Isolation of mMMP-3 CD/FL from *E. coli* inclusion bodies

I Expression

- Restreak single BL21(DE_3)pLysS + pRSET mM3-CD/FL out on a 2YT-amp (100 μg/ml) / chloramphenicol (34 μg/ml in 100% EtOH) plate. Let grow O/N at 37°C.

- Next morning inoculate 4 x 25 ml 2YT amp+Chloramphenicol cultures with two toothpicks worth of *E.coli*: let grow for ± $2^1/_2$ hrs at 37°C. Meanwhile, prepare 6 x 2L flasks with 500 ml 2YT-amp (100 μg/ml) / chloramphenicol (34 μg/ml in 100% EtOH): pre-shake at 37°C, so that the *E.coli* will not slow down their growth.

- Combine the 4 x 25 ml cultures and use 12.5 ml to inoculate each 2L flask with 500 ml medium. Let grow at 37°C until $O.D._{550}$ ~ 0.6 (cells will virtually stop growing once induced with IPTG).

- Induce mM3-CD/FL expression with 0.8 mM final concentration of IPTG (= 500 μl 0.8M IPTG stock). Switch the temperature to 30°C and induce for 4 hrs.

- Afterwards collect cells by centrifugation: 6,000 x *g* for 10 min. Resuspend each 500 ml pellet in 5 ml 40 mM Tris/HCl pH 8.0. Freeze cell slurry away at -80°C until further processing.

II Cell disruption + Isolation of inclusion bodies + protein extraction

- Thaw the ± 7.5 ml cell slurry and add:
  - 2 mM final concentration EDTA (30 μl 500 mM pH 8.0)
  - 1 mM final concentration PMSF (38 μl 200 mM in EtOh)
  - 100 μg/ml lysozyme (75 μl 10 mg/ml stock)
  - 0.1% Triton X-100 (150 μl 5% in 40 mM Tris/HCl pH 8)

- Rotate cell slurry at 37°C for 30 min (solution will become highly viscous). Stick on ice for 5 min and sonicate until solution is completely liquid.

- Sediment the recombinant protein as insoluble inclusion bodies by centrifugation: 12,000 x *g* for 15 min at 4°C (keep pellet, discard SN).

- Wash inclusion bodies 5 times with 5 ml inclusion body wash buffer to remove cellular proteins: each time vortex and/or sonicate to thoroughly resuspend the pellet. Recover the inclusion bodies by centrifugation: 12,000 x *g* for 10 min at 4°C. Include a final wash with just H_2O.

Inclusion body wash buffer: 50 mM Tris/HCl pH 8.0
10 mM EDTA

200 mM NaCl
0.25% Triton X-100

- Solubilize the recombinant protein in solubilization buffer: expect ± 10 mg/L culture and we want ± 2 mg/ml final concentration. Resuspend the pellet by vortexing and/or sonicating: rotate for at least 1 hr at RT.

Inclusion body solubilization buffer: 50 mM Tris/HCl pH 8.0
200 mM NaCl
8 M Urea
5 mM Imidazole

- Prepare a SDS-PAGE gel of the appriate % to estimate protein yield (see below).

- Remove any non-solubilized material by centrifugation: 12,000 x *g* for 15 min at 4°C. Add $^1/_3$ volume of 50 mM Tris/HCl pH 8.0, 200 mM NaCl, 5 mM Imidazole to reduce the urea concentration of the protein extraction SN to 6M. Run some of the protein extract out on SDS-PAGE together with known amounts of BSA to estimate the protein concentration. The sample can be stored at –80°C until further processing.

III Protein Purification

Ni-NTA from Invitrogen comes as a 50% solution in 30% EtOH, and can bind 5-10 mg of protein per ml of resin: calculate how much Ni-slurry you need. The following protocol is for a 5 ml bed volume column (10 ml of Ni-slurry) / batch-wise purification: do not allow the resin to dry at any point!

- Equilibrate the nickel-sepharose: (each wash is for 5 min)
  - 3 x 25 ml Milli-Q washes
  - 3 x 25 ml $NiCl_2$ (5 mg/ml) washes
  - 3 x 25 ml denaturing binding buffer

Denaturing binding buffer: 50 mM Tris/HCl pH 8.0
200 mM NaCl
6 M Urea
5 mM Imidazole

- Incubate the nickel-sepharose with the 6 M urea protein extract for 1 hr at RT by rotary shaking (take a 100 µl sample before start: **START**). Afterwards, let the nickel-sepharose settle by gravity and remove SN + store at – 80°C (take a 100 µl sample: **FLOW THROUGH**).

- Wash column 3 x with 25 ml denaturing wash buffer; each wash is for 5 min (take a 100 µl sample of each wash: **WASH I**, **II** and **III**).

Denaturing wash buffer: 50 mM Tris/HCl pH 8.0
200 mM NaCl
6 M Urea
20 mM Imidazole

- Elute bound protein with 25 ml of denaturing elution buffer (take a 100 μl sample: **ELUATE**).

Denaturing elution buffer: 50 mM Tris/HCl pH 8.0
100 mM NaCl
6 M Urea
250 mM Imidazole

The same resin can be used three to five times before EDTA stripping and nickel recharging are necessary (see 10.11.17 of Current Protocols in Molecular Biology). If resin is to be stored for longer than 1 day, wash with 10 bed volumes 20% EtOH and add 2 bed volumes 20% EtOH (make sure column is sealed to prevent evaporation). Only one protein should be purified on any given column.

- Concentrate eluate with a 3 kD cut-off Amicon concentrator to a final volume of ± 5 ml.
Dialyse the protein O/N at 4°C in 2L of Denaturing dialysis buffer (50 mM Tris/HCl pH 8.0, 100 mM NaCl, 6 M Urea) to get rid of the imidazole.

- Prepare SDS-PAGE gel and assess protein recovery by staining with Coomassie Blue (include BSA standard to determine final yield). Alternatively, you can also estimate from $O.D._{280}$ = 1, representing 1 mg/ml solution.

IV Protein refolding

I will assume a final yield of 30 mg protein (3L of culture) in 5 ml = 6 mg/ml!

- Rapidly dilute by drop wise addition to a final protein concentration of 100 μg/ml in 295 ml Refolding buffer I: 50 mM Tris/HCl pH 8.0
100 mM NaCl
1 M Urea
5 mM $CaCl_2$
1 μM $ZnCl_2$
Add the purified protein slowly from a pipet while stirring the refolding buffer to ensure thorough mixing and continue to stir at RT for 2 hrs. Store the refolding mixture at 4°C O/N without stirring.

- Concentrate refolding mixture with Vivacell 70 ml concentrators (5 kD cut-off) to ± 5ml: afterwards clean concentrators + store in 20% EtOH so that they can be re-used. Rapidly dilute concentrate by drop wise addition to a final protein concentration of 100 μg/ml in 295 ml Refolding buffer II:

50 mM Tris/HCl pH 8.0
100 mM NaCl
5 mM $CaCl_2$
1 μM $ZnCl_2$

Add the purified protein slowly from a pipet while stirring the refolding buffer to ensure thorough mixing and continue to stir at RT for 2 hrs. Store the refolding mixture at 4°C O/N without stirring.

- Concentrate refolding mixture with Vivacell 70 ml concentrators (5 kD cut-off) to ± 5ml. Dialyse the refolding mixture O/N at 4°C against 2L of Refolding buffer II to completely get rid of the urea.

- Perform a protein assay to determine the final protein concentration. Perform a α-casein zymogram and an enzyme assay to determine the specific activity (also run some out on a regular SDS-PAGE). Freeze 100 μl aliquots at -80°C.

! THE ABOVE REFOLDING PROTOCOL LEADS TO AUTO-DEGRADATION OF THE FULL LENGTH (CLEAVES OF ITS PRO- AND HEMOPEXIN DOMAINS) AND THE CATALYTIC DOMAIN (CLEAVES OF ITS PRO-DOMAIN): REPEAT IN THE PRESENCE OF EITHER A GENERAL MMP INHIBITOR SUCH AS 10 μM 1,10-PHENANTROLINE OR AN INHIBITING ZINC CONCENTRATION OF 5 Mm $ZnCl_2$. REMOVE INHIBITORS BY DIALYSIS ONCE REFOLDING IS COMPLETE!