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Protocol can be downloaded from: http://www.biochemistry.ucla.edu/biochem/Faculty/Hubbell/

Purification procedure for rat intestinal fatty acid binding protein

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Materials:

- Lysis Buffer: 50mM Tris-Cl pH 8.0, 1mM EDTA, 0.05% NaN_3
- Saturated Solution of Ammonium Sulfate $[(NH_4)_2SO_4]$ > 4.1M
- Low Salt Buffer for Ion Exchange: 20mM K-phosphate pH 7.3, 1mM EDTA, 5mM DTT
- High Salt Buffer for Ion Exchange: Low Salt Buffer + 1M NaCl
- Delipidation Buffer: 20mM K-phosphate buffer pH 7.3, 1mM EDTA, 0.05% NaN_3
- Lipidex-1000 (type VI)
- Reconstitution buffer: 100mM PIPES pH 7.3

Procedure:

1. Resuspend cell pellets in lysis buffer (4ml/g cell) and freeze at -20˚C. In order to thaw the cell suspension, place tube in the 37˚C water bath for ~15 minutes.
2. Place lysate on ice and then sonicate for 5 minutes (output control 50%, duty cycle 4), then keep in ice for 5 minutes, and repeat sonication.
3. Spin for 20 minutes at 15,000 rpm in the SS 34 rotor.
4. **Ammonium Sulfate Precipitation**: Measure volume of supernatant. *The following steps must be done on ice*. In order to bring the crude extract to 60% saturation, add a volume of 15ml of saturated ammonium sulfate solution per 10ml of supernatant dropwise while stirring. Incubate on ice for 30 minutes, then centrifuge for 20 minutes at 15,000 rpm. Keep the pellet and measure the volume of the supernatant. (rIFABP should be in the supernatant).
5. In order to bring the saturation point to 95%, add 261g/L of solid ammonium sulfate to the supernatant. Stir on ice and incubate at 4˚C overnight. Centrifuge the solution for 20 minutes at 15,000 rpm. rIFABP should be in the pellet.
6. Resuspend the pellet in low salt buffer. Dialyze overnight in the cold room against 4L of low salt buffer.
7. **Ion Exchange Chromatography**: Use a HiTrap SP HP (cation) and Q FF column (anion) in tandem. The pI of rIFABP is 7.3, so the protein will pass in the flowthrough. Filter the protein solution and inject. Elute contaminants with high salt buffer right after the flow through fraction has been collected. Assess protein purity *via* SDS-PAGE.
8. **Desalting and Spin labeling:** Prepared a stock solution of 200mM of MTSL. Check protein concentration. Run the rIFABP fraction through a HiTrap desalting column. Collect the protein peak in a tube containing 10-fold molar excess of MTSL and incubate overnight.
9. **Gel filtration**: Wash and equilibrate the gel filtration column (Superdex 75). Either filter the spin labeled protein or centrifuge the sample for 10 minutes at 10,000 rpm in order to remove any precipitated protein/particulates. Concentrate the protein to 250µl and inject. (This step will isolate rIFABP from other contaminants that will be present and from excess spin label reagent).
10. **Delipidation**: Lipidex-1000 is stored in 20% ethanol or delipidation buffer containing NaN_3. Wash the Lipidex-1000 beads with several CV of filtered water and then wash with several CV of delipidation buffer. Glatz et al. (1983) reported that the maximum binding capacity of Lipidex-1000 for palmitate at 37˚C is 12.8µg/ml packed gel. That corresponds to about 0.8mg of rIFABP/ml of pack gel. In order to remove bound endogenous fatty acids, add rIFABP to the

Lipidex-1000 and nutate for 1 hour in the 37˚C incubator. After the incubation, flow delipidation buffer over the column to collect the rIFABP fraction. Wash with a couple of column volumes of delipidation buffer. Concentrate the protein fraction to a final concentration of 400-800μM. This is the apo rIFABP protein. Save an aliquot (~50μl) for CW EPR experiments.

11. In order to remove the fatty acid from the column, wash with several column volumes of methanol, followed by several column volumes of water and finally wash with several column volumes of 20% ethanol or delipidation buffer containing NaN_3 and store.
12. **Reconstitution**: Exchange K-phosphate buffer for 100mM PIPES pH 7.3 and concentrate to 1mM. Prepare a stock solution of 20mM myristate in 0.1M KOH. Preheat water bath at 37˚C. Add 2-fold molar excess of fatty acid to rIFABP and incubate for ~20 minutes at 37˚C. Concentrate the holo protein to a final concentration of 400-800μM for EPR measurements.

$$\varepsilon_{280nm} = 16{,}900\ M^{-1}\ cm^{-1}$$

MW = 15,124 Da

1AU = 0.89mg/ml protein

Reference:

1 López, C. J., Fleissner, M. R., Guo, Z., Kusnetzow, A. K. & Hubbell, W. L. Osmolyte perturbation reveals conformational equilibria in spin-labeled proteins. *Protein science : a publication of the Protein Society* **18**, 1637-1652, doi:10.1002/pro.180 (2009).