Non-denaturing purification of alpha-Synuclein from erythrocytes

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Method Article

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Abstract

Pathogenic aggregation of α-Synuclein \(αSyn) is implicated in familial and sporadic Parkinson disease \(PD) and several other synucleinopathies. Choosing non-denaturing conditions, we developed a several purification schemes in order to obtain αSyn from human erythrocytes, enabling researchers to employ biophysical techniques in order to elucidate its native structure and properties.

Introduction

Here we introduce a novel way of purifying human α-Synuclein from human erythrocytes, leaving the physiological conformation of the protein intact. This methology has been also successfully applied to human neuroblastoma cells stably overexpressing wt human α-Synuclein as well as to COS-7 cells transiently overexpressing wt and mutant forms of α-Synuclein. The overall purification is divided in three general steps: An ammonium sulfate preciptation giving a crude protein solution, a bulk purification which can be adjusted to the exact sample properties \(volume, cell type, cross contamination) utilizing alternatively 3 different methods \(AX, HIC, covalent chromatography), and a polishing step giving pure protein after size exclusion chromatography \(Fig. 1). See figure in Figures section.

Reagents

General reagents/disposables: $(NH_4)_2SO_4$ \(Sigma, A441) (NH_4) acetate \(Sigma, A1542) PBS \(Sigma, P4417-100TAB) Protease Inhibitor \(Roche, 11 873 580 001) ACK Lysing buffer \(Lonza, 10-548E) Centricon Plus-70 centrifugal filters \(Millipore, UFC701008) Amicon Ultra centrifugal filters 10,000 MWCO \(Millipore, UFC901008) Filter paper \(Whatman, 1002-270) Sterile filter 0.22 µm \(Corning, 431098) Tris-HCl \(Sigma, T3253) NaCl \(Sigma, S3014) EDTA \(Sigma, ED2SS) Dipyridyl disulphide \(Sigma, D5767) DTT \(Sigma, 43815) Boric acid \(Sigma, B6768) Sodium tetraborate decahydrate \(Sigma, S9640) **Antibodies \(usage according to manufacturers recommendations)**: _anti α-Synuclein_: Syn211 \(Santa Cruz, SC-12767) Syn1 \(BD, 610787) LB509 \(Santa Cruz, SC-58480) C20 \(Santa Cruz, SC-7011) _anti Transthyretin_: Prealbumin rabbit monoclonal \(Epitomics, 2434-1) _anti Hemoglobin_: Anti-Hemoglobin alpha Polyclonal \(Thermo Scientific, PA1-9527) Rabbit Antiserum To Human Hemoglobin \(MP Biomedicals, 55129)

Equipment

Instruments Sorvall RC 5C Plus with SLA 1500 rotor or equivalent Äktapurifier UPC 10 or equivalent system Fischer Sonic Dismembrator Model 300 with micro-tip or equivalent **Columns**: Column packed with Superdex 200 or equivalent HitrapQ HP 5 ml HiTrap Phenyl HP 5 ml Column packed with Sepharose 6B or Sepharose 4B gel media

Procedure

Cell lysis _Erythrocytes_: 1. Spin down freshly drawn blood at 500 g for 10 min 2. Discard plasma and buffy coat 3. Wash erythrocytes 3 x in PBS 4. Lyse erythrocytes with addition of 3x volume of ACK lysing buffer \(leading to 4 x original volume), add 1x protease inhibitor \(PI) 5. Nutate lysate at 4 °C for 10 min, spin down at 14,000 g for 10 min \(pellet contains organelles and membranes and can be discarded), take aliquot _3D5 cells_: 1. Everything is done quickly on ice 2. Aspirate medium 3. Depending on confluence and size of dish , add 200-100 µl PBS \(higher confluence, higher volume) + PI 4. Scrape plates 5. Wash 3 times with PBS + PI \(meaning spinning cells down for 5 min at 3000 rpm, resuspend in 1 ml PBS + PI and repeat) 6. Last washing step: Take up in desired volume PBS + PI \(to get desired concentration, higher amount of cells leaving to higher amount of PBS + PI) 7. Sonicate each sample 3 x 15 s, using micro tip sonicator \(Fischer Sonic Dismembrator Model 300) Lysate is placed on ice between each run 8. Samples are centrifuged 10 min at 14000g to pellet nuclei, cell debris and mitochondria 9. Supernatant is transferred to new cups, pellet is discarded **Ammonium sulfate precipitation**: 1. Add 25% $(NH_4)_2SO_4$ SLOWLY at 4 °C \(calculation via http://www.encorbio.com/protocols/AM-SO4.htm) 2. stir lysate for 1 h at 4 °C 3. Spin down sample at 20,000 g for 20 min 4. Keep supernatant, take aliquot 5. Discard pellet, take aliquot 6. Bring up the supernatant rom 25% to 50% $(NH_4)_2SO_4$, by SLOWLY adding at 4 °C \(calculation via http://www.encorbio.com/protocols/AM-SO4.htm) 7. Stir lysate for 1 h at 4 °C 8. Spin sample down at 20,000 g for 20 min 9. Discard supernatant take aliquot 10. Only for erythrocyte lysate: Wash pellet 5-10x with 55% $(NH_4)_2SO_4$ in dd water, \(calculation via http://www.encorbio.com/protocols/AM-SO4.htm), until red color of sup is mostly gone. Washing means nutating the pellet in 55% $(NH_4)_2SO_4$ for 10 min at 4 °C, then spinning it down at at 20,000 g for 20 min, keeping the pellet and discarding the supernatant. 11. Bring washed pellet up in respective chromatography binding buffer \(see below) by nutating it for 1 h at 4 °C 12. Spin sample own at 20,000 g for 20 min, discard pellet 13. Filter supernatant sequentially trough Whatman filter paper and 0.2 µM sterile filter. After filtering, take aliquot **Bulk purification** _Hydrophobic Interaction Chromatography \(Äktapurifier and HiTrap Phenyl HP 5 ml)_ 1. Running buffer: 50 mM phosphate, pH 7.0, 1 M $(NH_4)_2SO_4$. Elution buffer: 50 mM phosphate, pH 7.0 2. Equilibrate HiTrap Phenyl HP 5 ml \(GE Healthcare) with binding buffer 3. Inject erythrocyte lysate after $(NH_4)_2SO_4$ precipitation with 0.5 ml/min flow rate until UV absorption is stable. Collect flow through for analysis 4. Elution of protein: Pressure Limit 0,30 MPa, Flow Rate 1,5 ml/min, Fraction Size 1,5 ml, linear gradient 0% to 100% elution buffer for 15 CV. Collect all fractions, take aliquots for analysis 5. Wash column with MilliQ water \(to UV-Absorption = 0) to reactivate column 6. System, sample loop and column can be washed with 1 x dd water, 1 x 44% formic acid, 1 x dd water in case excess hemoglobin could not be removed 7. Analyze fractions via Western blotting for α-Synuclein and Hemoglobin 8. Pool α-Synuclein positive / Hemoglobin negative fractions and concentrate using centrifugal filters _Anion Exchange Chromatography \(Äktapurifier and HitrapQ HP 5ml)_: 1. Equilibrate column with binding buffer \(20 mM Tris, 25 mM NaCl, pH 8.0) 2. Let sample \(erythrocyte lysate after $(NH_4)_2SO_4$ precipitation) run trough column at flow rate of 0.5 ml/min until UV absorption is stable, collect flow-trough 3. Elute sample at a flow rate of 1,5 ml/min, Fraction Size 1,5 ml, linear gradient for 10 CV from 0% to 100% elution buffer 4. Wash column with 1 M NaOH \(to UV-Absorption = 0) to completely elute any remaining protein 5. Analyze fractions via Western blotting for α-

Synuclein and Transthyretin 8. Pool α-Synuclein positive / Transthyretin negative fractions and concentrate using centrifugal filters _Covalent Chromatography \(Äktapurifier and Thiopropyl Sepharose 6B packed in a 150 ml XK 16/100 column)_: 1. Equilibrate column with PBS 2. Load erythrocyte lysate after $(NH_4)_2SO_4$ precipitation on Sepharose 6B column, flow 0.2 ml/min, keep flow-trough \(contains α-Synuclein) 3. Sample is concentrated using Centricon Plus 70 to approx. 10 ml \(avoid concentrating it too much since it will cause aggregation). Take aliquot 4. Elute column with 5 column volumes of 25 mM DTT in PBS, flow rate 2.5 ml/min 5. Equilibrate column with borate buffer pH 8.0 + 1 mM EDTA, flow rate 2.5 ml/min 6. Mix 320 mg dipyridyl disuphide with 400 ml borate buffer, stir at 4 C for 4h and filter \(0.2 µm) 7. Eluted column with the borate/dipyridil sulfide mixture for 2 CV at 1 ml/min 8. Wash column with 5 CV 20% EtOH **Size Exclusion Chromatography \(Superdex 200 10/300 GL)**: 1. Equilibrate column with 50 mM NH_4Ac pH 8.5 2. Inject 250 ul of α-Synuclein positive concentrated fractions, collect fractions at flow rate 0.8 ml/min 3. Freeze/lyophilize peak fractions, take aliquot

Troubleshooting

Cell lysis: _Erythrocytes_: A. Instead of freshly drawn blood, older samples can be employed. The ratio of α-Synuclein/Hemoglobin in those samples can be drastically reduced however, making the purification more challenging _3D5 cells_: A. It has to be made sure that the cell lysate does not heat up too much during sonication. Short sonication steps with incubation of the sample on ice in between are therefore recommended. B. In cell culture cell lysates, a 25% $(NH_4)_2SO_4$ pre-precipitation step can be beneficial to remove unwanted protein. If HIC is employed for the bulk purification, this is not necessary since the respective binding buffer already contains approx. 25% $(NH_4)_2SO_4$ and thereby eliminates contamination before the column is loaded **Ammonium sulfate precipitation**: In erythrocyte lysate, washing the 50% $(NH_4)_2SO_4$ pellet in 55% $(NH_4)_2SO_4$ solution is highly recommended. Since Hemoglobin stays soluble until 60-65% $(NH_4)_2SO_4$, the washing steps provide a cheap and easy method to eliminate large amounts of contaminating Hemoglobin. **Bulk purification** The exact method of bulk purification has to be decided by the nature of the sample. In general, the HIC gave the highest purity but lowest yield. If high trough-put is needed, the Sepharose 6B method should be applied. If large amounts of Hemoglobin are present, AX is recommended. If a particular sample has low purity with one of the chosen methods, an additional step utilizing one of the other methods can be employed. A: HIC advantages: Fast, cheap, best purity, $(NH_4)_2SO_4$ in binding/elution buffer stabilizes folding. Disadvantages: Low binding capacity, struggles with high amounts of contaminating Hemoglobin. Results can be inconsistent depending on the exact sample. Has sometimes to be applied twice to effectively purifiy all α-Synuclein out of solution. B: AX advantages: Fast, cheap, good binding affinity, Hemoglobin does not bind \(making it very effective in separating α-Synuclein and Hemoglobin). Disadvantages: High amounts of NaCl in elution buffer can destabilize α-Synuclein, causing it to precipitate. Transthyretin and α-Synuclein elute very close to each other making thorough removal of blood plasma prior to erythrocyte lysing necessary. Has sometimes to be applied twice to effectively purifiy all α-Synuclein out of solution. C: Instead of Sepharose 6B gel medium, Sepharose 4B can be used. Sepharose 6B has shown to have a better binding capacity, while Sepharose 4B packed columns can be

easier reactivated. Sepharose 6B advantages: Simple and consistent, good for large sample volumes, high yield, removes both Transthyretin and Hemoglobin from erythrocyte lysate samples very effectively. Since no high pressures/high flow rates, no buffer gradient and no monitoring of UV-absorption or conductivity are needed, it can be done with a simple peristaltic pump. Disadvantages: Expensive gel media, no pre-packed columns commercially available, regeneration of column time consuming. **Size Exclusion Chromatography \(Superdex 200)**: Instead of a Superdex 200 column, Superdex 75, Superose 12 and Sephacryl 200-HR were also successfully employed. The advantage of the Superdex 200 gel medium is the ability to separate Hemoglobin from α-Synuclein in contrast to the above mentioned media.

Anticipated Results

Yield From 40 ml of freshly drawn packed red blood cells, up to 0.5 mg α-Synuclein can be recovered. **\($(NH_4)_2SO_4$ precipitation** As seen in Fig. 2, α-Synuclein usually precipitates between 30 and 45% \($(NH_4)_2SO_4$. Human Hemoglobin should stay soluble until 60%. See figure in Figures section. **Chromatography** _HIC \(HiTrap Phenyl HP)_: α-Synuclein should elute between a conductivity of 135-110 mS/cm \(approx. 0.7 M \(NH4)2SO4~) \(Fig. 3A). _AX \(HiTrap Q HP)_: α-Synuclein should elute between a conductivity of 30-35 mS/cm \(~ approx. 300 mM NaCl) \(Fig. 3B). _SEC \(Superdex 200 10/300 GL)_: α-Synuclein should elute around 13.5 ml \(Fig. 3C). See figure in Figures section. _Covalent chromatography \(Sepharose 6B, XK 16/100)_: α-Synuclein should be completely found in the clear flow-trough. Hemoglobin should be completely bound to the column and then be eluted with DTT \(Fig. 4). See figure in Figures section. **Purity** Example Coomassie stains of the different purification steps for human erythrocytes are shown in Fig. 5. After applying the procedure the purity of the sample should be >95% \(measured by densitometry of Coomassie stain). See figure in Figures section.

Figures

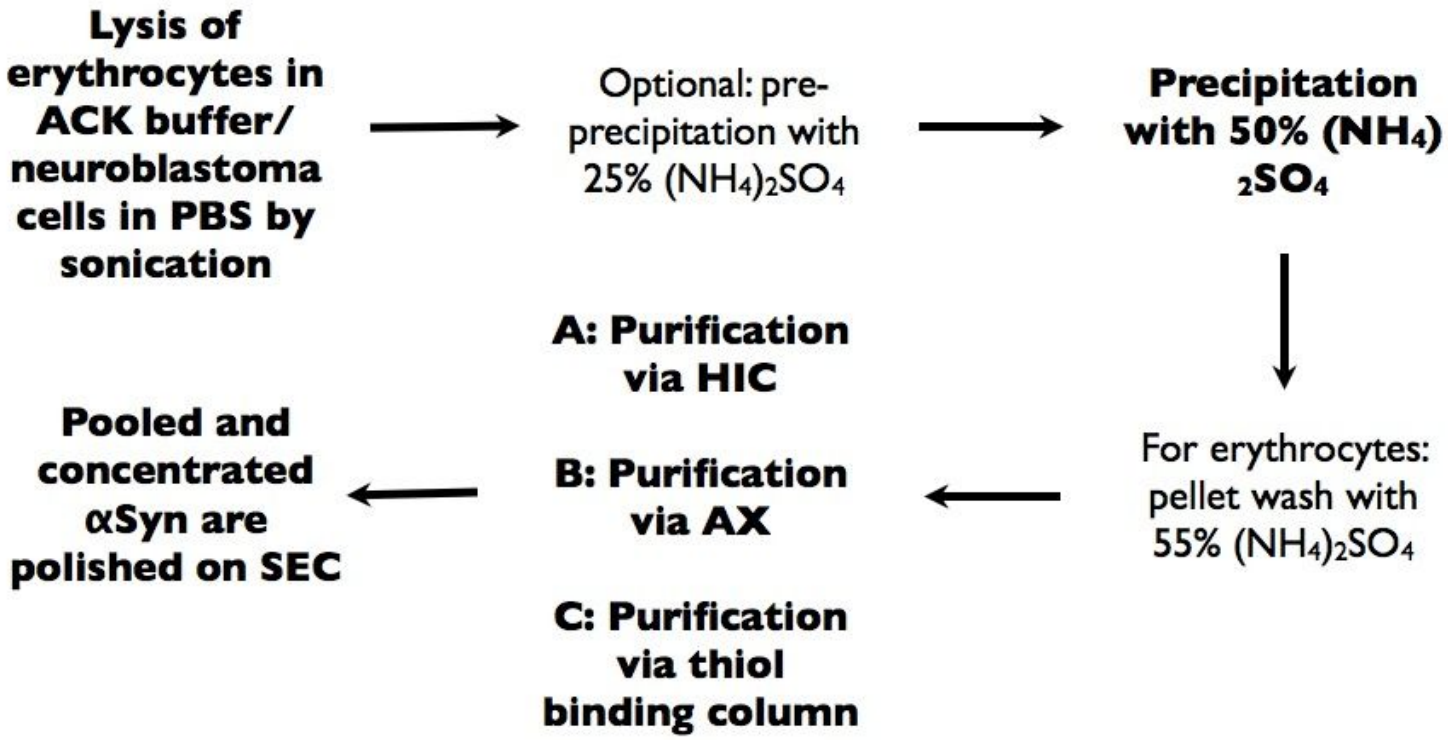

Figure 1

Flow diagram of the purification scheme

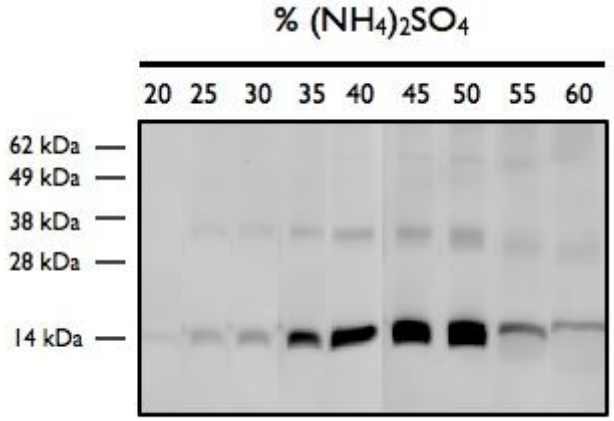

Figure 2

Ammonium sulfate precipitation of α-Synuclein from cell lysate SDS-PAGE Western blot (C20) of α-Synuclein. Cell lysate (erythrocyte) was treated step wise with increasing (NH~4~)~2~SO~4~ concentrations, the resulting pellet was taken up in PBS and analyzed.

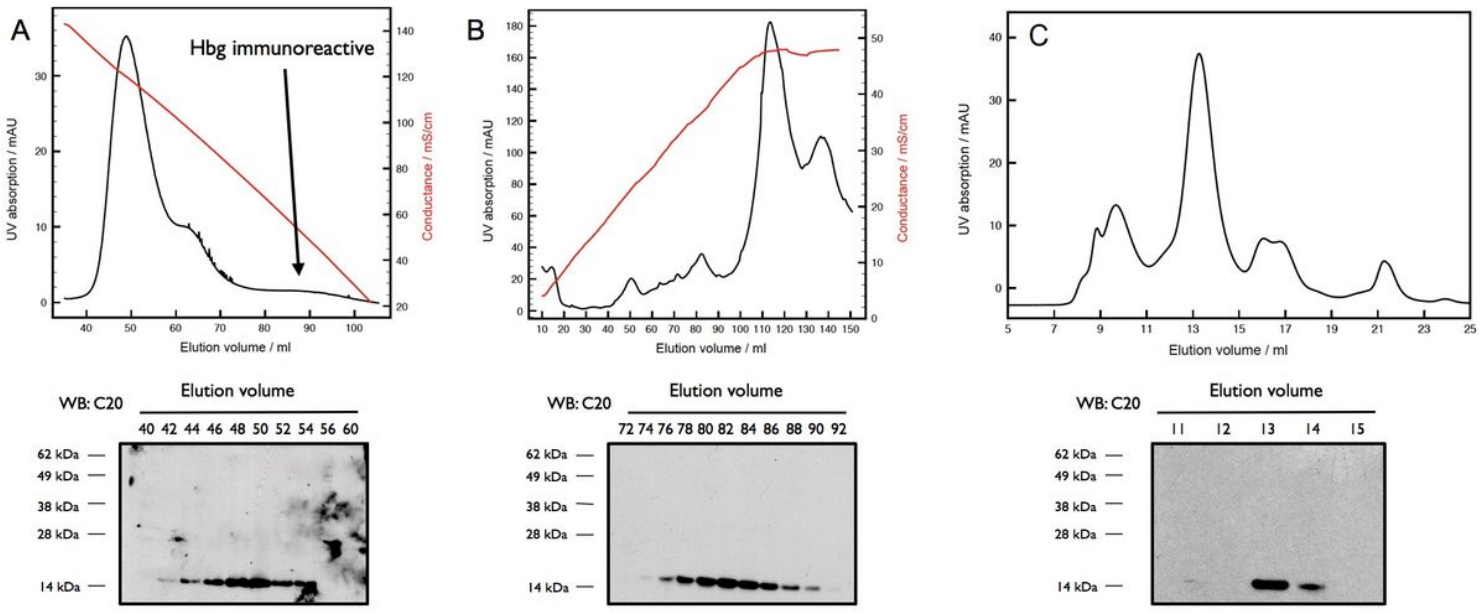

Figure 3

Example FPLC chromatograms of α-Synuclein purifications and WB analysis of fractions *A*: Chromatogram of HIC purification of α-Synuclein from human erythrocytes. Shown are the UV absorption at 280 nm during elution with a (NH~4~)~2~SO~4~ gradient. *B*: AX chromatogram of purification of α-Synuclein from human erythrocytes. Protein was eluted with a NaCl gradient. *C*: Example chromatogram of SEC (Superdex 200 GL 10/300) of α-Synuclein after Sepharose 6B purification. α-Synuclein eluted at a volume corresponding to a native molecular weight of 55 kDa (calibration with Bio Rad gel filtration standard, cat. No. 151-1901)

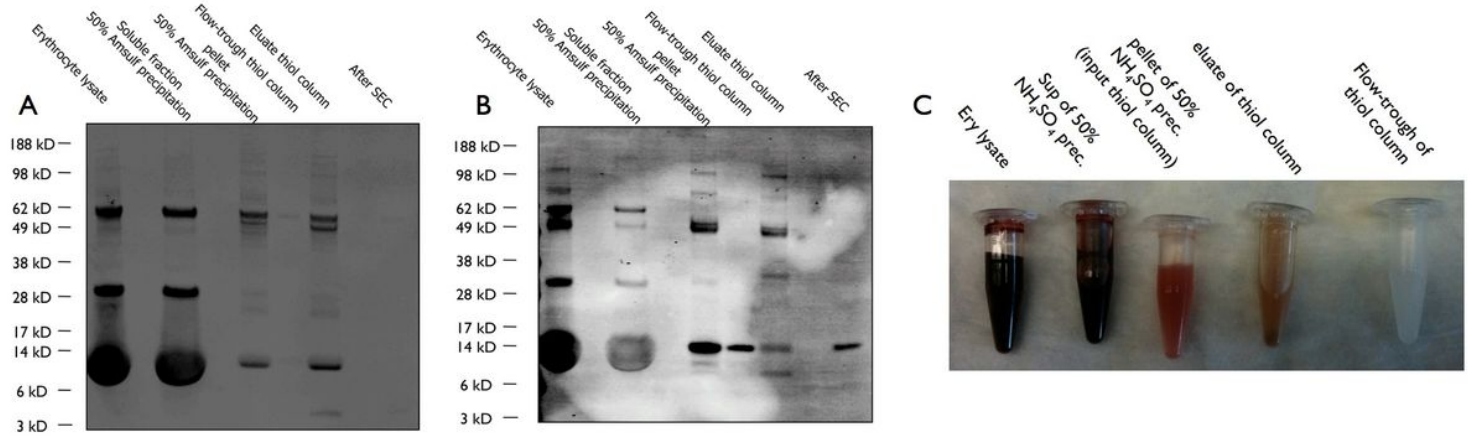

Figure 4

Separation of Hemoglobin and α-Synuclein via covalent chromatography *A*: SDS-PAGE Western blot analysis of different aliquots of purification scheme utilizing a Sepharose 6B column. Antibody anti-Hemoglobin *B*: Same Western blot as in A, but probed with C20 for α-Synuclein. *C*: Photography of

different aliquots of Sepharose 6B purification scheme visualizing the stepwise removal of Hemoglobin from the sample.

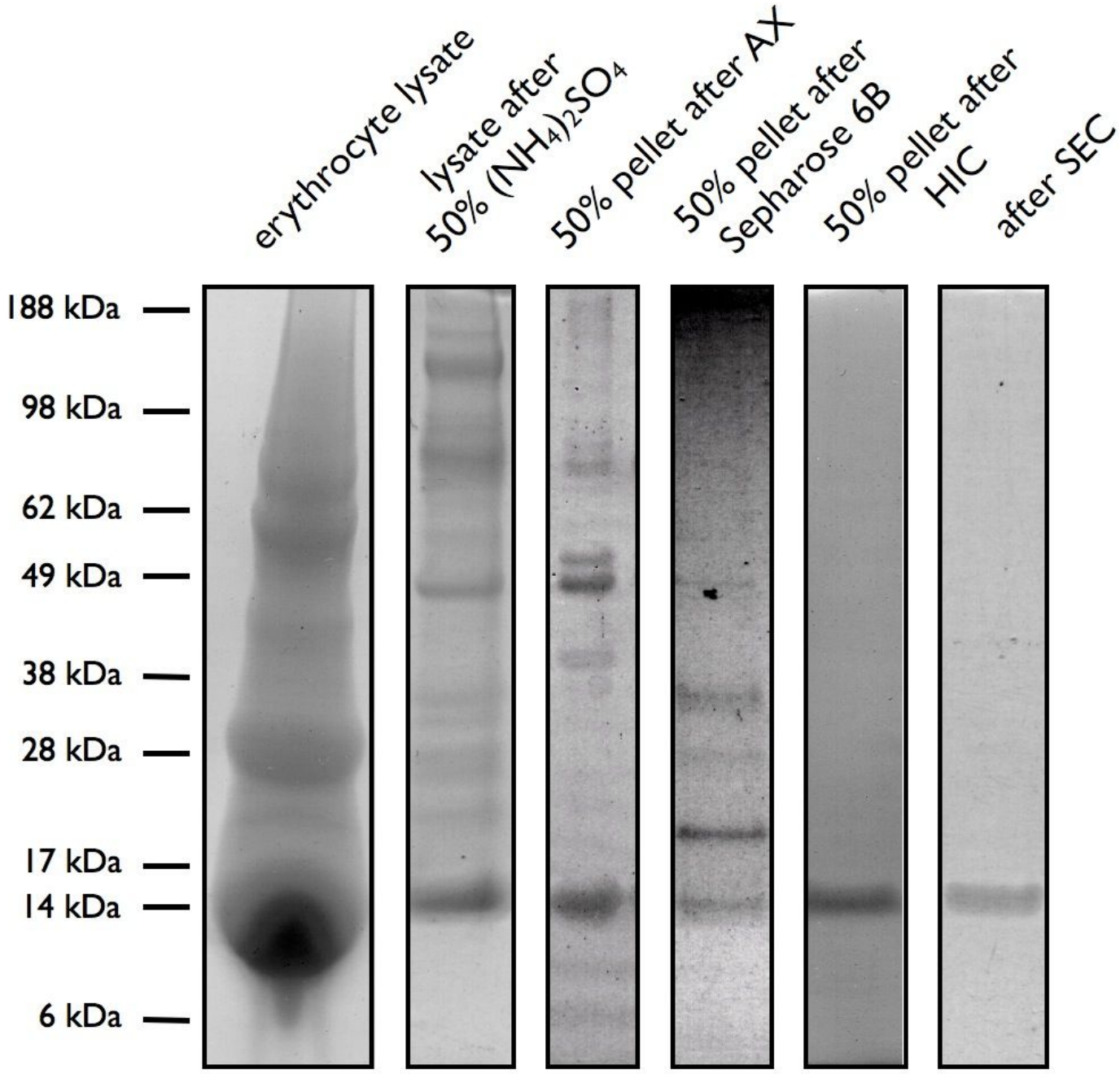

Figure 5

Coomassie stain of different purification steps SDS-PAGE Coomassie stain analysis of aliquots taken from different stages of purification.