate, and 0.1% pluronic F-68 is used.
4. To increase the yield of G protein subunit, the freshly amplified recombinant viruses are recommended. Among three G protein subunits, γ subunit usually expresses most efficiently. The excess expression of γ subunit may inhibit the expression of $G\alpha$ subunit. Therefore, if the expression level of the $G\alpha$ subunit to be purified is low, infecting less amount of 6-His- γ_2 (and/ or β_1) virus may be helpful to increase the $G\alpha$ expression.
5. The presence of AMF and the increase in cholate concentration facilitate the dissociation of a subunit from $\beta\gamma$ subunit on the column.
6. The yield of $G_z\alpha$ from 1500-mg membrane protein is approx 300 μ g. It was demonstrated that $G_z\alpha$ purified with this method directly inhibited specific subtypes of adenylyl cyclases (**12**). Such activity could not be detected using $G_z\alpha$ produced in *E. coli*. It is likely that the myristoylation that is present in $G_z\alpha$ from Sf9 cells, but not from *E. coli*, is critical for the interaction with adenylyl cyclase.
7. $G_q\alpha$ has slow GDP–GTP exchange rate and cannot be activated by incubation with buffer F. In contrast, endogenous $G_i\alpha$ subunit has faster GDP–GTP exchange rate and is activated with this procedure. Contamination of Sf9 $G_i\alpha$ increases the GTPase activity of the purified $G_q\alpha$ sample and interferes with GTPase assays, especially assays with receptor reconstitution.

8. The final yield of recombinant $G_q\alpha$ is approx 200 mg from 1500-mg membrane protein. The same procedure can be applied for the purification of $G_{11}\alpha$. However, the yield of $G_{11}\alpha$ is much less than that of $G_q\alpha$. $G_{15/16}\alpha$ could not be purified using this method because of its low expression in Sf9 cells and its inability to be activated by AMF (*II*).
9. In order to perform GAP assays for $G\alpha$, GTP-bound form of $G\alpha$ has to be prepared as a substrate for the reaction. Because the intrinsic GDP–GTP exchange rate of $G_q\alpha$ is low, it is quite difficult to generate enough amount of GTP-bound $G_q\alpha$ by simply incubating $G_q\alpha$ with GTP. The agonist-activated receptor is usually required to facilitate the GDP–GTP exchange of $G_q\alpha$ (*21*). $G_q\alpha R183C$ mutant in which arginine183 in switch I region is mutated to cysteine has much reduced GTP hydrolysis rate than wild-type. This helps to increase the fraction of GTP-bound $G_q\alpha$ subunit during incubation with GTP. Furthermore, this mutant can respond to GAP activities of RGS proteins. Since the receptor reconstitution system takes a lot of effort to establish, $G_q\alpha R183C$ mutant has been used as a convenient alternative tool to detect GAP activity of $G_q\alpha$ (*22,23*). Although the affinity of $G_q\alpha R183C$ to $\beta\gamma$ is lower than wild-type $G_q\alpha$, it can be purified on Ni-NTA column using the method as described previously for wild-type $G_q\alpha$. After the elution with AMF and 1% cholate containing buffer D, the peak elution fractions are concentrated, and the buffer was exchanged into buffer S with 100 mM NaCl and 5 mM GDP to remove AMF (final volume is <1 mL from 4-L culture). The final sample is not pure as wild-type $G_q\alpha$, but GAP assays of approx 20 tubes can usually be performed using 20–30 μ L of the purified sample.
10. The presence of glycerol prevents the aggregation of the protein during concentration. It is also recommended to occasionally mix the sample gently during concentration by Centricon. The yield of $G_{12}\alpha$ from 1500-mg membrane is approx 200 μ g.
11. Because $G_{13}\alpha$ tends to aggregate during concentration, it is also recommended to occasionally mix the sample gently during concentration by Centricon. The yield is approx 200 μ g from 4 L of Sf9 culture.
12. The yield of $\beta_1\gamma_2$ from 1200-mg membrane is 1–2 mg. The same protocol can be applied to purify different combinations of $\beta\gamma$ subunits, such as $\beta_2\gamma_2$, $\beta_1\gamma_1$, $\beta_1\gamma_3$, $\beta_1\gamma_5$, or $\beta_1\gamma_7$. The methods to purify β_5 subunit complexed with γ_2 or RGS proteins with G protein γ subunit-like domain have been described in *refs. 24–26*.
13. Hexahistidine-tagged $\beta_1\gamma_2$ can be further purified over Mono Q column as described for wild-type $\beta\gamma$ subunit. The same procedure can be applied to purify the combination of 6-His- β_1 and γ_2 . In both cases, the yield of hexahistidine-tagged $\beta_1\gamma_2$ is 1–2 mg from 1-L culture. The presence of hexahistidine tag at the amino terminus of β_1 or γ_2 does not interfere with the interactions with effectors. Mutant $\beta\gamma$ subunits that have less affinity for $G\alpha$ subunits can be purified with this method. Wild-type, nontagged $\beta\gamma$ subunit is recommended for receptor reconstitution assays. The imidazole bump fractions from Ni-NTA column of $G\alpha$ purification that contain hexahistidine tagged $\beta_1\gamma_2$ can also be used as sources to purify 6-His- $\beta_1\gamma_2$ with Mono Q column.

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