Isolating proteins for mass spectrometry analysis

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Reasons to purify proteins

- To characterize them
  - e.g., for their enzyme activity, chaperone function
- To determine structure
  - How they assemble in complexes
- To make antibodies
  - For immunoaffinity purification
- Their pharmaceutical properties

Purification fundamentals

- How much do we need and should it be pure?
- Do we want it in the native form?
  - Many of the recombinant proteins are misfolded
- How are we going to measure it during the purification steps?
  - Enzyme or other biochemical assay
  - Using an antibody
  - Using SDS-PAGE gel

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Where do we get the protein from?

- Used to be from tissue or body fluids
  - Some proteins are from animal parts obtained at slaughter houses
  - These can include liver, brain, kidneys
  - Some proteins are richly expressed in one of these tissues, e.g., tau in brain
- Since 1980, recombinant expression in bacteria and other systems have provided replenishable and unlimited amounts
  - But the proteins still have to be purified

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Limitations of recombinant expression

- Poor folding in bacteria
  - Large amount of proteins in inclusion bodies
  - Possible to coincidentally overexpress chaperone proteins
- Most of the posttranslational mechanisms in eukaryotic systems do not occur in bacteria
  - Phosphorylation on a serine can be simulated by mutating the serine to aspartate

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Assists using molecular biology approaches

- It is possible to add tags that assist in recovering the protein
  - 6xHis, HAT, maltose-binding protein, glutathione S-transferase
  - Biotinylation site
- But from a structural/functional point of view, is that a disadvantage?
  - Alterations in protein structure caused by the tag
  - Back to protein purification

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What to consider

- Choice of biological source
- How to maximize the tissue recovery
- How to monitor the protein
- How to develop a purification strategy
- Techniques to be used
- How to integrate the techniques

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Harvesting the protein

- Remember that what ever you do from this point on, you're likely to have remnants of any buffer component in your final purified sample
  - Avoid detergents!!!!!!
  - The reality is that proteins that will be studied by mass spec have to be water soluble
  - Grind tissue in liquid nitrogen to minimize degradation
  - Use BugBuster™ for bacteria

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For tissues choose the correct compartment

- Homogenize the tissue in an isotonic buffer
- Separate by differential centrifugation and with sucrose density gradients
  - Nuclear fraction (x800*g* pellet)
  - Lysosomes/plasma membrane (x10,000*g* pellet)
  - Mitochondria (20-35,000x*g* pellet)
  - Peroxisomes (Opticlear gradient)
  - Endoplasmic reticulum (100,000x*g* pellet)
  - Cytosol (100,000x*g* supernatant)
- For bacteria, the cytosol or the inclusion bodies

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Principles of protein purification

- Proteins should remain at high concentrations
  - Proteins stick to surfaces (*sic*, ELISA assays)
  - Early stages can use large surface areas, but miniaturize the system as the purification proceeds
- Consider the chemical and physical properties of the protein
- Most proteins benefit from being kept cold

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Characteristics of proteins that can be exploited

- Solubility in different solvents
- Balance of charged amino acids (Asp and Glu versus Arg and Lys)
- Molecular weight
- Thermal stability
- Specific binding regions
- Availability of immunoaffinity reagents

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Purification techniques

- $(NH_4)_2SO_4$ precipitation
- Ion exchange (anion and cation)
- Chromatofocusing (isoelectric point)
- Hydroxyapatite
- Hydrophobic interaction chromatography
- Reverse-phase chromatogrphy
- Small molecule affinity chromatography
- Immunoaffinity chromatography
- Gel filtration

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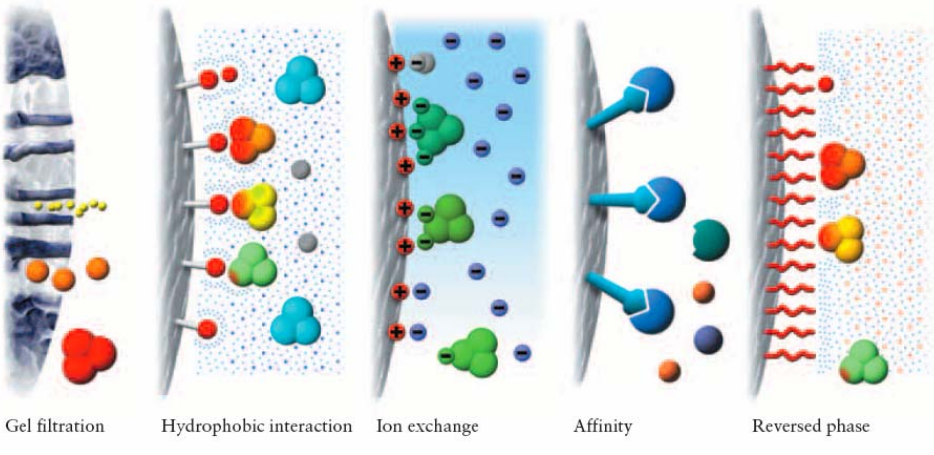
The techniques



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$(NH_4)_2SO_4$ precipitation

- High concentrations of $(NH_4)_2SO_4$ strip water from the hydration sphere of the protein and lead to its reversible precipitation
  - This occurs at different degrees of $(NH_4)_2SO_4$ saturation - for instance, a protein will not be precipitated at 30% saturation, but others will. However, the protein is precipitated at 40% saturation
- But is adding huge amounts of $(NH_4)_2SO_4$ such a good idea - depends on protein amount

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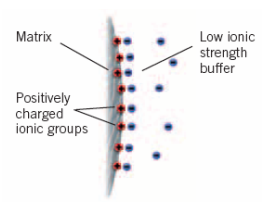
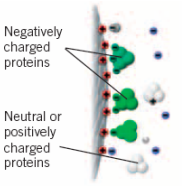
Anion exchange

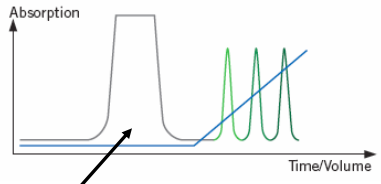


The phase

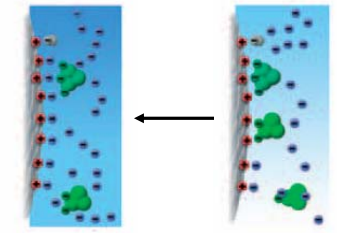
At low ionic strength the -ve charge proteins bind to the +ve beads



Increase the ionic strength with NaCl



Non-bound proteins



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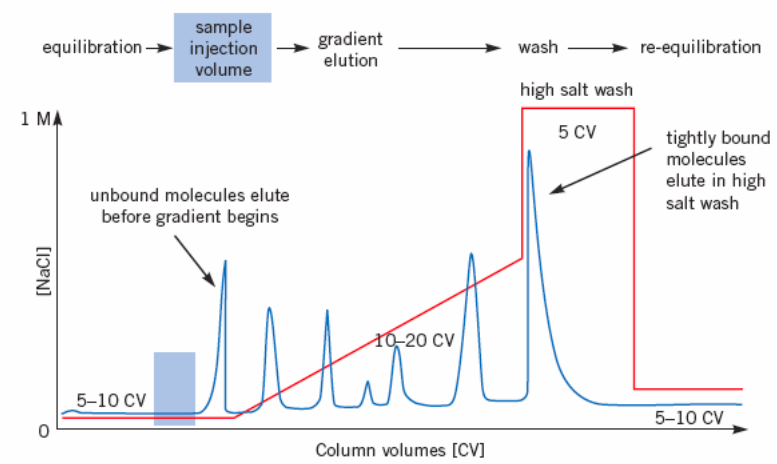
Gradient elution IEX



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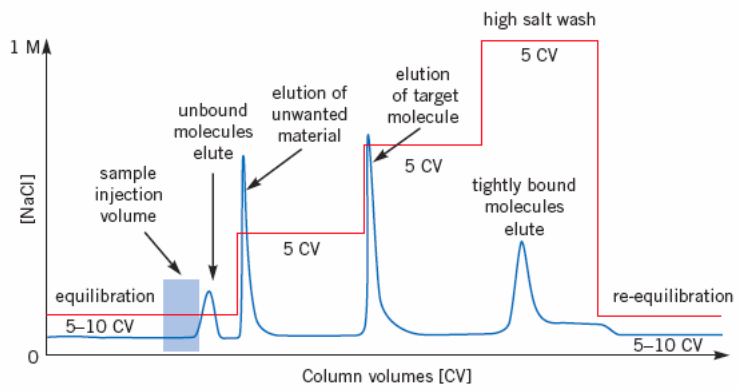
Step elution IEX



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More on anion exchange

- **The initial binding conditions are crucial**
  - **If you know the isoelectric point of the protein, use an equilibration buffer that is at least 1 pH unit higher - that will ensure that most of the protein is negatively charged**
  - **If the protein has a low number of charged amino acids, then use a low ionic strength buffer - and vice versa - a highly charged protein will stick to the anion exchange phase when others won't**

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The nature of ion exchange resins

Anion exchangers		Functional group
Quaternary ammonium (Q)	strong	$-O-CH_2N^+(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak	$-O-CH_2CH_2N^+H(CH_2CH_3)_2$
Diethylaminopropyl (ANX)*	weak	$-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$

Cation exchangers		Functional group
Sulfopropyl (SP)	strong	$-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$
Methyl sulfonate (S)	strong	$-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$
Carboxymethyl (CM)	weak	$-O-CH_2COO^-$

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Summary for ion exchange

- **A high capacity technique**
- **Typically can achieve a 10-fold purification**
- **Very high recovery of protein and its activity/function**
- **Often used to clean up a cytosol mixture prior to an affinity column**
- **But the purified fraction(s) can have high concentrations of salts (NaCl/KCl)**

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Chromatofocusing

- **Some similarity to ion exchange, but based on the balance of charges (isoelectric point), not the absolute amount**
- **The protein is bound to the positively charged phase as its negatively charged ion**
- **Bound proteins are eluted with a <u>linear</u> pH gradient**

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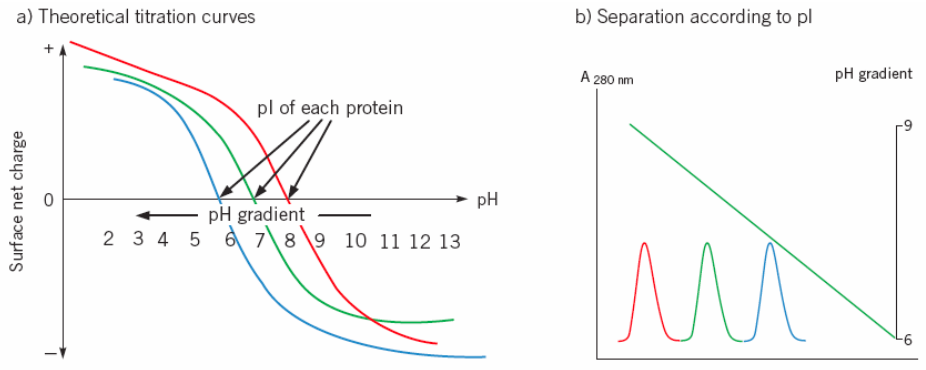
Basis of chromatofocusing



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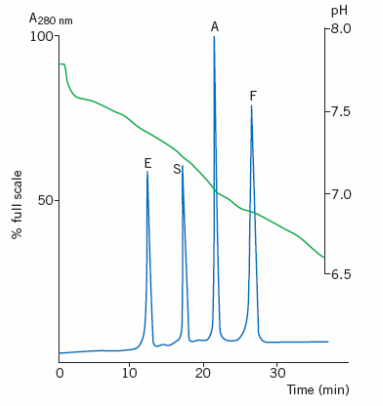
Chromatofocusing example



Variants of hemoglobin are first bound at pH 8.1 to a mono P column

The proteins are eluted by passage of polybuffer 96 with methane sulfonic acid, pH 6.65

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Summary of chromatofocusing

- **Quite high capacity**
- **Best for anionic proteins**
- **Choice of the polybuffer is crucial**
  - **pH ranges 4-7 (polybuffer 74), 6-9 (polybuffer 96), 8-11 (polybuffer 118)**
- **Getting rid of the polybuffer!**
  - **An affinity step next would be very helpful**

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Hydrophobic interaction chromatography

- Phenyl-, butyl- and octyl-Sepharose
- Protein is mixed with $(NH_4)_2SO_4$ at concentrations that are below the precipitation point
- The protein binds via its hydrophobic regions to escape the strong electrolyte environment
- As the $(NH_4)_2SO_4$ concentration is lowered, the hydrophilic parts of the protein dominate and the protein dissociates from the stationary phase

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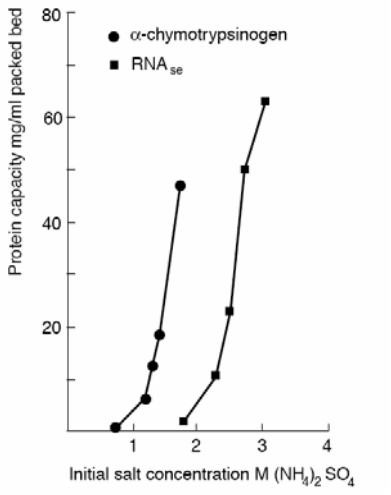
Binding and salt concentration



Binding of proteins to a Phenyl-Sepharose column

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Hydrophobic interaction and reverse-phase columns

- HIC is a high capacity method
  - Has low loading of hydrophobic groups
  - Usually short alkyl groups - C_4, C_8
  - Eluted without organic solvent
- Reverse-phase columns are low capacity, but high resolution
  - Longer alkyl groups - C_4, C_8, C_{18}
  - High loading
  - Elution requires organic solvent

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Hydroxyapatite chromatography

- A mixture of affinity and ion exchange
- Hydroxyapatite is a calcium phosphate insoluble matrix, often embedded in an agarose matrix
- Very sensitive to pH (pH 6.5-12)
- Elution occurs with a gradient of phosphate buffer (200-500 mM)
  - Use potassium salts - greater solubility in cold

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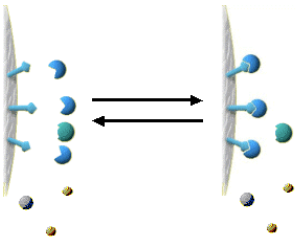
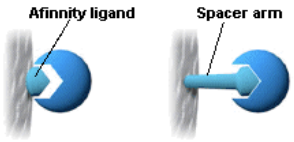
Affinity Chromatography



Very selective absorption of the protein based on local structure



The affinity group is often put on the end of a hook created by adding a spacer arm

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Affinity ligands

- The ligands can be small molecules (often inhibitors of an enzyme)
- Or antibodies
  - The affinities for the binding must >10^{-4} M, but not too high
  - Monoclonal antibodies are better than polyclonal antibodies
- Elution can occur with alterations of pH or ionic strength, or with specific elutants

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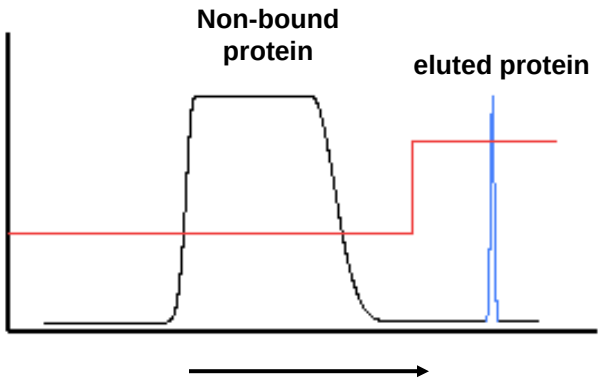
The ideal result



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Gel filtration

- Based on hydrodynamic radii of protein
  - See video at http://www1.amershambiosciences.com/aptrix/upp00919.nsf/4a0f132842ea4d354a25685d0011fa04/50c849d0d5b16ba0c1256e92003e865b/$FILE/GE_Gel%20Filtration.swf
- It always dilutes the sample whether conventional or HPLC forms are used
- The smaller molecules are retained by the phase - they tarry inside the spaces in the phase
- The large molecules are excluded from the phase and elute fast

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A purification table: important part of purifying a protein

- The goal is to obtain enough protein with highest possible activity and the greatest purity

Step	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min/mg	Fold purification
Homogenate	100	1000	0.1	1.0
Cytosol	90	600	0.15	1.5
DEAE column	80	80	1.0	10
Affinity column	75	2.0	37.5	375

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Examples from Barnes' lab

- Rat liver bile acid PAPS sulfotransferase
- Human bile acid CoA:amino acid N-acyltransferase (hBAT)
- Rat liver bile acid CoA ligase
- Recombinant hBAT expressed in *E. coli*

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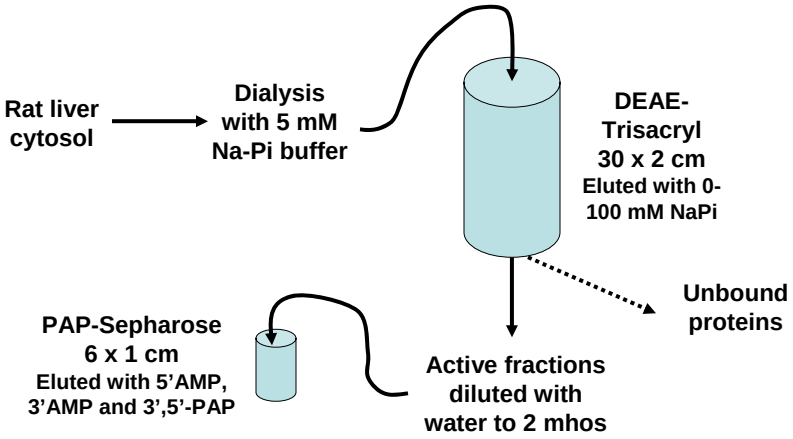
Bile acid PAPS sulfotransferase



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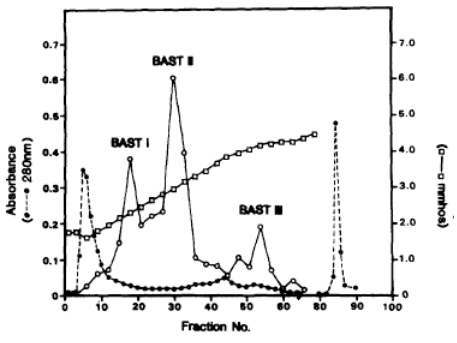
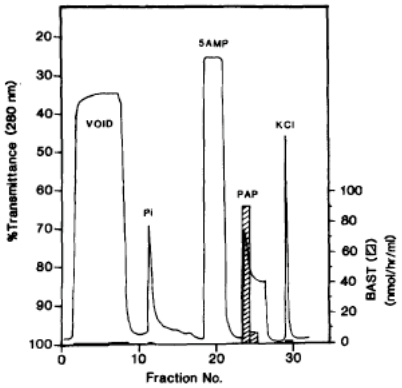
Purifying BAST



Anion exchange



PAP Affinity

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BAST purification data

Fraction	Protein (mg)	Activity (nmol/min)	Spec Act (nmol/min/ mg)	Fold purification
Cytosol	1206	144	0.119	1.00
DEAE peak	50	48	0.96	8.1
PAP-purified	0.385	7.2	18.7	157

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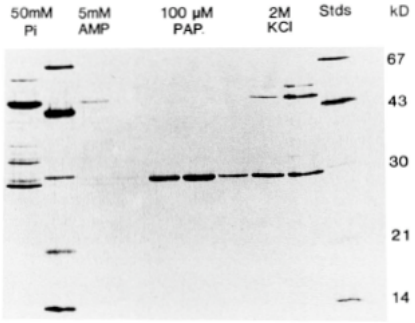
Purified BAST



Fractions from PAP-Sepharose affinity absorption and elution

N-terminal sequence:
pdytwfegipfpafgisketlqdv

pdytwfegipfpafgipketlqnv
Determined by cDNA cloning

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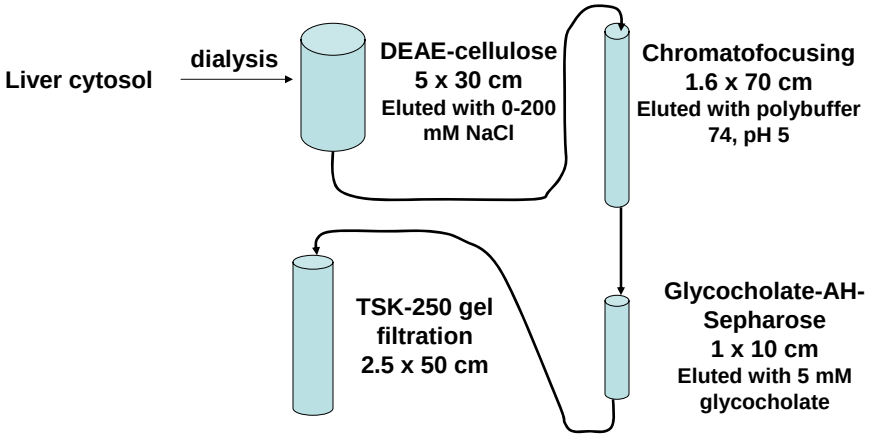
Human bile acid CoA:amino acid N-acyltransferase (hBAT)



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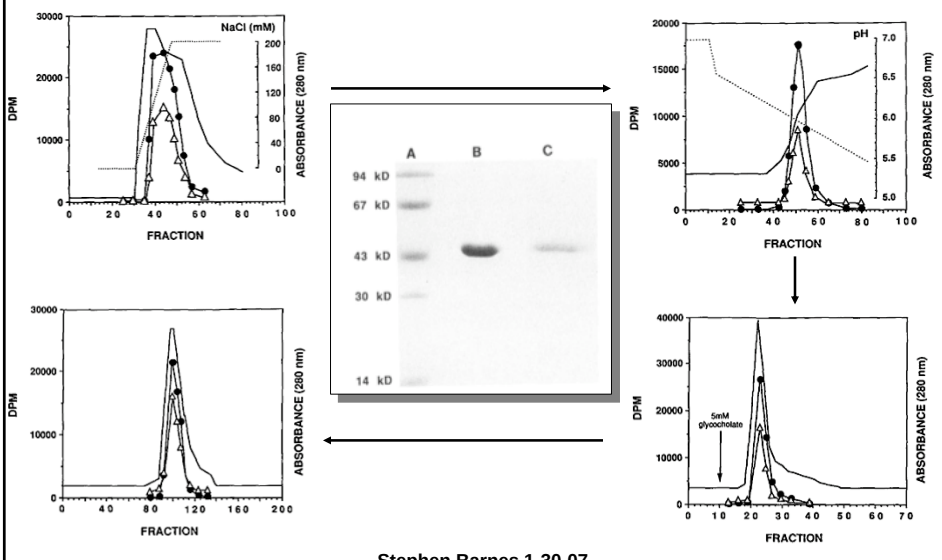
Purification of hBAT



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hBAT purification table

Fraction	Protein (mg)	Activity (nmol/min)	Spec Act (nmol/min/ mg)	Recovery (%)	Fold
Cytosol	18,000	1,200	0.067	100	1.0
DEAE-cellulose	1,764	987	0.56	82	8.4
Chromatofocusing	52	271	5.22	22	78.0
GC-Sepharose					
Gel flitration	7.7	246.7	31.87	20	475.7

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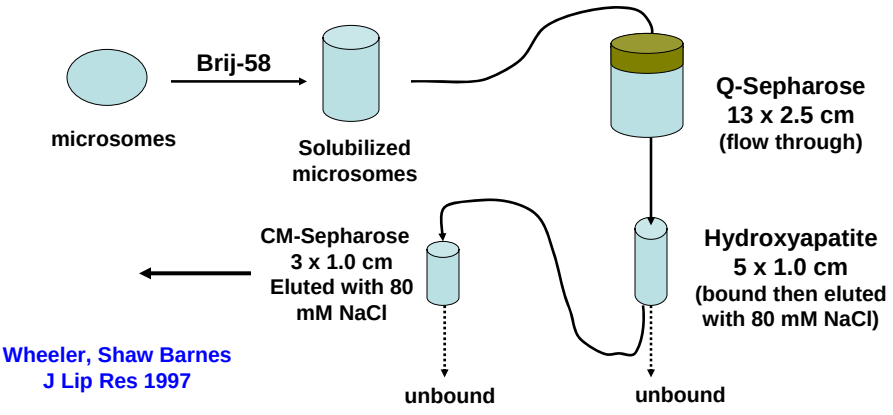
Purifying rat liver bile acid CoA ligase



Wheeler, Shaw Barnes
J Lip Res 1997

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Bile acid CoA ligase

Fraction	Protein (mg)	Activity (nmol/min)	Sp. Act. nmol/min/ mg	Yield (%)	Fold purified
microsomes	176	341.4	1.94	100	1.00
Sol microsomes	176	1010.2	5.74	295	2.96
Q-Sepharose pool	44	545.6	12.4	150	6.39
Hydroxyapatite	0.74	72.67	98.2	21	50.6
CM-Sepharose	0.055	21.18	385.0	6.2	198.4

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Affinity purification of rBAL

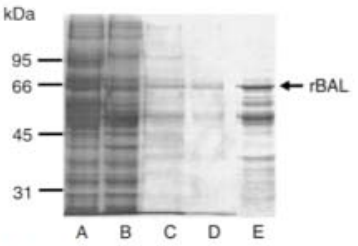
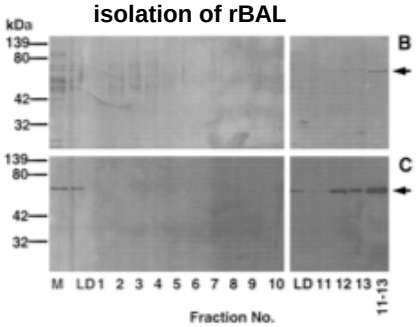


FIG. 1. SDS–PAGE analysis of reduced, denatured proteins in fractions from the purification procedure. Equal amounts of rBAL activity were loaded in lanes A–D. (A) Solubilized microsomes, (B) Q-Sepharose chromatography pool, (C) hydroxyapatite pool, (D) CM-Sepharose pool, (E) CM-Sepharose pool overloaded (rBAL calculated as being 33% of total protein loaded).

Generation of hybridomas and selection of anti-rBAL clone - attachment to IgG-coupled agarose beads - followed by affinity isolation of rBAL



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Recombinant hBAT

6xHis-tag BAT was expressed in *E. coli* but when the cytosol was passed over a Ni-affinity column, the imidazole eluate gave rise to 23 kDa and 56 kDa bands in addition to the 50 kDa hBAT band. p23 was shown to be peptidyl prolyl cis-trans isomerase, a protein with 14 His residues in a 30 residue C-terminal region. p56 is a bacterial GRoEL chaperone.

Mindan Sfakianos

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hBAT-avi

The recombinant *E. coli* contains both hBAT-avi and a biotin ligase. Once hBAT-avi is overexpressed, biotin (5 mM) is added to label hBAT. The biotinylated hBAT-avi is recovered by use of soft avidin resin affinity phase. The bound hBAT-avi is eluted with biotin. This generates two proteins, p50 and p56. Once again, the p56 protein is a GRoEL chaperone. We removed it by pre-incubating the cytosol with an ATP generating system.

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Erin Shonsey

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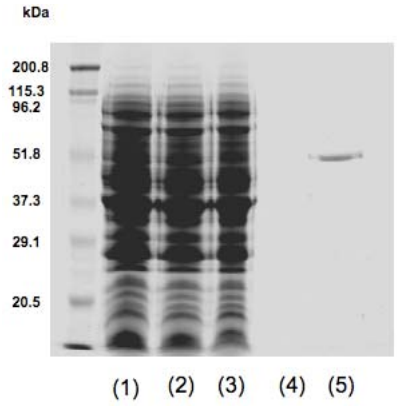
Pure hBAT-avi



(1) is the bacterial cytosol, (2) the DEAE column flow through, (3) is the avidin column flow through, (4) is the avidin column wash, and (5) is the biotin elution fraction from the avidin column. We optimized the recovery to over 80% in just two steps.

Erin Shonsey

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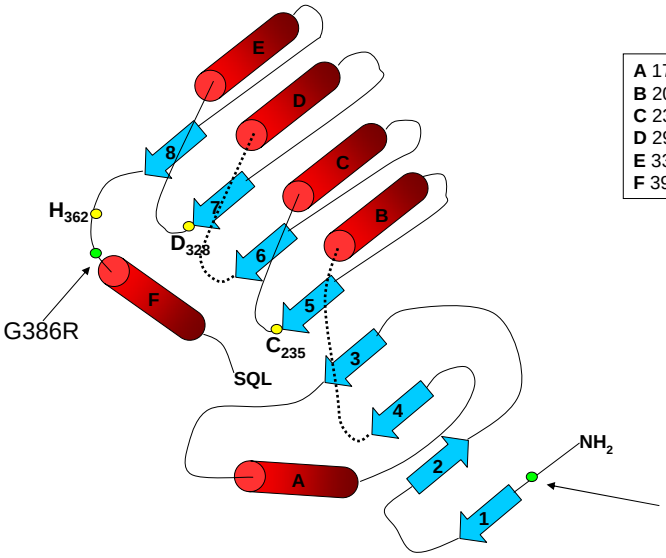
But is this what we want?



A 172-180 GLLEFRASL
B 208-219 LEYFEEAANNFLL
C 237-247 GVQIGLSMAIY
D 292-298 LLELYRR
E 337-346 AEQAIGQLKR
F 392-408 AAAQEHAWKEIQRFLRK

1 101-108 FQVQVKLY
2 149-153 RGALF
3 163-166 VIDL
4 ?
5 229-234 VGVVSV
6 252-257 TATVLI
7 320-324 FLFIV
8 353-357 TLLSY

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Thanks

- **Marilyn Niemann**
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- **GE Healthcare**

- **http://www.jp.amershambiosciences.com/catalog/pdf_attach/00097.pdf**

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