PROTOCOL FOR THE PURIFICATION OF RECOMBINANT Rhesus MIP-1α, MIP-1β and RANTES

MIP-1α, MIP-1β and RANTES are members of the CC family of chemokines. They are produced by T cells (MIP-1α, MIP-1β, RANTES), B cells (MIP-1α, MIP-1β), macrophages (MIP-1α, MIP-1β, RANTES), Langerhans cells (MIP-1α) and neutrophils (MIP-1α)[1-4] MIP-1α is an inhibitor of stem cell proliferation and chemoattractant for B cells, eosinophils and killer T cells. MIP-1β has stimulatory effects on myelopoietic cell growth and exhibits leukocyte chemoattractant activity. RANTES has chemotactic activity for monocytes, eosinophils and memory T helper cells, activates eosinophils and causes the release of histamin from basophils. The size of the expressed proteins is approximately 8 kD. More recently, MIP-1α, MIP-1β and RANTES have been shown to inhibit HIV-1 replication in vitro[5].

Cloning of mature MIP-1α, MIP-1β and RANTES .

Mature chemokines were cloned by amplification of rhesus macaque PBMC cDNA with primers designed based on human chemokines. The amplification product was cloned first into pGEM and then into the bacterial expression system pET32a (Novagen, Madison WI) in frame with an amino terminal hexahistidine tag, S-Tag and Trx tag. This construct was introduced into AD494 (DE3) pLysS E coli host for optimal expression.

Production and purification of recombinant MIP-1α, MIP-1β and RANTES .

The following protocol is intended for 100 ml of bacterial culture and should result in the obtention of up to 5 mg of protein using the protein purification method under denaturing conditions on the His-bind resin or S-Protein agarose.

1. Induction of protein synthesis:

a) From the glycerol stock of AD494 (DE3) pLysS containing the chemokine pET32a construct, streak an agar plate that contains 100µg/ml of ampicillin, 30µg/ml of kanamycin and 30µg/ml of

chloramphenicol and incubate overnight at 37°C. This plate can then be stored at 4°C for up to 2 months as a source of single bacterial colonies. From the plate pick a single colony and inoculate 2 ml of Luria Bertani (LB) broth containing the 100µg/ml of ampicillin, 30µg/ml of kanamycin and 30µg/ml of chloramphenicol and incubate at 37°C with shaking for 6-8 hours.
Then either store the culture at 4°C overnight or proceed by centrifugation of the bacteria for 5 min. at 5000 g.
Discard the supernatant and eventual toxins and resuspend the pelleted bacteria in 100 ml LB broth containing the 100µg/ml of ampicilin, 30µg/ml of kanamycin and 30µg/ml of chloramphenicol in a 500 ml Erlenmeyer flask.
Incubate with shaking at 37°C until the optical density $(OD)_{600}$ of the culture reaches 0.4-1.0 (0.6 recommended, after approximately 3-4 hrs.)

b). Add 1.0 ml of isopropyl β-D-thiogalacto-pyranoside (IPTG) from a 100 mM stock to a final Concentration of 1 mM and continue the incubation for 3 hrs at 30°C to induce protein production.

c). Place the flasks on ice for 5 min. and harvest the bacteria by centrifugation at 7000 g for 15 min at 4°C. Discard the supernatant and store the pelleted bacteria at -70°C.

2. Preparation of the His.Bind resin and column packing.

Resins containing activated Nickel cations can be obtained from a variety of vendors for the single step purification of proteins bearing a stretch of histidine residues. The protocol described below applies to the His.Bind resin from Novagen, similar reagents from other suppliers differ little in their protocol and efficacy.

a). Dilute the supplied stocks of Charge Buffer, Binding Buffer, Wash Buffer and Elution Buffer to 1x with sterile enterotoxin free water before use.

b). Gently mix the bottle containing His.Bind resin by inversion and transfer approximately 6 ml of slurry to a column (e.g. Sigma liquid chromatography column 2.5x10cm Cat#C4669). Allow the resin to

pack by gravity flow, this should result in an approximate volume of 2 ml of packed resin.

c). When the level of storage buffer drops to the top of the column bed use the following sequence of washes to charge and equilibrate the column with:

3 volumes sterile deionized water
5 volumes 1x charge buffer
3 volumes 1x binding buffer

3. Purification of recombinant chemokines on His-bind resin.

Purification under denaturing conditions is recomended, since in our experience chemokines were poorly released from bacteria under native conditions.

a). For purification under denaturing conditions, urea was added to a final 6, 4 and 2 M concentrations to the His.bind 1xbinding buffer, with verification of the pH to be 7.9.
Prior to loading the denatured protein on the resin, the column was equilibrated with 4 volumes of binding buffer containing 6 M urea.

b). The pelleted bacteria are resuspended in 10 ml ice cold binding buffer without urea, and are sonicated on ice for 3 twenty seconds periods. The paramaters for sonication largely depend on the type of sonicator probe and size and shape of the vessel holding the cells. As a rule of thumb, the sonication has to be pursued until the samples are no longer viscous to pass through the column.

c). Centrifuge the lysate at 20,000 g for 15 min. to collect the inclusion bodies.

d). Discard the supernatant and resuspend the pellet in 10 ml binding buffer containing 6 M urea and incubate on ice for 1 hour to completely dissolve the protein.

e). Centrifuge at 39,000xg for 20 min.

f). Collect and filter the supernatant through a 0.45 micron membrane and load the prepared extract on the His-bind column preequilibrated with 1x binding buffer containing 6 M urea.

g). Maintaining a flow rate of 10 column volume/hr, wash the column with 6 ml (3 vol.) of 1x binding buffer containing 6 M urea, then with 6 ml (3 vol.) of 1x binding buffer with 4 M urea followed by 6 ml (3 vol.) of 1x binding buffer with 2 M urea and a final 6 ml (3 vol.) of 1x binding buffer without urea.

h). Wash with 6 ml (3 vol.) of 1x wash buffer.

i). Elute protein with 6 ml (3 vol.) of 1x elution buffer containing 1M imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 7.9.

j). Exchange the buffer and concentrate purified recombinant chemokines by ultrafiltration/ centrifugation over a Centricon-10 system (Amicon, 10 kDa cut-off) by concentrating to approximately 0.5 ml, refilling with the desired buffer and concentrating again. Usually 2 refills are sufficient to exchange the buffer .

k). Digest with the enterokinase. Proteins should not be very concentrated, otherwise they tend to precipitate. Use about 7-10 units of recombinant enterokinase (rEK)/preparation and digest for about 8-16 hours at room temperature. The amino terminus containing tags is then removed by one passage over the His.Bind resin column or S-protein agarose and the eluate is further purified from contaminant rEK by one passage over a rEK capture agarose (Novagen). If desired, the purified protein can be concentrated by ultrafiltration (Amicon, 3 kDa cut-off).

l). Quantitate the amount of protein using a commercially available assay and assess the purity of chemokines on SDS-PAGE (15%). The protein should appear as a main band at approximately 8 kDA. It may be adviseable at this point to lyophilize the protein in aliquots for long-term storage of biologically active recombinant chemokines.

m). As with most proteins produced in bacterial systems, each preparation should be tested for the presence of endotoxin by the

limulus amoebocyte lysate assay prior to use in cell culture or administration in vivo.

4. Preparation of the S-protein agarose and column packing.

S-Protein agarose does not require special preparation. 50% slurry of the agarose could be packed directly into column or used in the batch-wise preparation of the desired protein. Generally 2 ml final volume of packed agarose (4 ml of 50% slurry) is sufficient for the purification of the protein from 100 ml of bacteria.

5. Purification of recombinant chemokines on S-protein agarose.

a). Release the proteins from bacteria as in 4)a-e, use 1x bind/wash buffer for S-tag purification instead of 1xbind buffer for His-tag resin purification. Collect supernatant from the final spin, filter through the 0.45 micron membrane and dilute 3 times with 1x bind/wash buffer without urea to get the final concentration of 2 M urea in the sample.

b). Load twice on the S-tag agarose and wash with 10 volumes of 1x bind/wash buffer without urea.

c). Elute protein with 2.5 volumes of 1x wash/bind buffer with 2 M guanidium thiocyanate.

d). Concentrate protein and exchange the buffer as in 3)j.

e). Further process the protein as described in 3)k-m.

Addendum

The His.Bind columns are easily regenerated by eluting the bound histidine tags using 3 volumes of strip buffer and stored at 4°C with PBS with 0.01% sodium azide.

The S-protein agarose could be regenerated by washing with 1xbind/wash buffer without Triton X-100 and stored at 4°C with 1xbind/wash buffer with 0.01% sodium azide.

Testing of biological activity can be done by several methods:

MIP-1α: inhibition of the proliferation of hematopoietic stem cells in a CFU-A assay, chemotaxis for eosinophils
MIP-1β: chemotaxis for memory T cells
RANTES: chemotaxis of monocytes, memory T cells, eosinophils

References

1. The Cytokine Facts Book. R Gallard and A Gearing. Academic Press 1994.
2. Properties of the novel proinflamatory supergene "intercrine" cytokine family. JJ Oppenheim *et al.* Annu. Rev. Immunol. 9:617 (1991).
3. A human T cell-specific molecule is a member of a new gene family. TJ Schall *et al.* J.Immunol 141:1018 (1988).
4. Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors. PF Zipfel *et al.* J.Immunol 142:1582 (1989).
5. Identification of RANTES, MIP-1α and MIP-1β as the major HIV-suppresive factors produced by CD8+ T cells. F Cocchi *et al.* Science 270:1811 (1995).

Sequences of MIP-1α, MIP-1β and RANTES

Rhesus MIP-1α:
FSASLAADTPTACCFSYTSRQIPQNFIADYLETSSQCSKPGVIFLTKRSRQVCADPSEE
WVQKYVSDLELSA*

Rhesus MIP-1β:
SAPMGSDPPTSCCFSYTVRKLPRNFVVDYYETSSLCSQPAVVFQTKRGKQVCADPSET
WVQEYVNDLELN*

Rhesus RANTES:
SPYSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWV
REYINSLEMS*

Note: Rhesus and mangabey mature MIP-1α and RANTES proteins are identical to human MIP-1α and RANTES.