The University Of Sheffield.

Department of Molecular Biology & Biotechnology

Protocols and tips in protein purification

or

How to purify protein in one day

Second edition
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Contents

I. Introduction 7

II. General sequence of protein purification procedures 9

Preparation of equipment and reagents 9

Preparation and use of stock solutions 10

Chromatography system 11

Preparation of chromatographic columns 13

Preparation of crude extract (cell free extract or soluble proteins fraction) 17

Pre chromatographic steps 18

Chromatographic steps 18

Sequence of operations during IEC and HIC 18

Ion exchange chromatography (IEC) 19

Hydrophobic interaction chromatography (HIC) 21

Gel filtration (SEC) 22

Affinity chromatography 24

Purification of His-tagged proteins 25

Purification of GST-tagged proteins 26

Purification of MBP-tagged proteins 26

Low affinity chromatography 26

III. "Common sense" strategy in protein purification 27

General principles and tips in "common sense" strategy 27

Algorithm for development of purification protocol for soluble over expressed protein 29

Brief scheme of purification of soluble protein 36

Timing for refined purification protocol of soluble over -expressed protein 37

DNA-binding proteins 38

IV. Protocols 41

1. Preparation of the stock solutions 41
2. Quick and effective cell disruption and preparation of the cell free extract 42
3. Protamin sulphate (PS) treatment 43
4. Analytical ammonium sulphate cut (AM cut) 43
5. Preparative ammonium sulphate cut 43
6. Precipitation of proteins by ammonium sulphate 44
7. Recovery of protein from the ammonium sulphate precipitate 44
8. Analysis of solubility of expression 45
9. Analysis of expression for low expressed His tagged protein 46
10. Bio-Rad protein assay Sveta's easy protocol 47
11. Protocol for accurate determination of concentration of pure protein 47
12. Calibration plot for gel filtration column 48
13. Ni-chelating cartridges : cleaning and recharging 48
14. SDS PAGE Sveta's easy protocol 49
15. DTNB reaction 50
16. Refolding protocol 50

V. Charts and Tables 51
Ammonium sulphate –Sugar refractometer 53
NaCl-Sugar refractometer 54
Calibration plot for Hi-load Superdex 200 column 55
Calibration plot at various salt concentrations for Superdex200 GL column 56
Ammonium sulphate table 57
Properties of amino acids 58

VI. Appendix 1 59
1.Determination of protein concentration 59
2.How to concentrate proteins 64
3.How to store proteins 67
4.How to estimate purity of a protein preparation 68

VII. Appendix 2 69
1. Prepare your solutions right! 69
2. Gel filtration 70
Separation by molecule's size 70
Calibration plot 71
Main reasons for "abnormal" mobility of some proteins during gel filtration 72
3. Time saving tips 73
4. Golden rules in protein purification 74

VIII. Show cases 75

1. Two step purification protocol and a lesson to learn (BPSL 1549 (BLF1)) 75
2. It is easy with the coloured proteins! 79
Single-domain haemoglobin cgb from *Campylobacter jejuni* 79
Flavohemoprotein HMP from *Escherichia coli* 81
3. Almost typical three step purification (Glutamate Dehydrogenase from *Clostridium symbiosum*) 85
4. Monitoring purification by enzyme activity (Glutamate Dehydrogenase from *Clostridium symbiosum)* 88
5. Power and failure of dye pseudo affinity chromatography 91
Phenylalanine Dehydrogenase from *Nocardia sp.* 91
Glutamate Dehydrogenase from *Clostridium symbiosum* 97
6. DNA binding proteins 102
Typical Heparin protocol: RuvC from phage bIL67 102
Playing with solubility: LrpC from *Bacillus subtilis* 104
Heparin *and* solubility: FrmR from *Escherichia coli* 105
Real power of Heparin: Human FEN 108
In need of nuclease treatment: SfsA 111
7. Tagged proteins 116
Proper use of a His-tag: Human protein PARP1 expressed in *E.coli* 116
Removable tag: SAG19 118
Cleaver use of tag: TssA 121
8. Special cases 124
Phosphoglycerate Kinase (PGK) from *Escherichia coli* 124
Low affinity chromatography: BPSL1958 127
BacM from *Myxococcus Xanthus* 130
Perfect purity: IGPD from *Arabidopsis thaliana* 134

Abbreviations

AA	acrylamide
APMSF	4-Amidinobenzylsulfonyl fluoride hydrochloride
AS	ammonium sulphate
CBV	column bed volume
CE	crude extract
CFE	cell free extract
CM	carboxymethyl
DEAE	diethylaminoethyl
DTNB	Dithionitrobenzole
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FF	fast flow
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIC	hydrophobic interaction chromatography
IEC	ion exchange chromatography
MES	2-morpholinoethanesulfonic acid
MW	molecular weight
MWCO	molecular weight cut off
PBS	phosphate saline buffer
PS	protamine sulphate
PSA	ammonium persulphate
PAGE	polyacrylamide gel electrophoresis
Q	quaternary ammonium
S	methyl sulphonate
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography or gel filtration
SP	sulphopropyl
TEMED	*N*,*N*,*N'*,*N'* tetramethylethylenediamine
TP	target protein
UV	ultraviolet

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I. Introduction

Why do we need to purify protein in one day?
After 25 years and 360 proteins it has become clear to me that for some proteins even one extra day under conditions normally used for protein purification can detrimentally affect their activity and crystallisation ability. Whilst the majority of proteins can survive long purification and can be kept and stored at 4ºC for days and even weeks with little loss in activity, it does them no harm to be purified in one day either. To achieve the goal of complete protein purification in one day you should move fast and choose appropriate protocols, avoiding long procedures such as dialysis, long centrifugations and slow chromatography. Below are some protocols and tips which help me to achieve this goal.

My approach is based on classical combination of ion-exchange, hydrophobic and size-exclusion chromatography for natively (no tags) over-expressed proteins. Nowadays the high throughput approach dictates increasing use of tags in protein purification and sometimes classical methods are considered to be defunct. This is not the case. The most justified use of tags is for difficult expressions, such as expression of eukaryotic proteins in E.coli, expression of the domains of the proteins, toxic proteins, in the cases when tags help polypeptide chains to fold, in the cases when correct fold occurs only occasionally and so expression yield is hardly detectable. In such the cases tagged proteins often cannot be purified to the desirable purity by using just one tag affinity chromatography, so more purification steps are required anyway.

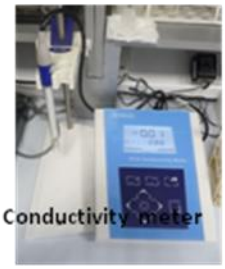
We also have to remember that tag as well as being often useful in some cases it may be an obstacle that prevents correct proteins folding. I consider the recent trend to do all expressions in a tagged form is misleading . For most of enzymes and similar cytoplasmic proteins the chance to be successfully expressed is greater in the absence of the tag. My recent experience also revealed that if the fusion protein or the tag has to be removed from the target protein you may face a great problem either with protease underperforming or unspecific proteolysis leading to damage of the target protein.
In this second edition I have included my recent experience with purification of the tagged proteins.

Please note that this brochure is not a complete guide to protein purification and you should still read serious books on the theory of chromatography to become familiar with the subject.


INFORS
INFORS
Incubator shaker


Sonicator
Soniprep 150


JA-25.50 rotor
J-25 Avanti
centrifuge

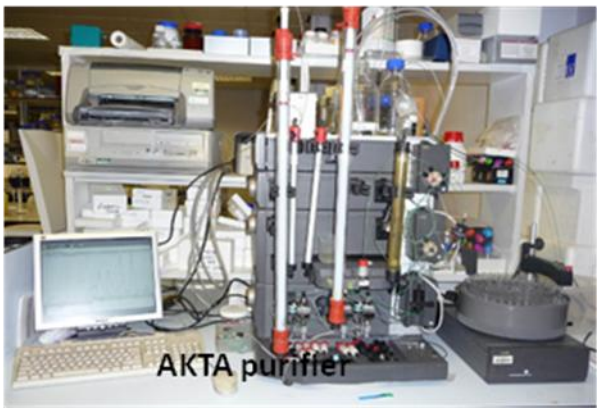

Conductivity meter


Nanophotometer


Sugar refractometer


Refrigerated low speed centrifuge


AKTA pure


AKTA purifier


SDS-PAGE equipment

II. General sequence of protein purification procedures

Preparation of equipment and reagents

For protein purification you need the **equipment and reagents** listed below:

- **Sonicator or French Press**
- **Centrifuge, medium speed** (30-70Kg, e.g. J-20 or Avanti J-25)
- **Appropriate centrifugation tubes**
- **Chromatographic system comprising of, as a minimum, pump and fraction collector** (normally system also includes UV monitor and recorder)
- **Gradient mixer**
- **Spare pump or second chromatographic system** (optional)
- **Chromatographic columns packed with different types of matrixes**
- **Spectrophotometer VIS (340-800nm) or better UV/VIS (190-800nm)**
- **Bio-Rad Protein Assay Reagent**
- **Plastic cuvettes** (1.6 ml)
- **Stock solutions of salts and buffer components**
- **Concentrators** (VivaSpin 20, 6 and 0.5ml)
- **Low speed refrigerated centrifuge**
- **Filtration Device** (such as Filter Holder or Stericup Filter Unit)
- **Apparatus and solutions for SDS-PAGE** (ideally precast gels)
- **Refractometer** (a pocket one for sucrose is fine)
- **Pippetors and tips**
- **Eppendorf tubes**
- **Tubes for fraction collector**

Preparation and use of stock solutions

It is very convenient to have stock solutions of the main salts and buffers used during purification. Correctly and carefully prepared stock solutions can noticeably improve accuracy and reproducibility of the purification procedures.

Stock solution preparation sequence

When preparing stock solutions, follow the sequence below:

Weigh powder → dissolve in about 80% of final required volume of ultra pure water→ adjust pH (for buffers and EDTA only**) → adjust to final volume with ultra pure water → filter (**also see poster in Apendix2**)**

The most useful stock solutions in protein purification are:

- **5M NaCl**
- **4M $(NH_4)_2SO_4$**
- **1M Tris-HCl, pH 8.0**
  - To prepare this solution you need to adjust the pH to 8.0 with concentrated HCl, this titration generates heat. However Tris buffer is temperature sensitive: raising the temperature by 3°C decreases pH by 0.1**.** To make a reproducible stock solution it is best to adjust pH on a water-ice bath with a thermometer placed in the solution, you are aiming for pH 8.0 at 20^{o}C.
    A significant volume of concentrated HCl is required to adjust pH, therefore Tris powder should be dissolved in 60-70% of the final volume, pH adjusted to 8.0, then water added to final volume.

Other stock solutions:

- **0.2M - 0.25M EDTA**.
  - The stock concentration is relatively low due to the low solubility of this compound. Add 5M NaOH to the solution until all EDTA is dissolved (pH is 7.5-8) before you adjust it to required volume.
- **Good's Buffers:**
  - **1M HEPES-NaOH pH 7.0 or 7.5**
  - **1M MES-NaOH pH 6.0 or 6.5**

Unlike Tris buffer, these buffers are not temperature sensitive so there is no need to control temperatures during their preparation.

It is best to keep stock buffers in the fridge.

Preparation of working buffers from stock solutions

By having stock solutions you can prepare any buffer for protein purification in seconds. Simply pour required volumes of stock solutions in to a Duran bottle and adjust the volume with ultra pure water to the top mark on the bottle.

Worked example: to prepare 1 litre of buffer *50mM tris-HCl pH 8, 100 mM NaCl, 2mM EDTA*:

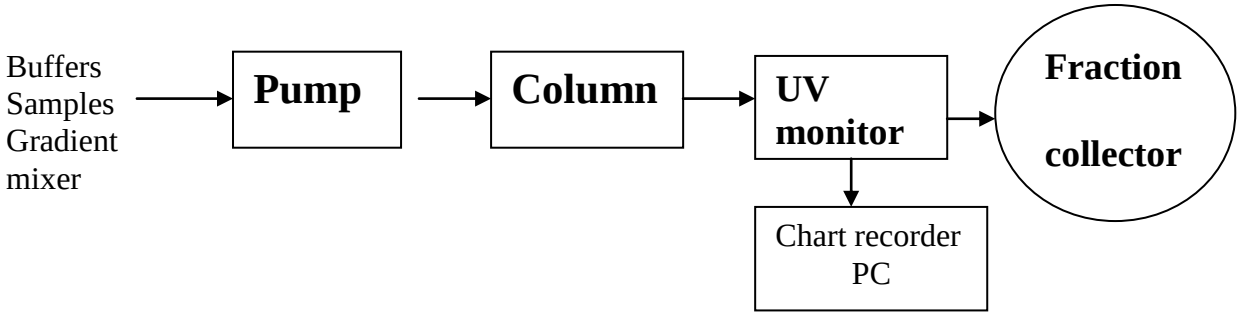
pour 50 ml of 1M tris-HCl, 20 ml of 5M NaCl and 10 ml of 0.2M EDTA in to a 1 litre bottle and add ultra pure water to 1 litre mark.

Please note that dilution leads to a decrease in buffer pH. Normally we use 50 mM solution for purification (made from the 1M stock) and the actual pH for tris, MES or HEPES buffer is about 0.3 pH units lower than in 1M buffer.

For 50mM tris buffer prepared from 1M stock pH 8.0 actual pH at 20^{o}C is about 7.7. However if we use it at 4^{o}C the pH is at about 8.

Chromatography system

- **Principal scheme of the chromatographic system:**

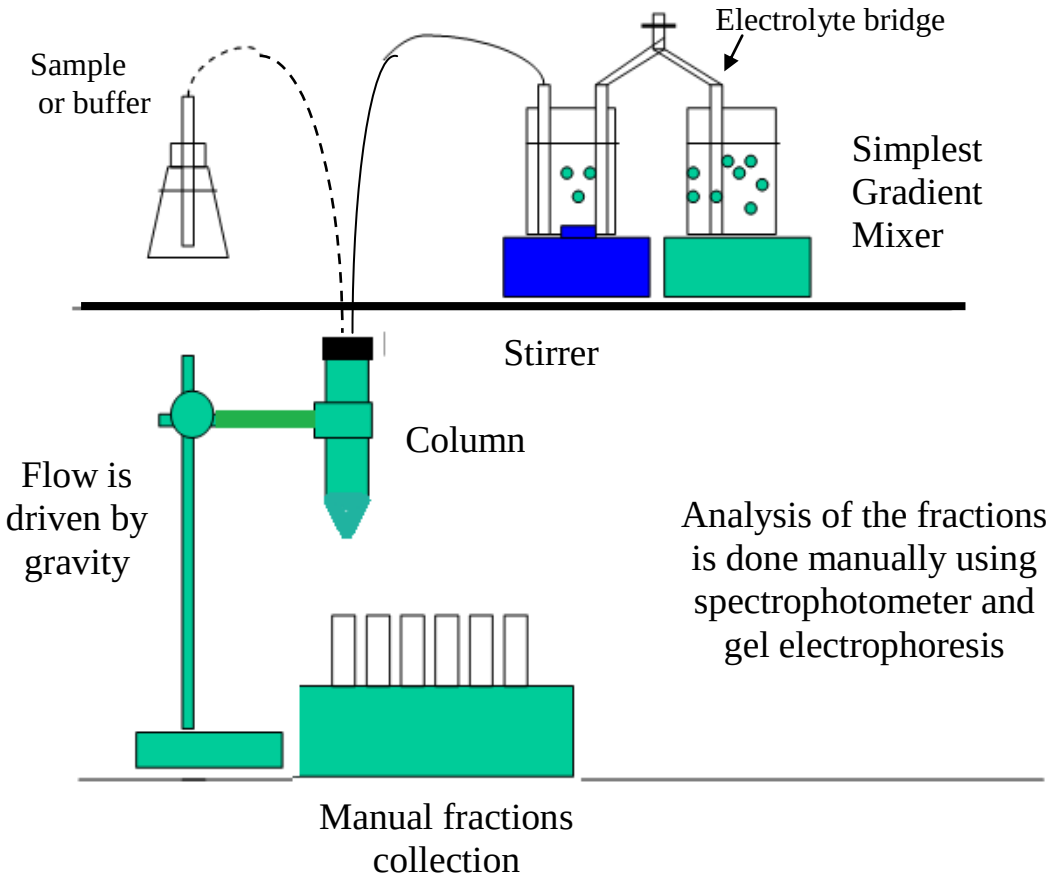


The simple chromatographic system consists of a peristaltic pump, a column and a fraction collector. A gradient mixer is required if gradient elution of the proteins is used. Fractions are analysed manually. More advanced systems include UV monitor usually monitoring an absorbance at 280nm.

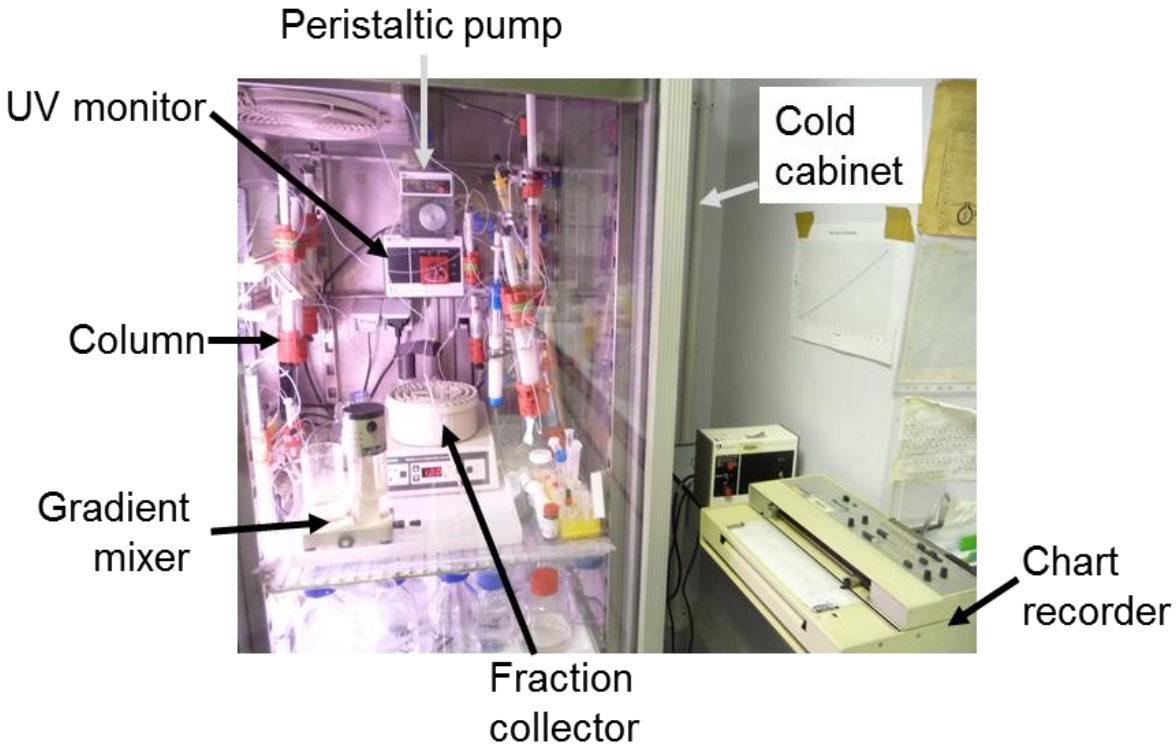
Modern systems such as the AKTA Prime and other AKTA types have a little bit different and more complicated arrangement of the same parts. All compartments are integrated and centrally controlled through a PC interface.

Please notice that the pump is placed before the column, not after as some people do. Don't place the horse behind the cart!

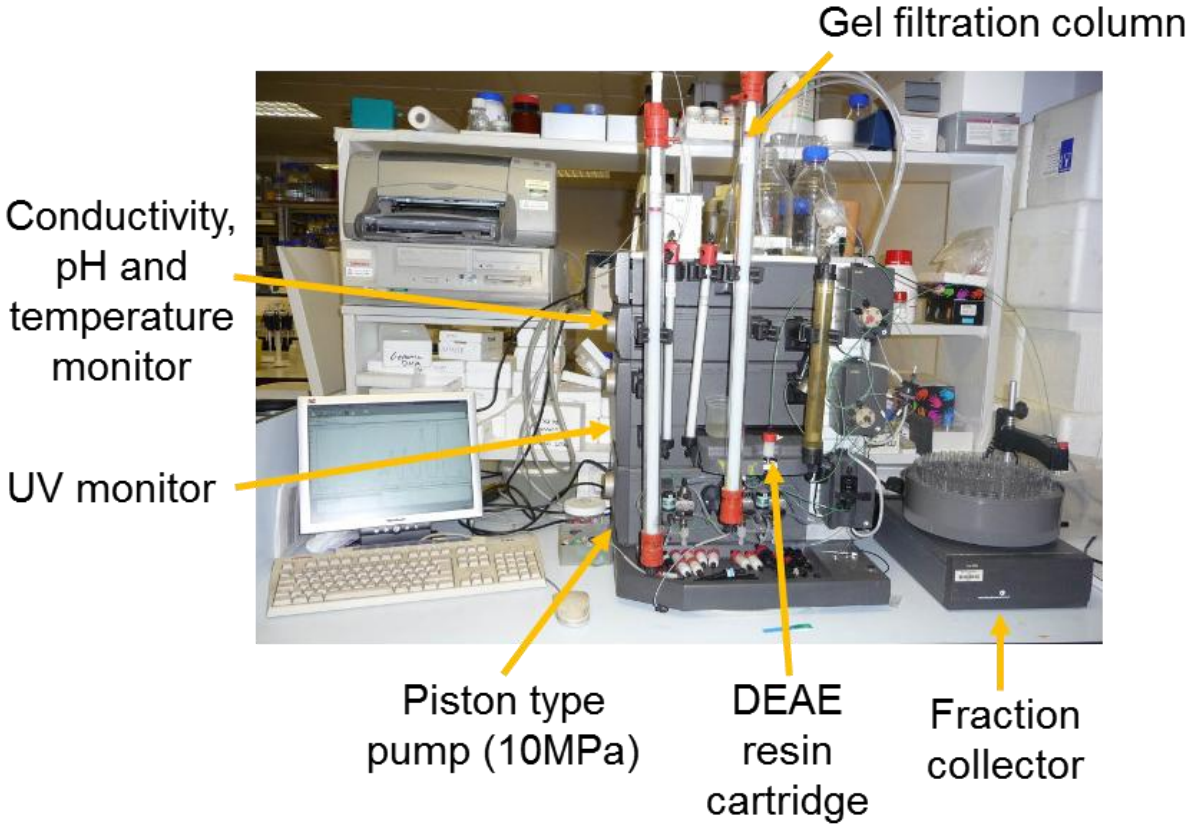
- **Evolution of chromatographic systems**
  - **Simplest chromatography system**



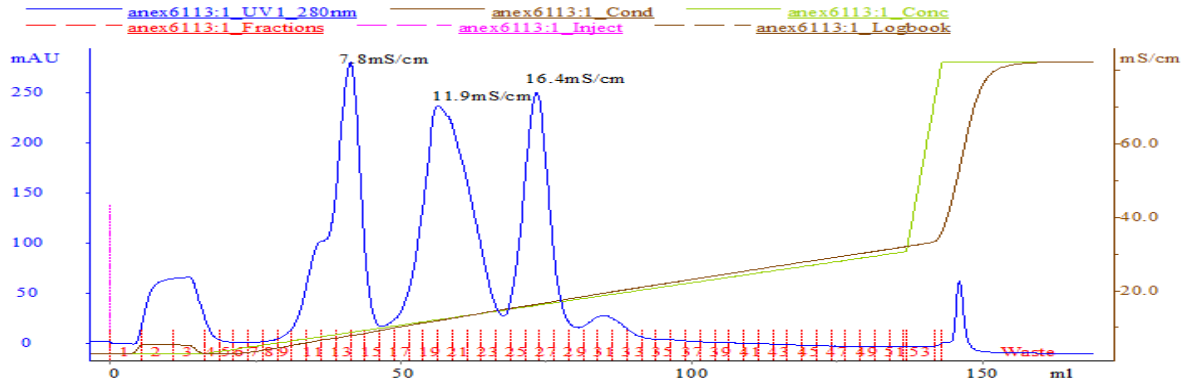
- **Semi-automated systems (1960-80)**



- **Modern system (AKTA purifier) (2000+)**



This system is an AKTA Purifier FPLC (Fast Protein Liquid Chromatography) machine. It is operated by PC through UNICORN software and is highly automated. The feature I like most is the way the chromatograms are saved and presented:



- **Cold cabinet vs room temperature**

You may notice that our AKTA machine is not in a cold cabinet, and that the chromatography is performed at room temperature while the earlier system is placed in the cold cabinet and chromatography is done at 4°C. Some labs still put AKTA machines at 4°C (putting it at risk of damage by condensation etc.). However, the reason that chromatography has historically been done in cold rooms was to protect proteins from proteolysis during the days-long purification processes. When proteins were purified from original sources purification procedure lasted for a few days, one chromatography took a day or longer. It was vital to perform chromatography in cold to slow down any possible proteolysis and bacterial growth. In contrast, the chromatography performed on modern systems with 5ml cartridges lasts 20-30 minutes, and combined with the use of low-proteolysis *E.coli* strains, the risk of protein degradation at room temperature is minimal. So the most rational way is to do chromatography at room temperature and place eluted fractions in the cold cabinet to prevent occasional bacterial growth in them.

- **Cleaning of chromatography system**

It is extremely important to keep clean all parts of system (tubing, pumps, sample loops etc.)

- Always pump water through all part of system after chromatography to prevent salt to be crystallised in the tubes and channels.
- To prevent bacterial growth 20% ethanol wash is usually recommended. However it is not 100% safe and sometimes bacterial growth still happens. In our lab we use **1-2mM EDTA** solution to keep AKTA systems between purifications. It never failed.

Preparation of chromatographic columns

Chromatographic matrixes

For the basic purification protocol you need a set of 3 columns: one packed with ion exchange matrix, one with hydrophobic matrix and one with gel filtration matrix.
I suggest the following option:

1. **DEAE-Sepharose Fast Flow** (weak anion exchanger)
2. **Phenyl-Toyopearl 630S** or **Phenyl-Sepharose** (medium hydrophobic matrixes)
3. **Superdex-200** (gel filtration**)**

You also may need columns with different variations of matrixes in case if any of above columns does not perform satisfactorily.
Other matrixes and columns I suggest to use are as follows:
Q-Sepharose Fast Flow (strong anion exchanger)
CM-Sepharose fast flow (weak cation exchanger**)**
DEAE-Toyopearl 650S (weak anion exchanger)
SP-Toyopearl 650S (strong cation exchanger**)**
Butyl-Toyopearl 650S (strong hydrophobic matrix)
Ethyl-Toyopearl 650S (weak hydrophobic matrix)
Heparin-Sepharose Fast Flow (pseudo-affinity**)**
Sets of HPLC (FPLC) columns (if you possess the appropriate system)
You may need other types of matrixes, such as Hydroxylapatite, Dye and tag affinity matrixes.

Chromatographic columns

Nowadays we have a choice **to pack columns ourselves** or **buy ready-to-use pre-packed** columns. Both options have their advantages and disadvantages.

Option1. Pack your own column.

For our lab scale protein purification we need columns 5-30 ml in volume..

We used to buy empty columns (mainly Amersham –Pharmacia (now GE Healthcare))

C10/10, C10/20, C16/20 and XK16/20 with adapters and pack them in the lab with the listed above loose matrixes.

Advantages: 1) this is considerably cheaper than pre-packed columns
2) column can be repacked if it gets badly contaminated or dried

Disadvantages: 1) it takes time to pack columns
2) often in house made columns are underperforming the pre-packed equivalents

Column preparation (packing) (except the gel filtration one):

1. Check that all parts of column are in place (according to specification)
2. Fix empty column on a stand in a vertical position
3. Fill bottom outlet with a few ml of ultra pure water
4. Prepare slurry of any matrix taking 1 part sediment matrix and two parts water, warm to room temperature if matrix has been kept in the fridge. De-gassing under vacuum is recommended but not overly crucial.
5. Open the bottom outlet of the column, pour the slurry into the column, adding the slurry portion by portion, the matrix should settle at the bottom of the column and the water should run through. When all matrix is in the column, continue to add water until matrix has settled, then close the bottom outlet of the column.
6. Fill the column with water to the top. Using a peristaltic pump, fill an adapter with water and fit it onto the top of the matrix bed. Press down slightly and close the adapter. Alternatively you could leave a 5-7mm gap between the top of the matrix and an adaptor to trap any occasional air bubbles.

Option 2. Buy pre-packed columns

Advantages: 1) No trouble with packing
2) Good performance

Disadvantages: 1) Significantly more expensive than self packed columns
2) They are not re-packable. If they get dried or badly contaminated the only good option is to buy a new (expensive) one.

For gel filtration it is the best to use a pre-packed column. This is because a self-made column never gives as good a separation as a readymade one**.** Gel filtration is a zonal separation method and because of that the perfection of packing is absolutely essential**.** In my hands the best result was 8000 theoretical plates/meter, while pre packed columns show at least 13000.

A 1.6x60cm Hi-Load Superdex 200 column (GE Healthcare) is the best option for the majority of proteins. For smaller scale purifications and analytical runs we use

1x30cm Superdex200 Increase column (GE Healthcare)

GE Healthcare cartridges to use with AKTA systems

Over recent years, we have moved over to the AKTA purifier and AKTA pure systems due to their advantages for recording and analysing chromatograms. **5ml cartridges** are very convenient option to use with AKTA systems as they can be combined together to make the equivalent of 10-25ml columns.

The cartridges contain **Sepharose HP (high performance) matrix** which consists of smaller and more uniformly sized particles than Sepharose Fast Flow (FF) and so provide sharper peaks and better separation. There is a wide range of matrixes available in form of cartridges, including ion-exchange, hydrophobic and affinity.

GE Healthcare also sells pre-packed (**HiPrep) 20ml columns with DEAE-, SP-, Q- Sepharose FF,** but I do not think that they are cost effective, better to pack such columns in-house.

Unfortunately Toyopearl manufactures failed to produce suitable and convenient cartridges for the use with AKTA systems and these good matrixes are now only occasionally used in our lab.

More information on columns and matrixes is provided in ***Chromatographic steps*** part.

Columns care

- During chromatography **do not allow air to enter the column**.
- Also be careful with **samples which you apply on a column**:
  - Make sure that sample is properly clarified and free of any particles which could block the net on the top of the column (see Cell free extract preparation below)
  - Do not overload columns (see corresponding information on chromatography matrixes below). Proteins tend to be bound on the top of column and if overloaded it may completely block the flow. If it happens the only solution is to open the column and mix matrix following by protein elution.
- **Column cleaning**
  - General recommendation is to wash columns after chromatography with a few column volumes of water and 20% ethanol. This is very safe method but also it is very impractical. It only worth doing if there are a long (a few months) gaps between purifications. In our lab we leave ion exchanger columns in 1-2M NaCl and hydrophobic matrixes in water or no salt buffer.
  - If column is contaminated the best way is to wash it with 2-3 CV of 1M NaOH. Always wash column with 1M NaOH after chromatography if there was possible protease contamination (for example, if protease was used to cleave tag from the target protein prior purification).

Preparation of crude (cell free) extract

Disruption of bacterial cells

Methods for cell disruption can be divided into two groups:

1) Non-mechanical methods, including
- **freezing-thawing**
- **osmotic shock**
- **lysozyme treatment**

2) Mechanical methods, including
- **ultrasonication**
- **liquid extrusion** (**French Press, flow-through press**)

Liquid extrusion

The most powerful method is liquid extrusion. If a machine (such as a French press) works well (all parts are in good order, right pressure is applied etc.) and you have passed the material twice though the press, the result is typically very good. This method usually outperforms ultrasonication for fresh living cells such as *E.coli*. The disadvantage of this method is that the operating of the equipment involves heavy bomb to handle and important teflon ball is prone to wear so can fail to provide the right sized channel.

Ultrasonication

In contrast to liquid extrusion, ultrasonication is easy to perform if you have a properly working machine (no heavy lifting involved). The problem is that it is not as powerful as liquid extrusion; ultrasonication would not effectively destroy intact living *E.coli* cells so you have to use it in combination with non-mechanical methods.

Non-mechanical methods

In the literature, it is common to see lysozyme treatment prior to ultrasonication. I believe this method is still very popular mainly because people tend to repeat protocols uncritically. In my experience, there are at least two major disadvantages:

1. You have to add a significant amount of lysozyme which could compromise purification of your target protein.

2. It involves 30 minutes long incubation at 37^{o}C exposing proteins to possible protease degradation and still needing ultrasonication because lysozyme does not destroy cells but only makes holes in them.

Lysozyme-free method for cell disruption

I've found a combination of freezing-thawing, osmotic shock and sonication simpler and quicker than lysozyme based protocols.

1. Cells are collected and pelleted by centrifugation, medium removed and pellet is frozen at -20^{o}C. Frozen cells can be kept for years before protein purification.
2. On the day of purification, cells are defrosted with 8-20ml of lysis buffer (typically 50mM Tris-HCl, pH 8.0, but see below) per gram of cells and then sonicated on ice. To prevent protein denaturation due to the heat released by the probe, sonication should be performed in 2-4 cycles with cooling between sonication sessions. The exact regime depends on the machine you use and so you need to find the right time and power empirically. For example, with a Soniprep150 machine we perform 20-25sec cycles at 16-micron amplitude (full power) using a medium probe.

***Tip**: To save time on this step it is useful to divide cell suspension into 2-4 portions and treat the portions one after another thereby allowing each portion to cool while you treat the other portions.*

Also, you can add small pieces of ice into the cell suspension between the sonication sessions for more effective cooling.

Lysis buffer

Lysis buffer has to be chosen accordingly to the particular protocol.

50mM tris-HCl pH 8.0 (referred as **Buffer A** in this brochure) is typical for most of the protocols

- For a basic protein with the first column being a cation exchanger, choose a buffer with pH below 7 e.g. **50mM MES-NaOH pH 6.0**
- It is useful to include **1mM EDTA** as a good bacteriostatic, but it is **not** suitable for proteins containing divalent cations or for His-tagged proteins.
- For His-tagged proteins, add **0.5M NaCl** to inhibit anion binding on Ni^{2+}
- **Protease inhibitors (PI)** are sometimes required and are particularly important if you have to purify protein from the native organism, or for eukaryotic proteins expressed in *E.coli*, as they often have extended loops attractive to proteases. Use Roche "cOmplete EDTA-free" tablets.

  On other hand it is ***not necessary to use protease inhibitors routinely*** for purifications of the proteins over expressed in special strains of *E.coli* as they have low level of protease activity.

Removal of cell debris (insoluble part of cells)

Practically the only method used for the removal of debris is **centrifugation.**

A method called **differential centrifugation** can be applied to separate a homogenate's components by size.

- To spin down unbroken cells and other large bits apply 10 000g for 5-10 minutes.
- To pellet inclusive bodies apply 10 000g for 10 min.
- To collect membrane fractions, 1 hour at 100 000g is required.
- To pellet ribosomes apply 100 000g for 3 hours.

Soluble cytoplasmic proteins

Normally to purify soluble cytoplasmic proteins we do not need to remove ribosomes or all the membranes from the crude extract, we only need to remove big particles (bigger than 10μm - the mesh size of the net used in a chromatographic column adaptor).

Empirically the optimal protocol has been found to be as follows:

- For JA-20 rotor in J-20 centrifuge: 19000 rpm (~43000g) for 15 minutes at 4˚C
- For JA-25.50 rotor in J-25 Avanti centrifuge: 24000 rpm (~70000g) for 10 min at 4°C.

Membrane proteins
For purification of membrane proteins you only need to spin down the large pieces (this is done by centrifugation at 10000g for 10 minutes) and then collect membranes by ultracentrifugation at 100000g for 1 hour.

Sometimes it is useful **to filter CFE** prior of loading on an especially precise column. However it is not easy. It is better to use 0.45µ filter because 0.22µ is quickly blocked. **Do not do it for routine purifications** when first column is Sepharose Fast Flow or HP (ion exchange or affinity).
See **Protoco**l number 2 for more details

Pre-chromatographic steps

Some protein purification protocols include a treatment of the crude extract (CFE). This could be dialysis, differential ultra centrifugation, addition of a reagent (such as substrates or inhibitors, etc.) or precipitations of some components of crude extract using different precipitants.

- **Dialysis** is normally required if CFE was obtained in the buffer which is not suitable for the first chromatographic step
- **Differential ultra-centrifugation** is applicable if the target protein (TP) is associated with a certain part of the cell or with certain organelles. It is not necessary for purification of soluble cytoplasmic proteins.
- **Fractionation of the cell free extract using various precipitants** was a very popular and powerful method in the early days when chromatographic techniques were not properly developed. Ether, chloroform, ethanol, isopropanol and polyols were widely used. Nowadays **ammonium sulphate** is practically the only precipitant that is frequently used for fractionation of the CFE (in a so called **ammonium sulphate cut** procedure).
    - **Ammonium sulphate cut** (see **Protocol number 4** for the procedure) For this application ammonium sulphate concentration is usually expressed in a % of saturation. Saturated solution (100%) at room temperature is about 4.1M. Using the ammonium sulphate table (see **Tables and Charts** section) you can find out how much ammonium sulphate powder you should add to the solution to get a desirable concentration. During precipitation the protein solution should be kept on ice or at 4˚C to prevent proteins from denaturing due to heat produced by the ammonium sulphate dissolving. After this step the protein sample will contain significant amounts of ammonium sulphate and cannot be used directly for ion exchange chromatography but can be used for hydrophobic chromatography (after adjusting ammonium sulphate concentration to required level) or applied on the gel filtration column for separation or desalting
    - **Clarification of crude extract by protamin sulphate treatment** is another method that can sometimes be useful**.** Protamin sulphate (PS) is used to precipitate out nucleoproteins such as ribosomes and DNA/protein complexes. In my work it has been particularly useful for clarification of fungal extract. After PS clarification the sample can be used directly for any kind of chromatography (see **Protocol number 3** for the procedure).
- **Denaturing of contaminating proteins by heating** has become a rather common procedure that is especially effective if you are dealing with recombinant protein from a thermophilic organism expressed in *E.coli*. If you know the denaturing temperature of the TP, you can heat the crude extract for 10-20 minutes at temperature 5-10°C lower than denaturing temperature. This process depends on pH, salt concentration and protein concentration. Small-scale trials are required to optimise all conditions.

Chromatographic steps

Among about a dozen kinds of chromatography the most useful in protein purification are:

	Purification (fold)
Ion-exchange chromatography (IEC)	2-10x
Gel filtration or size exclusion chromatography (SEC)	2-3x
Hydrophobic chromatography (HIC)	2-10x
Affinity and pseudo affinity chromatography	>100x

To become familiar with the basic theory and methods in chromatography, please read the useful booklets from GE Healthcare. They are accessible on the website and at minimum you need to read these five:

- **Affinity chromatography. Principles and Methods**
- **Ion exchange chromatography, Principles and Methods**
- **Gel filtration, Principles and Methods**
- **Hydrophobic interaction chromatography, Principles and Methods**
- **Protein Purification Handbook**

Sequence of operations during ion exchange or hydrophobic chromatography:

1. Connect chosen column to the system
2. Wash with 1-2 column bed volumes (**CBV or CV**) of starting buffer
3. Apply sample. Collect unbound material in the separate container or start to collect fractions. Collect full tubes on this stage.
4. Elute proteins using either continuous or stepwise gradient of elution agent. The duration of continuous gradient should be 10-20 CBV (up to 30CV for HPLC columns). With stepwise elution apply 3-4 CBV of each concentration. Collect fractions (25-50% of CBV).
5. Clean column with 1-2 CBV of high concentration elution agent (1-2M NaCl for ion exchange matrix or water for hydrophobic matrix)
6. Check protein concentration in fractions by method of Bradford.
7. Analyse protein containing fractions using SDS-PAGE.
8. Combine fractions containing TP, check volume and protein concentration then calculate yield of protein after the step.

Tip: *To save time on the chromatographic step do not wait until the elution is totally completed. Start to analyse protein concentration in the fractions after collection of 15-20 fractions or after about 1/3 of the gradient has been applied on the column*.

Ion exchange chromatography

Please read "Ion exchange chromatography" booklet for theory and matrix characteristics.

Anion exchange matrixes and their standard columns:

Weak anion exchangers: DEAE-Sepharose Fast Flow, DEAE-Toyopearl 650S

Strong anion exchangers: Q-Sepharose Fast Flow or HP, Resource Q (HPLC), Mono Q (HPLC).

- The latter two columns are high resolution columns which have fine beads and some pressure is required to run them, therefore they are used with AKTA systems or the FPLC system and often applied as a last polishing step.

Anion exchange chromatography is favoured as a first chromatographic step.

Normally we use DEAE-Sepharose FF and sometimes Q-Sepharose FF columns.

With **AKTA system** use **HiTrap DEAE FF or HiTrapQ HP** cartridges, just screw 2 to 4 of them together if required.
Cell free extract prepared in Buffer A (50mM tris pH 8.0) can be applied on the column directly, no extra preparation needed.

Altering column pH
The typical pH range for anion exchange chromatography is **from 6 to 9.**
Note that it is not easy to change pH of an ion exchange matrix. It would not work if you simply try to wash column with the starting buffer of 50mM concentration at different pH. To change pH in the column apply about ¼ CBV of 1M buffer solution of the desired pH, following by 2 CBV of the starting buffer (with the same pH as the 1M buffer solution).

Main parameters of chromatography are:

- Column size and geometry
- Column's capacity
- Flow rate
- Shape and slope of elution gradient
- Fraction size

For standard first-step anion exchange chromatography parameter settings are as follows:

- **Column Bed Volume**: 5-40ml, diameter 1.6cm, cross area 2cm^2, bed height 3-20cm.
- **Capacity:** up to **40 mg** of total protein **per ml** of matrix. Typical loading on the above column is **100-800 mg** of total protein.
  One **5ml HiTrap cartridge** could take up to **150mg** of the cell free extract. Higher loading affects separation.
- **Flow rate:** for Sepharose FF and Toyopearl 650S it is 2-3ml/min/cm^2. So for the above column with cross-area 2cm^2 typical flow rate is **4-6 ml/min**.
  For **HiTrap cartridges** flow rate is **5ml/min**.
- **Linear elution gradient** is the best option for the first step anion exchange chromatography.
  - For weak anion exchange matrixes (DEAE) the final NaCl concentration should be **0.5M**.
  - For strong anion exchange matrixes (Q) it should be **1M**.

- Length of gradient is typically 10-30 CBV.
  - For the first step **10 CV** is optimal which corresponds to 100-400ml for a 10-40ml column.
    For **5ml cartridge**s it is **50ml**.

    So the slop is **0.1M salt/1CV** for weak (DEAE)
    and **0.2M/1CV** for strong exchanger (Q) matrixes.
  - For high resolution FPLC columns (e.g. **MonoQ, ResourceQ**) the slop can be reduced to **30-50mM/1CV** to achieve better separation

- **Fraction size** should be **25-50%** of the column volume.
  The sharper the gradient the smaller fractions are to be collected.

Cation exchange matrixes and columns:

Weak cation exchangers: CM-Sepharose FF, CM-Toyopearl 650S

Strong cation exchangers: SP-Toyopearl 650S, SP-Sepharose FF and HiTrap SP-HP cartridges

High resolution ready made columns: Resource S, Mono S

Typical pH range for cation exchange chromatography is **from 5 to 7.5**.
It is good first step for basic proteins.

All other parameters are similar to anion exchange chromatography.

Hydrophobic interaction chromatography

Please read "Hydrophobic interaction chromatography" booklet for theory and matrixes.

Hydrophobic matrixes and columns

In our lab we use the following matrixes:

Ether Toyopearl 650S; weak hydrophobic matrix

Phenyl Toyopearl 650S; medium hydrophobic matrix

Butyl Toyopearl 650S; strong hydrophobic matrix

Phenyl-Sepharose FF is readily reported in literature, but I found that Toyopearl 630 matrix (TOSOH) is significantly over performing it in respect with peak sharpness and resolution.

With **AKTA systems** we use Hi Trap Phenyl HP cartridges because TOSOH does not manufacture Toyopearl 630 cartridges suitable for use with AKTA systems. However Phenyl-HP cartridges perform much better than Phenyl-Sepharose FF and so have been successfully used for purification of a number of proteins in our lab.

GE Healthcare also produces 1ml and 6ml Resource ETH, Resource ISO and Resource PHE FPLC columns. I have not find them very useful mainly because HIC is very rear used as a polishing step.

Salt concentration in the sample is critical

To allow proteins to bind to a hydrophobic column the sample should contain a high concentration of salt. Usually ammonium sulphate (AS) is used. More rarely potassium chlorate, sodium sulphate or other salts are used.

The typical **ammonium sulphate concentration in the protein sample should be 1.5-1.8M**, which is OK for majority of proteins.

For most hydrophilic proteins AS concentration should be increased to the point when it bind.

For most hydrophobic proteins use of 2M KCl instead of AS can be suggested.

Hydrophobic chromatography parameters

- **Capacity** of Toyopearl matrixes is up to 30 mg of total protein per ml of matrix. Optimal loading is 1-20 mg of total protein per ml of matrix.
  It also applies to Hi Trap Phenyl-HP cartridges
- **Flow rate** is 1-1.5ml/cm^2 for the Toyopearl matrixes
  For Hi Trap Phenyl HP cartridge flow rate is 5ml/min
- **Elution gradient** is typically linear, **10-20 CV**, from high salt (AS) concentration to buffer without salt or sometimes to a lower concentration of salt.
  Optimized *starting buffer* should normally have a concentration of salt (AS) which is about *0.2M higher* than the concentration of the salt at the protein elution point and the *final buffer* should have no AS or have an AS concentration which is about *1M lower* than its concentration at the elution point.
  If protein elutes from the hydrophobic column at a low concentration of AS, you should wash column with the buffer without AS after the completion of elution gradient in order to push protein out the column and tubing into the collection tubes. Apply at least 4-5CV of buffer.

It is very useful to **check AS concentration** in the fractions with the TP to be able to compare different runs of purifications of the same protein and so to work out *optimal starting and final buffer* for the gradient. In our lab we use a convenient pocket sugar refractometer and Chart "Sugar refractometer for ammonium sulphate" (see **Tables and Charts** section)

Gel filtration (SEC)

Please read "Gel filtration. Principles and methods" booklet for theory and matrixes.
See also Appendix 2.2

The main feature of this type of chromatography is that it is **zonal separation,** so columns are long and samples are small to allow zones to be separated properly.
Purification power of this step is not high. Generally speaking we could expect about half of the contaminating proteins to be separated from the TP during this step. However for small (<20kDa) and big (>400kDa) proteins, gel filtration is more effective and often provides a significant purification.
It makes gel filtration a **suitable polishing step** that also provides additional information on the **oligomeric state of the TP and quality of the preparation**.

In our lab we use **Hi-Load Superdex 200 1.6x60cm** columns from GE Healthcare

- Total volume: 120 ml
- Void volume (V_0): 45ml
- Separation range MW: 600KDa – 10KDa
- Sample: volume 0.5-2ml, no restrictions on buffer composition
- Loading: up to 50 mg of total protein (20 mg per protein for the best result)
- Flow rate: 1-1.5 ml/min
- Elution with continuous buffer (pH 5-10, salt concentration 0.1M or higher)
- Collect 2ml fractions after void volume or start collection later if you know elution volume (Ve) of the TP peak.

Take care with these expensive columns

- Do not allow air to get into the column. If you do accidentally dry the column, even so badly that cracks appear, do not panic and wash column with plenty of water at flow rate 2-3 ml/min until all the air has been pushed out of it.
- Time to time, after couple of dozen of runs, the net on the top adapter get partially blocked and has to be replaced with the new one. Please, use the instruction in the leaflet provided with the column (or find one on the Web). This is very important to do it properly, do not damage all important top layer of the column bed. After drying event especially and if the net replacement was not done to a perfection, a column performance could decline a little bit (say losing 1000-3000 theoretical plates), but normally still remains good.

Never ever try to repack those expensive columns!

According to my experience self packed columns never come close in performance to the ready made ones. My best attempt to pack the column with the fresh loose Superdex200 prep grade matrix was yielded in producing the column with resolution power of 8000 theoretical plates/meter while for commercial columns the figure reproducibly is above 13000. Back then I was not happy with my column and decided to repack it and had it done following all rules to the scratch. The result was 5000 theoretical plates/meter.

- **Standard buffer for gel filtration** in our lab is 50mM tris-HCl pH 8.0, 0.5M NaCl, which is good for the majority of proteins.
  Any other buffer at reasonable pH could be applied, just avoid low salt conditions. This prevents absorption of proteins on the matrix.

- If required, gel filtration can be used to exchange buffer in the protein sample for the next purification step.

- **To estimate MW (molecular weight) and oligomeric state of** your protein you can use chart “Calibration plot for Hi-Load Superdex 200 column” **(Tables and Charts** section). This chart was produced for the "standard buffer" (50mM tris pH 8.0, 0.5M NaCl).
- The slope of a calibration plot depends considerably on the salt concentration in the elution buffer. See chart "Calibration plot at various salt concentration".
- **Gel filtration matrix may change its properties**
  Above calibration plot is typical for the 16x60Hi-Load Superdex 200 column and using it for your MW estimation you most likely will get a fair result. Nevertheless be aware that when column is in use for long time, after drying event or net replacement the separating properties of the column could change. The most drastic change I noticed in a separation properties of the Superdex 200 column was so big that the calibration plot of the column became more like that of Superose 6 matrix. The only explanation I could give for this is that dextran component of the beads was destroyed (probably by one of the “proteins of unknown function” which was purified on the column).
  - **Do calibrate your column each time when you want to estimate MW of your protein**. Run at least one mix of 4 calibration proteins in the same buffer as your protein.
    It is convenient to use **Low Molecular weight and High Molecular weight Gel Filtration Calibration Kits from GE Healthcare.** Just follow the instructions in the booklet. However these kits are expensive and you could use some other suitable proteins for calibration. See “Calibration of gel filtration column” protocol.
  - **Gel filtration is not an accurate method to measure MW** or oligomeric state of the protein. Proteins are separated by their size and not by MW. Proteins with the same MW could have a different shape and compactness therefore their sizes and so called Stock’s Radius could be different and so could the elution volume (Ve) and apparent MW. Another possibility for misidentification is if the protein has some affinity to the matrix and so elutes later than it should according to its MW.

See also Appendix 2.2 for more information.

Affinity chromatography

Refer to "Affinity chromatography. Principles and methods" for theory and applications.
In our daily protein purification routine we either use **Pseudo affinity chromatography** or **tag affinity chromatography** to purify tagged proteins.

Pseudo affinity chromatography

We refer to Pseudo affinity chromatography when matrixes with cross-linked ligands similar in structure to substrates of some enzymes are used for their purification.

- **Heparin matrix for DNA binding proteins**
  Typically Heparin chromatography is used as a first step in DNA-binding proteins purification protocols

In the lab we use

- **Heparin-Sepharose Fast Flow columns (10-20ml)**
- **5ml Hi-Trap Heparin HP cartridges for use with AKTA**
  - **Capacity** of Heparin matrixes is about 1-5mg of DNA-binding protein per ml of matrix.
  - **Flow rate** 3-4ml/min/cm^2.
  - **Elution** normally is performed by 10 CBV of a linear gradient of NaCl concentration. Starting and final concentrations of NaCl should be determined for each individual protein, but typically the gradient is 0 - 1M NaCl.

- So called **Red and Blue matrixes for purification of NAD/NADP dependant proteins**
  - Dye cross-linked to the matrix is somehow similar to NAD or NADP. Under the certain buffer conditions (these need to be found for each NAD/NADP dependant protein) the enzyme binds to the column and then can be eluted either by high NaCl concentration or by NADH/NADPH solution in the binding buffer.

"Dye" chromatography is widely used for purification of dehydrogenases.

My personal experience with this type of chromatography I would describe as a disappointing and even frustrating. This chromatography has no universal approach and thus specific conditions have to be found for purification of every particular dehydrogenase. These conditions include type of immobilised dye, binding buffer (pH, composition and concentration), as well as sample volume and concentration, elution mode and buffer, etc. Generally speaking "dye" chromatography has to be slow (0.5ml/min/cm^2 or less), sample has to be rather concentrated, and column has to be relatively big, as capacity of a dye matrix as a rule is low. Elution of the target dehydrogenase from the column is not straight forward either. The best option is to use bio specific elution with NAD(H), but it is expensive option, as for the good recovery concentration of NAD(H) has to be relatively high. More often elution is performed by increasing concentration of salt or changing pH. Step or gradient elution could be employed.
Purity of the dehydrogenase after this chromatography rarely is really high (depends on level of over expression). Often another purification step is required.
Yes, yield/purity combination could be better if you employ properly developed "Dye chromatography" protocol, but you never can be sure in the reproducible result. If you go for the new batch of the matrix, different expression protocol, or simply have a long time gap between purifications, then be prepared for the trouble. See examples in **Show cases** part.

Purification of His-tagged proteins

Among a number of tags in use the most common is **6xHis tag**. Recently **10xHis tag** is developed, which is good news as it has higher affinity to Ni-matrix.

- **Ni^{2+} charged IDA (imminodiacetic acid) or NTA(nitriltriacetic acid)** crosslinked to agarose or Sepharose beads are used to fish His-taged protein out from the cell free extract .
    - There is a wide range of Ni^{2+} charged matrixes, readymade columns and cartridges available from many companies. Among 3-4 brands which I have tried I've found **His Trap cartridges (GE Healthcare)** to be the best with respect to their capacity and separation power. They are convenient for use with the AKTA system, but are relatively expensive and not easy to clean. In my practice typical life time is about 30 runs.
    - It is at least twice as cheap to buy **bulk imminodiacetic acid-Sepharose from Sigma**, make own columns of any size and charge them with Ni^{2+}. It is good option for making very small or big columns or for batch method.

Properties of the different brands of Ni-columns and matrixes differ significantly

- Refine the elution conditions for your TP for any different brand or type of the Ni-column used.

Conditions for Ni-beads chromatography

- **Buffers:** mainly two types of buffer are used, **PBS pH 7.4 and Tris-HCl pH 8.0**. Also, to suppress ion-exchange properties of Ni^{2+}, 0.5M NaCl is normally included. Prepare cell free extract in the same buffer.
- **Capacity** of different brands of the Ni-beads noticeably vary from one to another, but the most serious factor which contributes into Ni-beads capacity is His-tagged protein itself. It seems that **accessibility of His tag to Ni** in the great degree depends on the **protein fold** and so is amount of the TP which binds to the column. The highest capacity I have observed was 30mg per ml of the beads, and lowest was just 2mg/ml. This also affects the elution concentration of Imidazole.
- **Elution**

  To elute protein from a Ni-column continuous or stepwise gradient of **Imidazole** concentration is used. According to my experience most of the His-tagged proteins elutes with Imidazole concentration in range 0.05-0.5M. Most of them are eluted at 150-200mM. In a couple of cases the elution concentration of Imidazole was higher than 0.5M.
    - Stepwise elution

      People who have no chromatographic equipment have to purify His-tagged proteins either by batch method or using syringe with cartridges. They only can use stepwise elution, which could yielded in pure protein if level of TP expression is high and concentrations of Imidazole on each elution step is optimized.
    - Continuous gradient elution

      Much more reliable is a continuous gradient elution. It is convenient to use **AKTA system and His-Trap cartridges**. Typically for a new protein it is useful to apply a gradient of Imidazole concentration from 0 to 0.5M in 10CV. There are a significant number of proteins in the cell extracts which have some affinity to Ni. They elute from the cartridge with about 50-80mM Imidazole as a sharp peak. Typically His-tagged proteins are eluted with higher concentration of Imidazole as a second, wider peak. Some of the His-tagged proteins elute just after contaminations and so the gradient has to be optimized, normally keeping 10CV, but finish at 0.25-0.35M Imidazole. In some cases we have to apply 2-3CV of 30mM Imidazole wash to remove majority of the contaminating proteins before applying the gradient or add 20mM Imidazole in lysis buffer.

Latest development on His-tag front is 10His tag. It may significantly improve binding of a TP to a column and so improve purity of the preps on this stage.

- **Cartridge care**
  After a number of runs (10-30, depends on amounts of the cell free extract applied) a cartridge became contaminated and flow rate significantly reduced. These cartridges could be cleaned up using 1M NaOH (see Protocol "Ni-chelating cartridges: cleaning and recharging").
  - In some labs people strip Ni with EDTA after each run or after a few runs. I do not think this method is effective as contaminations stick to beads most likely hydrophobicaly.

Purification of GST-tagged proteins

The second widely used tag is GST. **Be careful when you choose GST as a tag. GST is a dimer, so, if TP is oligomeric as well, it may lead to formation of big conglomerates.**
Glutathione –Sepharose (bought from GE Healthcare) is used to purify GST-tagged proteins. We can use loose Glutathione-Sepharose in batch method, pre packed gravity driven columns, spin columns or cartridges. Usually PBS buffer or Tris buffer pH 8.0 is used. Capacity of the matrix is at least 3mg/ml of matrix (25mg/ml according to manufacturer). In my experiments there was no trouble with the binding, the main trouble I had with purification of GST-tagged proteins was an incomplete elution. The recommended 10mM glutathione concentration elutes just fraction of the protein from the matrix and after increasing concentration to 50mM significant amount of the protein still remained bound to the matrix.
Check and adjust pH in elution buffer!!! Reduced glutathione is very basic.
Having GST-tagged protein with the protease cleavage site appeared to be more preferable and increase recovery significantly if "in column" cleavage is used. However cleavage is a tricky procedure, which is not very reproducible. Also it is not uncommon to have target protein nicked or even destroyed by a protease.

Purification of MBP-tagged proteins

Maltose binding protein is a good tag which also can help with protein folding.
Amylose-Agarose is used for purification. Typically it is gravity forced columns. I use PD-10 (GE Healthcare). Buffer composition is not critical, but better to avoid extreme pH or salt. Capacity is about 5mg/ml. However, it seems that incomplete binding is a common event. Also Amylose-Agarose matrix is losing some amylose after each run and capacity get significantly reduced after 5-6 purifications. Taking all this in consideration, I would recommend repeating purification of MBP-TP from the unbound material to increase yield. 10mM maltose has to be used for elution. Collect fractions of 25% CV. Elution is complete under these conditions.

Low affinity chromatography

Low affinity chromatography is a special technique which can produce very good result (See **show case**). It may be applied with any type of matrix, ion exchange or affinity. You can found that protein have low affinity to given type of matrix if it appeared in flow through material and also in first fractions of washing and elution. In this case try the following protocol.
Prepare protein sample or CFE in as small volume as possible. If it is a CFE, use 3-4 volumes of lysis buffer per 1 volume of cells. In this case apply 3-4 cycles of sonication and longer centrifugation.
Column has to be at least 3 fold bigger than the sample volume. Ideally to have 1:5 ratio. Apply sample and start to collect fractions (about 20-25% CV), wash column with 2-3 CV of starting buffer continuing to collect fractions. TP will appear as a separated peak just after unbound material. Wash column with high concentration of elution agent to clean up the column.

III. “Common sense” strategy for protein purification

General principles and tips in “common sense” strategy

Common sense is the best guide in protein purification

For structural studies our goal is to purify protein to a reasonable purity (90+%) with the best possible yield in the shortest possible time and with a minimum effort.

Taking all this into consideration common sense tells us that the best possible strategy for protein purification would be one started with affinity or pseudo affinity chromatography. This can give you purification fold of tens or even hundreds and results in almost pure protein even if the level of TP expression is not high. An affinity matrix could be a matrix with cross-linked substrate, pseudo substrate or inhibitor. The problem is that we need to create a special matrix and to optimise conditions for chromatography for each protein individually; this may take a lot of time.

The alternative is **Tag Affinity Chromatography,** which is now widely used. The protein gets expressed with a tag genetically attached to it. The most common tags are His_6 (fished out on a Ni immobilised column) and GST (fished out on a Glutatione column). There are, however, some restrictions to these methods. Firstly, it is not always possible to express tagged protein in a properly folded, soluble form. Secondly, proteins with tags are useful for many applications, but not for all. Often tags should be removed with the “specific” proteases and so further purification steps are required and also complications may occur with the protease presence.

In certain cases pseudo affinity chromatography could be considered as a first chromatographic step in the purification protocol:

Heparin chromatography should be used for **DNA-binding proteins**,

Dye chromatography may be used for **NAD/NADP binding proteins**

For purification of the **majority of enzymes and other soluble cytoplasmic proteins**, we propose **“Common sense protocol”** based on using 3 different types of chromatography separating proteins by their

charge (ion exchange chromatography**, IEC**),
hydrophobicity (hydrophobic interaction chromatography, **HIC**) and
size (gel filtration, or size exclusive chromatography, **SEC**).
Common sense dictates the sequence of the steps:

1. **IEC**. It is logical to prepare cell free extract in low salt buffer pH 7-8 and apply it directly on an ion exchange column. For acidic proteins and some basic proteins I propose to use anion exchange chromatography on a **DEAE-Sepharose FF column as a default first purification step applying 10CBV of a 0-0.5M NaCl gradient in tris-HCl buffer pH 8.0**
2. **HIC.** After IEC it is easy to prepare the sample for hydrophobic chromatography by addition of ammonium sulphate. At this stage **an ammonium sulphate cut** could also be considered.
3. **SEC.** After hydrophobic chromatography, the protein can be concentrated by precipitation with ammonium sulphate or by a milder method, using concentration by ultra filtration (pressure unit or spin concentrators) and applied onto a gel filtration column typically equilibrated in buffer 50mM tris-HCl pH 8.0, 0.5M NaCl. Gel filtration could also be performed using a buffer suitable for the further use of TP (for example, 50mM phosphate buffer pH 6.5, 50mM NaCl for NMR experiments) but low salt conditions should be avoided.

As a rule, these 3 steps are enough to purify over expressed protein to purity of 90+% (this is in cases where the level of target protein (TP) expression is higher than 10% of total cell protein). If the level of TP expression is high (20+%) it may be that only 2 chromatography steps are required to reach an acceptable level of purity (IEC – SEC, HIC- SEC or IEC-HIC).

In some cases, especially if the level of TP expression is low, a fourth step should be considered which could be HPLC ion exchange chromatography as a first choice, alternatively, low pressure IEC on a different type of matrix (at the same or different pH as that of the first IEC), HPLC hydrophobic chromatography, chromatofocusing or possibly even more exotic solutions can also be employed, but I never need to do it.

Hydrophobic chromatography is not so easy to adapt. Unfortunately unlike the universal first purification step (IEC on DEAE-Sepharose column with uniform parameters) HIC has to be optimized for every TP.

In the majority of cases a **Phenyl-Toyopearl 650S column** can be used successfully with gradient **of 10CV from 1.5-0M AS** in tris-HCl buffer pH 8.0. Also **Phenyl-HP cartridges** are useful if AKTA system is employed. However in some cases purification could benefit significantly from using a more specific gradient or different type of matrix (strong Butyl-Toyopearl or weak Ether-Toyopearl).

A serious problem is that **for some proteins hydrophobic chromatography is not at all suitable** as they bind to a hydrophobic matrix irreversibly. During development of the purification protocol I suggest to use about 20% of TP sample obtained after IEC to test HIC compatibility in order to avoid losing the whole pool of the protein on a hydrophobic column.

In the case when HIC can not be applied **second ion exchange chromatography** could be considered as a second or third (after SEC) purification step. This could be **HPLC ion exchange** or low pressure IEC on **a different type of matrix** at the same or **different pH** as that of the first IEC.

HPLC ion exchange chromatography was proved to be a powerful method and in some cases may replace gel filtration or hydrophobic chromatography. Particularly I found it **useful for basic proteins**. However, be aware that Resource and Mono matrixes used for HPLC IEC seems to be rather aggressive with respect to non-specific irreversible protein binding. It varies from protein to protein, so sometimes you can get your TP with excellent purity but very disappointing yield.

About 60% of 360+ proteins on my list were successfully purified using above strategy. Further 10% were DNA-binding proteins purified by Heparin chromatography and 20% were His-tagged proteins purified using Ni-NTA chromatography

About 5% of purifications failed due to protein instability or lack of soluble expression

In some cases, especially if level of TP expression is high it may only takes one day to develop a proper "one day purification protocol". Often, when optimisation of hydrophobic chromatography is required by running and analysing the test HIC or if TP expression level is low the development may take two or, in the most complicated cases, three days to finish.

Once developed and refined a three step purification protocol can be completed in one working day (8 hours or so). As a rule there is no need to run SDS-PAGE after each step, you can rely on previous purification to choose fractions with TP after each chromatography.

To achieve smooth quick purification you should have things prepared beforehand:

- Stock solutions
- Columns
- Sonicator
- Chromatography systems (it is convenient to have a separate system for gel filtration). In case of unavailability of a second system, use spare peristaltic pump to equilibrate gel filtration column while first and second purification steps are in progress. The second system is not required if you use default buffer (such as 0.5M NaCl, tris pH 8.0) for gel filtration.
- Bradford reagent, spectrophotometer and cuvettes
- SDS-PAGE equipment and solutions (or ready made gels) Apart from using the optimal sequence of procedures to save time during protein purification there is a number of **Time saving tips (**see **Appendix2.3)** Also always follow the **Golden rules** (see **Appendix2.4**)

Algorithm for development of purification protocol for soluble over-expressed protein

BLOCK I. **Preparations**

1. **Prepare stock solutions, columns and chromatographic systems.**
2. **Perform a solubility test** on the batch of cell paste that you are going to use and confirm that TP was expressed as a soluble protein (see Solubility test protocol in **Protocols** section, number 8). Estimate the level of expression.

If during this test you discover that the TP reversibly precipitates in low salt conditions you may consider using this property at the first step or later in the purification procedure.

3. Using **ExPasy** database find information on the target protein (TP). Using **ProtParam** Tool produce a primary structure analysis. **Work out MW, amino acid composition, isoelectric point (pI) and extinction coefficient of the protein.**
4. If the protein has Cysteines, you may consider adding 1mM DTT to all buffers. However in my practice I've found that it makes no difference on the redox state of the cysteins in the protein whether buffers contain DTT or not.
5. **Prepare cell free extract (CFE)** (see Crude (cell free) extract preparation protocol in **Protocols** section, number 2).
    - For acidic and neutral proteins (pI<8) use Buffer A (50mM tris-HCl pH 8.0).
    - For basic proteins (pI >8) use Buffer A' (50mM MES-NaOH, pH 6.0)
    - For our scale the optimum amount of total protein in CFE is 75-500 mg (1-10g of cell paste).

Save 0.1ml of the CFE for gel analysis

BLOCK II. **Acidic and neutral proteins**. **Anion exchange chromatography**

For self-made columns:

- Wash 20-30 ml DEAE-Sepharose FF column with buffer A. Flow rate 4-5ml/min.
- Apply CFE sample on the column. Collect flow through material.
- Elute proteins with 300 ml of a linear gradient of NaCl from 0 to 0.5M in buffer A.
- Collect 8 ml fractions.

For AKTA system:

- Depending on total protein in CFE use one (total protein <150mg) or two 5ml cartridges DEAE FF.
- In program equilibrate column with 2CV of buffer A. Flow rate 5ml/min.
- Sample applied and column washed with 1CV of buffer A. Collect whole tubes.
- Gradient: 10CV 0 to 50%B (B=A+1M NaCl).
- Fractions: 2-2.5ml for 1 cartridge or 4ml for 2 cartridges.

6. A TP with **pI below 6.5** most likely is bound to the column.

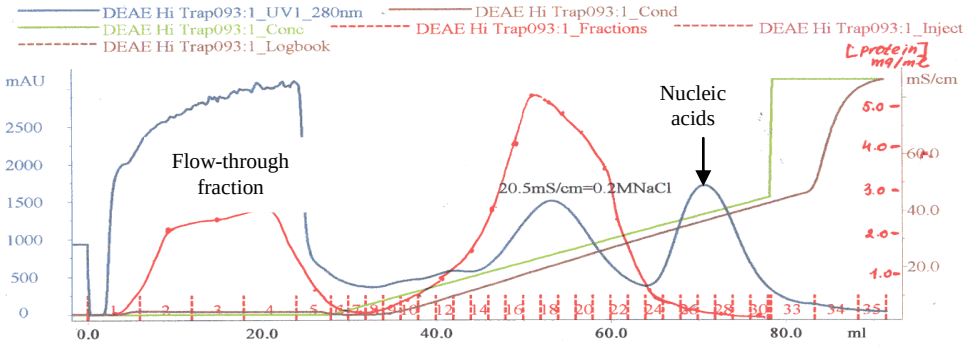
However, **collect flow though fraction** in the separate container or start to collect fractions, **do not discard** it.

- If TP has activity which can be easily assayed (time required for assay is shorter than time required for SDS-PAGE analysis) go to step **7**,
- If assay is not possible go to step **8**.

If TP has **pI close to 7**, then it's probable that it does not bind to the DEAE-Sepharose column. However, some proteins despite having a high calculated pI have acidic domains and so they can be bound to the column.

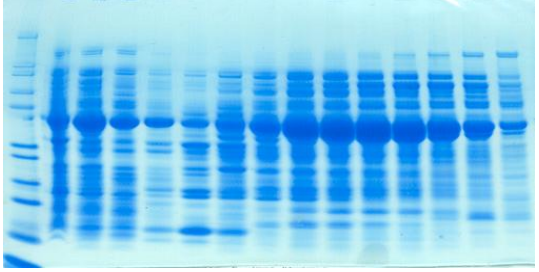
- If you are monitoring TP by activity, calculate total activity in the flow-through fraction to be sure that all activity is there.
- If an activity assay is not available, rely on protein concentration in flow-through fraction. If it is lower than 30% of the protein concentration in CFE, it is likely that protein stays on the column. To be on save side, run gel to find TP (step **9**)
- If TP does not bind to the column, go to step **11**.

7. **Analysis for activity**. Analyse every third/fourth fraction for activity.
   - When a fraction with activity is found, assay each fraction around the active fraction to find every fraction that is active.
   - Check protein concentration in those fractions using Bradford method and calculate specific activity.
   - Combine 2-4 fractions with the highest specific activity, taking about 80-90% of all activity. Only take fractions with higher specific activity than in CFE.
   - If specific activity of pure TP is known estimate its purity in the sample if it is higher than 60% go to step **10**, if it is lower go to step **11**.
   - If specific activity of pure protein is unknown, go to step **11.**
8. **Analysis of protein concentration**.
   - Analyse protein concentration in every second fraction. If expression of TP was higher than 20%, you will probably find a very distinct protein peak, normally in 4-6 fractions which most likely is a TP.
   - To find a TP for sure run SDS gel, step **9.**



Typical DEAE FF chromatogram

9. **SDS-PAGE analysis**.
   - Only fractions containing protein should be analysed, including the flow through fraction. It is not necessary to check each fraction, every second fraction is enough.
   - Prepare samples for a gel analysis as follows:
     - Calculate and take volume of CFE containing 15-20µg of CFE
     - Suspend cell debris in the same volume of water as it was for CFE (the best way to obtain fine suspension is to sonicate it briefly). Take the same volume from suspension as for CFE
     - Take the same volume from flow-through fraction
     - From the fractions take equal volumes so that protein in each sample remains between 2 and 25µg. For example, the highest protein concentration in fractions is 1mg/ml, so take 20-25µl samples from fractions with protein concentration ≥ 0.1 mg/ml. Normally in this step there are about 15-25 fractions containing any significant amount of protein, so a 15 well gel normally suits the analysis.
     - Add reducing agent and SDS sample buffer in the tubes and boil for 1 minute in the preheated heating block. Apply samples on gel and run.
   - Once the gel run is completed, stain gel for 5 minutes with fresh stain and destain it for about 10 minutes. Even better if you can use InstantBlue stain (Experion) or similar stain, then you can see the bands in 5 minutes. Analyse gel to find fractions with the TP.



Typical gel analysis for DEAE FF chromatography

- Combine 3-4 fractions with the highest content of the TP
- Check volume and protein concentration and calculate total protein in the sample
- If purity of the sample ≥ 60%, go to step **10**, if it is lower go to step **11**.
- If TP is found in the flow through fraction, go to step **18**
- Sometimes protein has low affinity to DEAE FF. In this case you can see protein in flow-through fraction and in the first fractions of wash and elution. In this case try **low affinity chromatography** protocol (page 26).

10. Concentration.

- Concentrate protein to 1-2 ml using a Viva Spin 20 concentrator with appropriate MWCO. Go to step **17**.

BLOCK III. **Ammonium sulphate cut**

11. AS cut. Perform analytical AS cut (see **Protocols** number 4).

- If analytical experiment shows that it is possible to obtain more than 60% pure TP using AS cut, perform preparative AS cut (see **Protocols**) then go to step **17** for SEC

Alternatively take simplified approach.

- Take 0.1ml of 4M AS in the eppendorf tube and add 0.1ml of the protein sample (2M AS precipitation).
- Spin down at 13-20000g for 2 minutes.
- Separate supernatant fraction, check protein concentration and work out how much of the total protein has precipitated out.
  - If you have more than 50% of total protein precipitated (presumably TP is in the pellet) go to step **12**,
  - if less than 15% precipitated (you can presume TP is in supernatant fraction) go to step **15**,
  - if more than 15% but less than 50% has precipitated, add 70µl of 4M AS (to 2.5M) in to supernatant fraction and spin down pellet again. Check protein concentration. If more than 50% of protein precipitated go to step **12,** if less, go to step **15**
  - If you use activity to monitor TP, apply assay to reveal TP distribution between fractions.

12. Test of AS pellet solubility. Dissolve pellets in 50-100µl of buffer A,

- Spin down any insoluble pellets in the bench top centrifuge at maximum speed for 2min.
- Check protein concentration in the supernatant fraction and work out how much of the protein has been recovered from the AS pellets.
  - If there is poor recovery of protein from the pellet, go to step **13**.
  - If there is not much insoluble material and recovery of protein from the ammonium sulphate pellet is reasonable, go to step **14**.

13. Rare event when AS precipitation is irreversible:

- Take 0.3 ml of the TP sample and add 0.1 ml of 4M AS (this makes 1M AS in the sample).
  - If protein precipitates, go to **DC(3)**.
  - Otherwise keep adding 4M AS in 10-20 µl portions to find maximum AS concentration at which the TP still stays in solution. Consider this AS concentration as a starting buffer for test **HIC**. Take about 20% of the whole TP sample, add appropriate volume of 4M AS to keep TP in solution and run test HIC as in step **15** with appropriate corrections for the starting buffer. There is a high possibility that TP will not bind to the column, in which case go to **DC(3)**.

14. AS precipitation.

- Take the whole TP sample and add equal volume of 4M ammonium sulphate. Add 1.7 volumes if TP precipitates with 2.5M AS.
- Spin down pellets for 5 min at 19-24K rpm.

- Separate supernatant fraction and dissolve pellet in 1-2 ml of buffer A,
- Spin down any insoluble material, check protein concentration and calculate total protein in the sample.

You should have a good recovery of protein in the sample (70-80%), go to step **17**, run SEC (gel filtration) and you will have your protein purified.

Do not discard any of the pellets or supernatant fractions, keep them for gel analysis

BLOCK IV. **Hydrophobic chromatography (HIC)**

15. Test HIC.

- Take 20% of the TP sample and add 0.6ml of 4M AS per ml of the sample (to 1.5M AS).
- *For self-made column:*
  Wash 10-20ml Phenyl Toyopearl 650S column with 20-40ml of buffer A + 1.5M ammonium sulphate. Flow rate 3-4ml/min.
  - Apply sample supplemented with 1.5M AS onto the column.
  - Collect flow-through fraction in the separate container.
  - Elute with 100-200 ml (10CV) of a reverse gradient of ammonium sulphate in buffer A (1.5-0M AS). Then wash column with 1CV of buffer A and 3 CV of water Collect 5 ml fractions
- *For AKTA system:*
  Use 5ml Phenyl-HP cartridge
  - In program equilibrate column with 2CV of buffer B. Flow rate 5ml/min.
  - Sample applied and column washed with 1CV of buffer B (A+1.5M AS). Collect whole tubes.
  - Gradient: 10CV 100%-0 B, then 3CV of 0%B
  - Fractions: 2.5ml
- Check protein concentration in every second fraction, including flow-through fraction, buffer wash and water wash.
- Run gel to analyse the elution profile. The gel should include: MW markers, CFE, TP sample after DEAE and before addition of AS, flow-through fraction (if there is a significant protein concentration), fractions containing protein, including buffer wash and water wash if there is any protein detected. Quickly stain-destain gel to reveal fractions with the TP.
  - If there is no TP revealed, it means that you probably cannot use hydrophobic chromatography for this protein – go to **DC(2)** (difficult cases).
  - If you can find TP on the gel, work out the AS concentration required for its elution. Using refractometer and **Ammonium sulphate-Sugar refractometer** chart, measure AS in the fractions with the TP.
    - Refine AS gradient: it should start with AS concentration being 0.2M higher than the concentration at elution point. If protein elutes early in the gradient (AS concentration higher than 1.2M) it is useful to get the final AS concentration in the gradient to be 3 times lower than the starting one. If it elutes later than 1.2M, it is better to have buffer A without AS as a final buffer. Go to step **16**.
  - If TP does hot bind to the column, increase AS in the sample and in the starting buffer to 2M and repeat chromatography.

16. Main HIC. Take rest of DEAE sample and add 4M ammonium sulphate solution to bring AS in the sample equal to refined starting buffer. *Save 0.1 ml of the sample before addition of AS for later gel analysis*.

- To calculate volume of 4M AS to be added to the sample (V_{AS}), use formula:
  $V_{AS} = (V_S \times SBC)/(4 - SBC)$,
  Where V_S is volume of the protein sample and SBC is AS concentration (M) in the starting buffer
- Clarify sample by centrifugation for 5-10 min at 45000-70000g.
- Apply sample on a column and elute proteins with 10CV of refined AS gradient.

- Check protein concentration in fraction.
  - If you find a distinct protein peak, combine 2-4 fractions with highest concentration and go to step **10** (concentrate protein on Viva Spin concentrator).
  - In the relatively rare event that there isn't a distinct protein peak or there is more than one peak revealed, run gel to analyse each fractions with protein. Also take for gel: CFE, DEAE sample and MW markers. Combine 2-4 fractions with purest TP, concentrate it by ultrafiltration or precipitate with AS to prepare sample for SEC
  - If protein was already purified by gel filtration, it should be pure - go to **F**. In the unlikely case that TP is still not pure, go to **DC(1)**.

BLOCK V. **SEC (Gel filtration)**

17. SEC.

- Apply 1-2.0ml sample of TP onto a 1.6x60cm Hi-Load Superdex-200 column pre-equilibrated with buffer A + 0.5M NaCl.
- Run gel filtration at flow rate 1-1.5ml/min.
- After 45 ml start to collect 2ml fractions.
- Check UV elution profile. When UV peak is out of the column check protein concentration in each fraction across the peak.
- Run gel to analyse fractions containing the protein.
  - On this gel there should be present: MW standards, CFE sample, DEAE sample, sample applied on the SEC column and each fraction across the protein peak (typically 6–10 fractions). Take 10-25μg of total protein/sample.
  - When gel is completely destained, estimate purity of the TP in the fractions.
    - If you have fractions with the TP of a suitable purity (≥ 80% for crystallization if there are no major contaminants) combine 2-4 fractions with the most pure protein. Go to **F**.
    - If protein is not pure enough and was not subjected to AS cut or HIC chromatography, go to step **11**.
    - If TP is not pure after a three step purification, go to **DC(1).**

BLOCK VI. **Neutral proteins**

18. If all TP comes unbound trough DEAE column:

- Perform analytical AS cut on a flow-through DEAE-Sepharose fraction (see **Protocols**).
  - If TP reversibly precipitates with AS concentration of 2.5M or less, go to step **14**.
  - If it stays in solution at 2M AS go to step **15** (test HIC)
  - If precipitation is irreversible, but TP precipitates at AS concentration higher than 1.5M, go to step **13**.
  - If TP irreversibly precipitates with 1.5M AS, go to step **19**.

19. TP does not like ammonium sulphate. Try pH 5.5 precipitation:

- Take 1-2 ml of flow-through fraction and dialyse it against 100 ml of 20mM Na Acetate buffer pH 5.5 overnight.
- Clarify sample in refrigerated centrifuge, 15000-25000g for 10 min.
- Separate supernatant fraction from the pellet.
- Check volume of supernatant fraction and suspend pellet in the same volume of Buffer A.
- Check protein concentration in supernatant fractions.
- Take a sample for gel analysis from the supernatant fraction (10-20 μg of total protein). Take the same volume of the pellet suspension. Run gel and analyse distribution of TP between pellet and supernatant fraction.
- In the best case, the TP would be found in the supernatant fraction at pH 5.5 and many contaminations would be precipitated.
- Consider pH 5.5 for cation exchange chromatography (CEC) and go to step **20**. If the TP is precipitated at pH 5.5 go to **DC(3)**.

20. Prepare sample for cation exchange chromatography(CEC)

- Dialyse whole flow-through fraction against 0.5-1 litre of buffer C (starting buffer, 20mM NaAc pH 5.5), change to fresh buffer after 2-3 hours and leave overnight.
- Check pH in the sample, and consider another 3-4 hours dialysis with fresh buffer if the right pH has not been reached or adjust pH by addition of diluted acetic acid.
- Clarify protein sample by centrifugation (45000-70000g rpm for 10 min).
- Check volume and protein concentration and perform CEC as described in **22**.
- Check protein concentration in flow-through fraction to find out if the TP binds to the column.
  - If most of the protein is found in flow-through fraction, go to **DC(3)**.
  - If there is significant binding, elute protein by 10CV of gradient 0 to 1M NaCl in buffer C.
  - Check protein concentration in fractions and run SDS-PAGE. On the gel there should be represented: sample before dialysis, pellet obtained after clarification of the sample after dialysis, sample applied on the column, unbound fraction and protein containing fractions obtained after chromatography.
    - If TP is revealed in the fractions and is pure enough – go to **F**,
    - if it is not pure enough, go to step **21**.

21. SEC. Concentrate protein on a Viva Spin concentrator to 1-2ml. Run SEC in the "standard buffer". Perform SEC as in step **17**

BLOCK VII. **Basic proteins.**

22. Cation exchange chromatography.

Prepare CFE in 50mM MES-NaOH pH 6-6.5 and proceed as for acidic proteins, but use SP-Toyopearl, CM-Toyopearl or SP-Sepharose columns instead of DEAE-Sepharose column.

***With AKTA* use SP-HP cartridge(s).**

Be aware that basic proteins sometimes fail to bind to cation exchange columns with no apparent reason. In this case handle them **as neutral proteins**.

If protein binds to the column, elute it with an appropriate NaCl gradient (0-1M in 10 CV). Usually the optimisation of the gradient is required for the best purification.

Purity of the TP most likely could be high enough to consider SEC as a final step to obtain pure TP even if initial expression level was not very high.

23. Run SEC in the standard buffer or alternatively consider a buffer that is at least 1 pH unit lower or higher than pI and has 0.15-0.5M NaCl. Try 50mM MES pH 6.5, 0.5M NaCl.

DC. Difficult cases.

1. The TP is not pure after three chromatography steps.

Consider a fourth step.

- If there is a FPLC system available, try Resource Q (Mono Q or any other anion exchange column) for acidic proteins and Resource S (Mono S or analogues) for basic proteins.
  - Prepare sample in a low salt buffer by dialysis, diafiltration or dilution.
  - First perform a test run with 10-20% of the sample.
  - For the test run consider a 10 CV gradient of NaCl from 0 to 1M in 50mM MES pH 6.5 for both acidic and basic proteins.
  - Collect fractions of 40-50% of CV.
  - Check protein concentration in peak fractions.
  - Run gel to see what level of purification has been achieved.
- If you are not happy with the result, optimise gradient by choosing appropriate start and final salt concentrations as well as slope of the gradient. Typically, start NaCl concentration should be 0.1M lower and finish concentration 0.1M higher than the TP elution point and the gradient length should be 10-15CV. Alternatively try different pH.

- If there is no FPLC system, use low pressure equipment. Try anion exchange chromatography on a DEAE-Toyopearl 650S column or a Q-Sepharose column in buffer A. Also, you can change pH and run chromatography on either of the anion exchange columns at a lower pH. 50mM MES-NaOH pH 6.5 can be considered. For basic proteins increase pH to 7-7.5. Do not discard flow-through fraction as there is a high possibility that protein will not bind to the column.
- If protein is still not pure (and you haven't actually lost all of it by this point!) further options are FPLC HIC, chromatofocusing, preparative PAGE etc. In all my 25 year practice there has not been a single protein which needed a fifth purification step.

2. TP has not been revealed after HIC. There are two ways to go:
- The first way is to do a second ion-exchange chromatography, as described above (**DC 1**).
- The second way is to try a different type of HIC.
  - Take 10-20% of the sample for the tests.
  - The options are:
    - use an Ethyl-Toyopearl column instead of Phenyl-Toyopearl;
    - Use 2M KCl in buffer A as a loading and starting buffer on a Phenyl-Toyopearl column or on a Butyl-Toyopearl column. The risk of losing the TP on above columns is high, but if the protein is bound and eluted from them successfully, there is a high chance of achieving a good purification.

3. TP precipitates with less than 1.5M AS or
basic TP precipitates with pH 5.5 or
TP does not bind to any column

This most likely means that TP is associated with small pieces of debris which are small enough to stay in the supernatant fraction during CFE clarification. Only particles smaller than 2MDa can enter the beads and be bound. If there is a significant insoluble component of the TP revealed in the solubility test this is another sign that, despite the appearance of TP as a soluble protein in the CFE, in reality the TP was expressed as an insoluble one. Try to optimise growth condition during TP expression or try refolding from the inclusive bodies.

However there is small chance that the TP is one of rare proteins which reversibly precipitates with 1.5M AS. In this case you should be able to dissolve it in 1-2ml of buffer A, clarify by centrifugation and perform gel filtration.

F. **Finish.**

Congratulations, you have now got a purified protein.

Run a gel to analyse purification step by step.

Present on the gel should be:

MW markers, CFE, samples after each purification step and all pellets and supernatant fractions which were obtained during purification. Take 15-20µg of total protein per sample.

Analyse the gel pattern and work out the optimal purification protocol for the TP.

Prepare protein for use. I strongly recommend using freshly purified protein when and where it is practical and possible.

For example: concentrate protein to 10mg/ml, change buffer to low salt 10mM tris –HCl pH 8.0 or 50mM NaCl using diafiltration cup on VivaSpin or using desalting spin columns (eg Zeba columns, Pierce) and use it for crystallisation on the same or next day.

Alternatively consider how the pure TP should be stored. (See **Appendix1.3**).

Brief scheme of purification of soluble protein

Cell paste with over expressed TP

↓

Suspend in appropriate buffer ↓ 50 mM buffer pH 8.0

Disruption (sonication or French-Press) ↓

Spin down debris ↓ Centrifugation 45000-70000 g, 10-15 min.

↓

Crude extract

↓

Ion exchange chromatography

anion exchange for acidic protein (column with DEAE-Sepharose or Q-Sepharose)
cation exchange for basic proteins (column CM-Sepharose or SP- Sepharose)

↓

analysis of elution profile (Bradford, activity, SDS-PAGE**), pooling of fractions containing TP**

↓

Ammonium sulphate precipitation analytical trial

Protein precipitates with lower than 2M $AmSO_4$

↓

Precipitate protein with $AmSO_4$

(using lowest possible concentration of $AmSO_4$)
Collect precipitate by centrifugation and dissolve in small volume of buffer

Protein precipitates with higher than 2M $AmSO_4$

↓

Hydrophobic chromatography

Perform test HIC .
Analyse elution profile by SDS-PAGE.

If TP revealed

↓

Run full scale hydrophobic chromatography
Collect TP, concentrate using concentrator or precipitate with $AmSO_4$

If TP is not revealed

↓

$AmSO_4$ cut

↓

Gel filtration

Run gel filtration in appropriate buffer
Analyse elution profile by SDS-PAGE

If purity is higher than 85%

↓

Prepare protein for use or storage
For example:
Concentrate protein using concentrator and exchange buffer on the concentrator, spin column or by dialysis to low salt

↓

Crystallisation trials

If purity is less than 85%

↓

Try *Ion exchange* chromatography on a column with *different matrix* at a *different pH or HPLC column*

Timing for refined purification protocol for soluble over expressed protein

Preparation of the crude extract (CFE)	0.5-1 hour
↓	
Ion-exchange chromatography on DEAE-Sepharose Fast Flow	1 hour
↓	
SDS-PAGE (optional)	1-1.5 hours
↓	
Ammonium sulphate cut or preparation of sample for hydrophobic chromatography in 1.5M $AmSO_4$	0.5-1 hour
↓	
Hydrophobic chromatography	1-1.5 hours
↓	
Concentration on VivaSpin concentrators or Ammonium sulphate precipitation for storage or to prepare a sample for gel filtration	0.5-1hour
Gel filtration	1.5 hours
↓	
SDS-PAGE analysis	1.5 hours
↓	
Concentration and preparation for storage or use	1 hour

The whole procedure takes from 4 hours (for two step procedure) to 8-10 hours (for a 3 step procedure with SDS-PAGE after first step)

Purification of DNA-binding proteins

DNA-binding proteins have a number of specific feathers:

- As a rule they have affinity to Heparin
- As a rule they are not tolerate low salt conditions and can reversibly or sometimes irreversibly precipitate in buffer A.
- Sometimes when cells are disrupted DNA-binding protein could stay bound to DNA.

According to my practice majority of DNA-binding proteins (at least major part of a pool) actually do not bind to DNA. So, the most useful protocol is as follows:

- Destroy cells in buffer A containing 0.1M NaCl
- Apply on a Heparin column (Heparin-HP cartridge for AKTA system) and elute with 10 column volumes of NaCl concentration from 0.1 to 1-2M NaCl
  - Most likely TP is bound and elutes with 0.3-0.8M NaCl. Run gel to analyse purity of TP in peak fractions.
    - If purity is high enough, the second step is gel filtration
    - Alternatively apply IEC (preferably on FPLC column).
    - Specific chromatography on Phospho-cellulose (or Phospho-Cellufine (CHISSO Corp. Japan) were found to give good purification for some of DNA-binding proteins
  - If all or significant of TP is found in flow through fraction most likely it is bound to DNA. In this case use alternative protocol

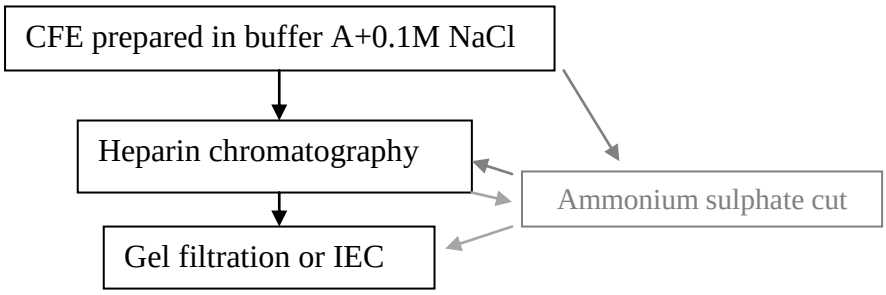
If majority of TP is bound to DNA the protocol is as follows:

- Cells should be suspended and disrupted in a buffer favourable for the DNase activity, (50mM tris-HCl, pH 7.5-8.0, 4-5mM $MgCl_2$). Add 5µg of DNase I per millilitre of cell suspension. Alternatively you can use Bensonase. You may found that some time is required to release TP from DNA. Incubate CFE overnight at 4^oC for the best result.
- However, if the protein is being purified to be used with DNA, DNase treatment should be avoided and cell paste disruption should be performed in 50mM tris-HCl buffer with 0.5-1.0M NaCl.
- In some cases ammonium sulphate cut was found useful as a first step of purification if the TP precipitates with less than 2M of Ammonium sulphate. It may separate protein from DNA and also give some purification. Precipitated TP is dissolved in buffer A and then purified by Heparin chromatography.
- If AS cut is not applied, add 1/10-1/5 of sample volume of 5M NaCl to bring salt concentration in the homogenate to 0.5-1.0M before centrifugation.
- Dilute CFE to bring salt to 0.1-0.2M and apply on Heparin column.
- Use gel filtration or IEC or both for further purification

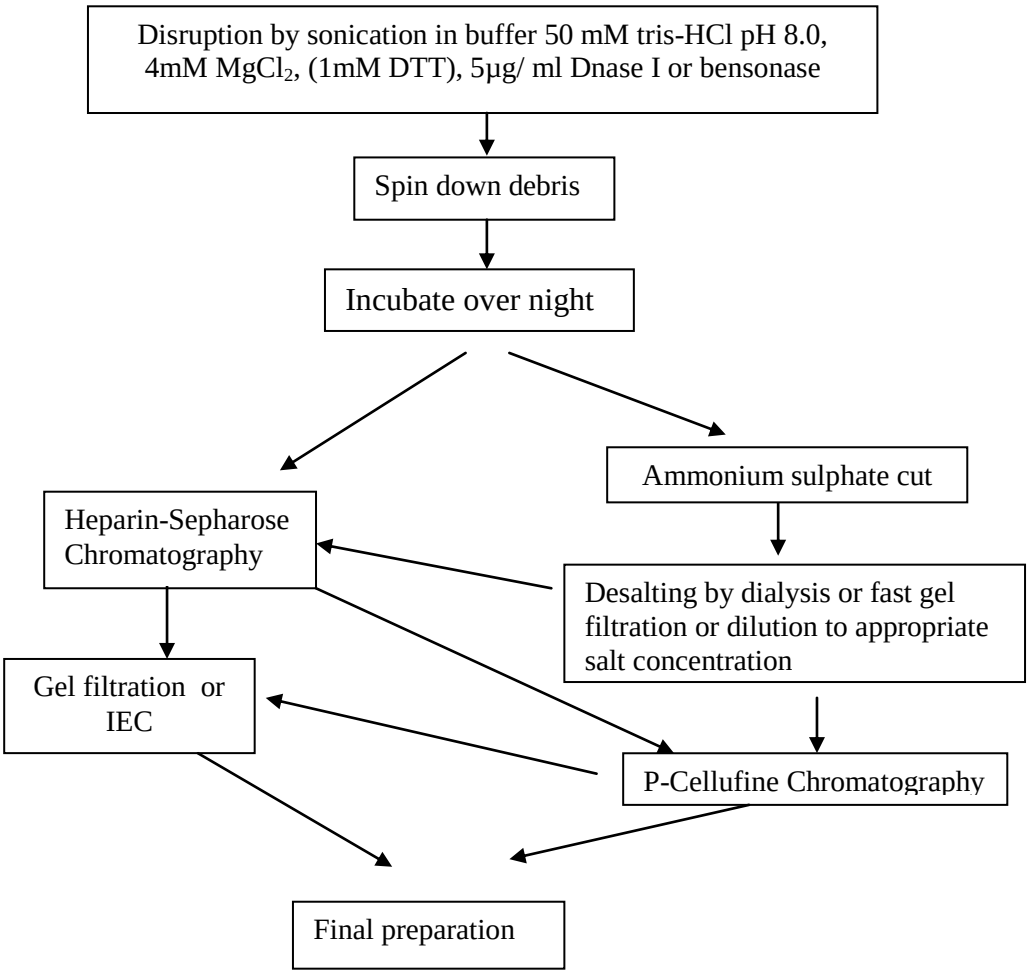
For some DNA binding proteins it was found very effective to use big difference in solubility at different salt concentration.

- Cells can be destroyed in Buffer A and after centrifugation TP is found in the pellet.
- Then it can be extracted using 1M NaCl.
- Next step could be AS cut
- Further purification is performed by gel filtration or Heparin chromatography.

Protocol 1



Protocol 2



Protein purification statistics

3 step: IEC-HIC-GF or IEC-IEC-GF

IGPD I A.t	Malate Syntase
IGPD II A.t.	EngB S .a.
IGPD Rice	YlaN S.a.
IGPD B.p.	YmfM S .a.
GDH C.s.	FtsZ S.a.
GDH P.i	DctP
GDH P.asac.	ICL A.n.
GDH S .s.	Thermochik33
GDH C.g.	Xeed
GDH N.crassa	BPSL:
GDH TADI	2626
GDH M.smeg.	2442
PheDH Nocardia	0672
LeuDH T.int.	0919
AlaDH Sh.sp.	2416
AlaDH PA-43	2169
Ths	0177
LRP	0666
Ap4Ahydrolase	0588
ApaM	BPSS0211
1-ATSquash	Bex B.s.
1-ATSpinach	YqeH B.s
ASS	YqiK B.s
COES	YtfmB.s
GyrB domainE.f.	YdiB B.s.
NifS	IspE B.s
RpoE	TrmU B.s.
ENR e.c.	lipA
ENR B.n.	MglA
βKR Rape	ThiD
βKR B.n.	QueF E.c.
βKR E.c.	AHA1844NTD
ACP	SdhA
BchH	YdaU e.c.
SynI	AHA1844SD2
SynD	EngB B.s.
orfB	Trx reduct. B.p
ortyB	POR
PPase	Urease H.p.
Chitinase	MurI S.p.
Calmoduline	RacE B.s.
PGK e.c.	FabK
PGK B.s.	MAL
HMP	ODH
PNP	Cgb

2 step: IEC-GF or IEC-HIC or HIC-GF or IEC-IEC

IGPD P.f	SbcD
IGPD Yeast	FtsZ b.s.
IGPD A.f.	LuxS B.s.
IGPD M.sm	MenE B.s.
IGPD M.tb.	LysDH
IGPD Acanameba	MurI E.c.
HisB E.c.	MurI A.p.
GDH P.f.	DHOase
PheDH B.sph.	YjeE B.s.
ValDH	YwlC B.s.
AlaDH P.1.	OBG B.s.
GlyDH	EngB B.s.
GlucoseDH	YkuR B.s.
Ths rub.	YukR E..c.
AdHA	YxeP B.s.
hAp4A Hydrolase	YtfD B.s.
GalK P.f.	YiaO B.s
PFK P.f.	YloQ B.s.
Catalase	YacM B.s.
Cot J.c.	YacN B.s.
T24	YacL B.s.
GyrB E.f.	PhoH B.s.
GyrB S.p.	YbfK B.s.
GyrB P.a	YsaA b.s.
GyrB E.c.	YlqF B.s.
GyrB S.a.	TrxA B.p.
GyrB S.pn.	TrXB B.p..
GyrB domain	L1 B.p.
PGI P.f.	BPSL:1958
Yube1	3175
PerR	0515
KasA	2479
KasB	0945
BchI	1449
AconitaseA	1204
AconitaseB	1549
SynH	BPSS:0211
p-23	0212
Rsf-1	0213
Cox2A protease	IspF B.p.
PDF	OueF B.p.
EctC	TssK B.p.
YqiD E.c.	Plu1961
ICL E.c.	Sac1
NorV	TssK CTD
FabG	PGM
Ea10	Lysostaphin
LytN S.a.	TssA
M.smeg3288	TssL
G39P	TssM
G40P	TssK B.c.
PGK B.st	Ysc84 F.1
AhlB	TSL2428
RuvB	SSL3451

His tagged: Ni or Ni-GF or Ni-IEC or Ni-HIC or Ni-IEC-GF

OxdB	Upf 36
OxdRE	Reductase
Xylanase	SlyA
GenD S.a.	FOX
PrsA S.a	P238
IsdA S.a	Ysc84
YjeE S.a	HlyE
RibA B.p.	AHA1844
CbbA B.p.	AHANTD
NugG B.p.	ParA2-GFP
PyrH B.p.	ParB2
BPSL 0667	C5a
VgrG B.c.	Barr1, 2
TssANTD B.c.	TEV protease
TssK B.c.	UTP3
M.smeg:5007	PARP1
6050	TDP1
0515	PARG
5456	Cor
CJ: 0241c	RidP
1224	DnaD
0045	Rpa:3723
0421	3724
0241	3725
0422	2805
0424	2806
NDH2	hASNS
Trb1	ORF100
Protease 3C	ORF124
PLAT	LysM
CT2	KEG 15107
NinH	Rv: 1288
IF4A-I	1675c
Ref2-1	WhiB1
Tap-P15	WhiB4
Hac-1	AhlA, B, C

DNA-binding proteins

RadB	67RuvC
SarA	Reg G e.c.
RuvAe.c.	RegG B.s.
RuvA mycopl.	EnrI
RuvA B.s	A2-3
RuvC	D5
RusA	T5 FEN
Hjc	hFEN
RecU	Tryp. FEN
UDF	FEN S.pn
FLP	SeqA
YneS s.a	DnaBX-p16
Fur S.a	SfsA P.f.
RdgC	SfsA e.c.
BPSL 0128	ParA2
1840	Taq polymerase
0606	CsoR
Rap	LrpC
FrmR	G40P
	AsnC

Non-typical purifications (4 step, unusual columns, unusual procedures)

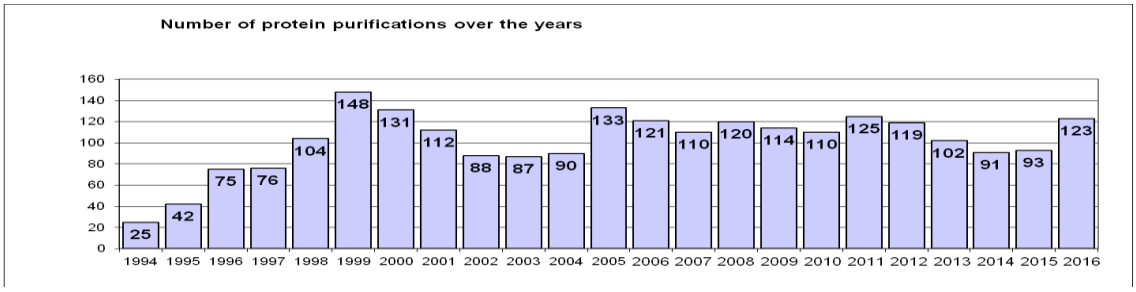
CEOS	Whl
CD9	Bacm
Carnein: P50,	BFL1-FAB
P36, P80	EK
SA0447	IF4A-I
Fab fragment	YdiC s.a
Glyl	YdicE
CotY	LH2
NheA	N2O reductase
NheB	bd-oxidase

Fused proteins with cleavage

Sagl5	GST: α1, α2 peptides
Sagl9	CD81
BcDef	Rho
TssA CTD	GAP
	Cdc42

NodF	BPSL	TssE
S4, P63	2999	SH2
radI	2265	Mytanlysin
hGalk	CBD	NUMA
Arp	Cyp51	MenF
YmdaS.a.	ProH	BhcP

Purifications failed because of lack of soluble expression or protein instability



IV. Protocols

1. Protocol for preparation of the stock solution

Salts stocks preparation

1. Take a 1 litre glass beaker with a big magnetic follower bar and pour in the weight of salt powder that is required.
   To prepare 1litre of 1M solution of any substance take amount in grams equal to its MW.
   For 5M NaCl weight 292g. For 4M Ammonium sulphate weight 528g.
2. Pour ultra pure water into the beaker to approximately the 900-950ml mark.
3. Place on a stirrer and switch on. At the start you should help the magnet to start rotating by stirring the slurry with a rod or a spatula.
4. When the salt has dissolved pour the solution into a 1 litre volumetric flask.

- To make sure all the salt has been washed into the flask, rinse the beaker with 2-3 small volumes of ultra pure water and add them to the flask.
- Make the volume up to the 1 litre mark with additional ultra pure water.
  *You may have a problem with dissolving all the **ammonium sulphate** because 4M is close to the saturation point. To encourage dissolving, you can gently heat the mixture up to 40-50^{o}C under constant control (to prevent overheating). Alternatively you can wait until most of the salt is dissolved, switch the stirrer off and gently pour the clear solution in to the volumetric flask. Add a little bit of ultra pure water to the rest of the salt in the beaker, stir it briefly and add any clear solution to the volumetric flask. Do this 2-3 times until all the salt has dissolved. **Be careful not to exceed 1 litre!!!***

4. Filtrate solution through a 0.22μm filter using Filter Holder with Cellulose Nitrate Whatman filter or Stericup Filter Unit connected to a vacuum pump. **Always wet the membrane with a few drops of ultra pure water before you start filtration.**
   Using the vacuum pump wash Filter Holder's porous disk with plenty of grey tap water immediately after use to prevent salt crystallising in the pores and therefore blocking the filter.
5. Pour solution into a clean bottle. Write the date and your initials on it.
6. For a control measure, check the refraction of the solution. For 4M $(NH_4)_2SO_4$, it should be 37% and for 5M NaCl it should be 28% on a sugar refractometer.

Buffers stocks preparation

1. Weight required amount of buffer powder in to 1litre glass beaker with the magnet in.
   For 1M solutions take Tris-121g, MES- 195g, HEPES-238g.
   Add ultra pure water to about 600-700ml and place beaker on the stirrer next to pH meter.
2. To adjust pH in MES and HEPES solutions use 5M NaOH or 5M KOH solutions. When pH is OK, adjust volume to 1 litre using volumetric flask and filtrate solution through 0.22μm filter.
   To adjust pH in Tris buffer place the beaker with the solution in to the ice-water bath and on the stirrer next to pH meter preferably under fume hood. Deepen pH probe and thermometer in the solution. Use concentrated HCl for titration. Take care, wear gloves and do not inhale fumes. First adjust pH to about 9. Wait while temperature droops to 20˚C and continue to add acid slower. Finally it should be desirable pH (usually 8.0 or 8.5) at 20˚C.
3. Proceed with adjusting volume and filtration.

2. Protocol for quick and effective cell disruption and preparation of the crude extract (cell free extract)

1. Cell harvesting
   - Spin down cells first in the big bottles
   - Re-suspend pellets in about 50 ml of culture medium, place in a 50 ml Falcon tube and spin down for 10-15 min at 5000 rpm.
   - Remove medium and put tube with cell pellet in to the -20°C or -70°C freezer.

   Alternatively suspend cells in Lysis buffer (see p16) (5-10 ml per gram of cell paste), pour into a Falcon tube and put it in to the freezer.

   Remember to use Virkon for decontamination of discarded medium

2. On the day of protein purification:
   - take cell paste from the freezer, add 8-15 ml of lysis buffer (e.g. Buffer A) per gram (ml) of cell paste
   - let it thaw for 5 minutes, briefly suspend cell paste with spatula
   - divide suspension into 10-15 ml portions placing them into 20 ml plastic vials using a plastic pipette . If there is small amount of cell paste (1g or less) you may use small 5 ml containers to place 3-4 ml of cell suspension in each.
   - Place vials on an ice bath.

   Tip: for more effective cooling put small pieces of ice into the vials.

   If cells were frozen with buffer, for quick defrosting place tube in to warm water or hold under a hot water tap, mixing by tipping upside down until it thaws. Then divide into portions as above.

3. Before you start sonication, put rotor (JA-20 or JA-25.50) into the Avanti centrifuge, close the lid and set pre-cooling at 4°C

4. Mount medium probe on a Soniprep 150 machine. Tighten it properly with the tool, but do not over-force.
   - Lower probe into the vial with the sample, leaving 2mm between the probe and the bottom of the vial; the probe should not touch the bottom of the vial but neither should it be close to the surface of the sample. If the probe is too close to the surface, foam will form, this should be prevented to avoid oxidation and denaturing of proteins.

5. Set sonicator for maximum force. This corresponds to 16 micron amplitude.
6. Sonicate portions one after another for 20 seconds each. Carry out 2-4 cycles without a break. The samples will cool down while you treat other portions. If you use small (5ml) containers, treatment should last for 8 seconds. If you have got just two portions to treat, be more careful and put ice pieces into containers between the sonication cycles.

   If power does not reach 16 micron, try reattaching the probe (tighten it more).

7. After completion of sonication unscrew the probe, wash it with distilled water and dry with tissue. Place back in the box.

8. Pour homogenate in to the centrifugation tubes, balance them and spin down in JA-20 or JA-25.50 rotor at 4°C
   - for JA-20 rotor in J-20 centrifuge set 19000 rpm (about 43000g) for 15 minutes
   - for JA-25.5 rotor in J-25 Avanti centrifuge set 24000 rpm (about 70000g) for 10 min

9. Once centrifugation has finished pour supernatant fraction into the cylinder (if you use peristaltic pump for chromatography) or in the beaker (for AKTA system application) and keep it cold. Check volume. Check protein concentration (see protocol for Bradford assay in Appendix). Calculate total protein.

The **Crude extract (Cell Free Extract)** is now ready for the next step.

3. Protocol for protamin sulphate (PS) treatment

1. Measure volume of the crude extract
2. Calculate an amount of protamin sulphate taking 2mg for each ml of the crude extract, weigh this amount into an eppendorf tube and suspend it in 1 ml of water. It takes a long time for protamin sulphate to dissolve, so do not wait and use the suspension instead.
3. Place a beaker with the crude extract into an ice bath or in the cold cabinet or cold room at 4ºC on a stirrer and set one to an appropriate speed (the vortex should appear in the middle of the beaker).
4. Add protamin sulphate suspension drop by drop. Stir for about 10 minutes.
5. Spin down precipitate at 40000g for 15 minutes or at 70000g for 10 minutes.
6. Recover supernatant fraction, check volume and protein concentration.

4. Protocol for analytical ammonium sulphate cut (AS cut)

1. Take six 1.5ml eppendorf tubes and mark them as follows:
   (I) 1.5M pellet, (II) 2.0M pellet, (III) 2.5M pellet,(IV) 3.0M pellet, (V) 3.5M pellet, (II) (VI) 3.5M supernatant.
2. Add 0.3ml of 4M AS solution in the tube (I) and 0.2ml in the tube (II). Weight 66mg of AS powder into each of tubes (III), (IV) and (V), leave tube (VI) empty.
3. Place 0.5 ml of the cold crude extract in tube (I) and mix by pipetting in and out. Leave for 10 minutes on ice. Spin down for 2 min in the bench centrifuge (13000rpm) or, better still, in a refrigerated centrifuge. Carefully take out supernatant fraction and add it into the tube (II).
4. Repeat the above operation with tube (II), then tubes (III), (IV) and (V). Each time dissolve AS powder in the supernatant fraction from the previous tube, incubate on ice and spin down the pellet. Last supernatant fraction goes to the tube (VI).
5. Add 0.1ml of Buffer A (50mM tris-HCl pH 8.0) into each of the tubes (I), (II), (III), (IV), (V) and dissolve the pellets.
   Check protein concentration in each tube, including tube (VI).
   You will probably find a very low protein concentration in tube (VI) (the 3.5M supernatant fraction).
6. Run gel to see which fractions contain the target protein. Take equal volumes from all fractions to prepare samples for the gel. You may have a problem with 3.5M supernatant fraction as it will have a very high AS concentration and low protein concentration. If you recover less than 10% of total protein from this fraction it is best to exclude it from the analysis. If there is a significant amount of protein, dilute it 4 fold with buffer A and concentrate to 0.1 ml using VivaSpin6 concentrator.
7. Analyse the pattern on the gel and develop preparative AS cut strategy.

5. Protocol for preparative ammonium sulphate cut

1. Pour protein solution or crude extract in to a beaker with a magnetic bar in it. Choose a beaker volume 2.5-3 times larger than the volume of the sample.
2. Place beaker on ice (or at 4˚C) and on the stirrer. Set stirrer to mix solution with a speed which allows a vortex to form in the middle of the sample.
3. If the first precipitating concentration is 2M ammonium sulphate (50% saturation) or lower (this would be found during the analytical experiment), use 4M AS stock solution to bring the sample up to the desirable AS concentration. This is a much gentler way than using powder. Calculate the volume of the 4M AS solution needed and add it to the beaker. Stop stirrer and incubate for 15 minutes.
4. Spin down pellets in JA-20 or JA-25.50 rotor at 19000 rpm for 10 minutes.

5. Measure the volume of the supernatant fraction and pour it back into the beaker that is on the stirrer (in ice bath or at 4˚C). Check protein concentration to control the process.
6. Using table (see **Charts and Tables**), work out the amount of AS powder that is needed to precipitate the target protein.
7. Weigh out the required amount of powder and add it to the sample (with stirring) in small portions, allow previous portion to dissolve before the next one is added. When all the salt has dissolved, turn off stirrer and leave sample for 15-30 minutes.
8. Collect pellets by centrifugation as above, remove supernatant fraction completely and dissolve protein pellet in the appropriate buffer. Check volume and protein concentration in the sample.

Remember that precipitation of protein depends on precipitant as well as protein concentration. Carry out the preparative and analytical experiments using the same protein concentration.

NOTE: a significant concentration of AS is still present in the sample!

6. Protocol for precipitation of proteins using ammonium sulphate

1. Measure the volume of the protein solution, pour it into a beaker with a magnet bar and place it in an ice bath or at 4^{o}C on a stirrer.
2. Calculate the required amount of ammonium sulphate, taking 0.6 grams of salt per millilitre of the protein solution and weigh the amount out.
3. Start stirring the solution and add salt to it in small portions, allow salt to dissolve before adding the next portion.
4. When all the salt has been added take beaker off stirrer and leave it at 4^{o}C for some time, ideally overnight.

As a rule, you can store protein this way for a very long time.

7. Recovery of protein from the ammonium sulphate precipitate

1. Place an appropriate volume of the ammonium sulphate precipitate suspension into a centrifugation tube.
2. Collect precipitate by centrifugation at 45000 g for 5 min at 4^{o}C.
3. Remove supernatant fraction carefully, try to take out every last drop of ammonium sulphate solution.
4. Re-suspend pellet in a small volume of appropriate buffer and dialyse it against some 10-100 volumes of buffer. Make 2-3 changes, the last one should be left overnight for full equilibration. After 2 changes you can expect there to be about 1-2mM of ammonium sulphate left in the sample, with 3 changes it is close to 0. As an alternative to dialysis you can use desalting columns.
5. It is useful to clarify sample from possible insoluble contaminants by centrifugation at 25000g or higher for 15 min.

Remember that some proteins can not be recovered from ammonium sulphate precipitate

8. Analysis of solubility of expression

A. "Bug buster" method

Preparation of the solutions:

Reagent A: BugBuster Protein extraction Reagent from Novagen

Reagent B: Benzonase Nuclease (product 70746 from Novagen), 25u/µl, 0.2ml.

- Divide this 0.2ml sample in to 20x10µl portions in 0.5ml eppendorf tubes. Store at -20˚C (not -70˚C).
- Prepare 4ml of the buffer 50mM tris-HCl pH 8.0, 20mM NaCl, 5mM $MgCl_2$, 20% glycerol and make 20x190µl aliquots in 0.5ml tubes, keep them frozen at -20˚C.
- Prepare working solution: Take buffer from the one tube and add it into the tube with 10µl of benzonase making 20 fold dilution). Make 50x4µl aliquots in 0.5ml tubes and store them at -20˚C. Mark these tubes "reagent B".

Method:

1. Take one tube of reagent B from the freezer and add 0.1 ml of reagent A (stored at room temp.)
2. Prepare about 20 µl of cell paste pellet in a 1.5ml tube. To get this, spin down 1-1.5 ml of culture or take a bit of frozen cell paste.
3. Add 0.1 ml of reagent A+B to the cell paste. Suspend it properly using a small plastic rod. **Do not** pipet up and down to avoid bubbles.
4. Place on a rotating platform and incubate for 10 min.
5. Spin down debris at 20000g for 5-10 min in the refrigerated centrifuge.
6. Take out supernatant and place into another tube. Mark it 'S'
7. Add 0.1 ml of 2% SDS solution to the tube with pellet and suspend it properly with a rod. Mark it 'P'.

B. Sonication method

1. Suspend 30-50 µl of cell paste in 0.3-0.5ml of Buffer A in 1.5 ml eppendorf. Place on ice.
2. Using thin probe apply sonication at 16 micron amplitude for 2-3 sec. It is most convenient to hold tube in hand to control temperature to avoid overheating. Place tube on ice for 20sec to cool down.
3. Repeat sonication 3-4 times.
4. Spin down debris in the refrigerated centrifuge at 4^oC, for 10 min, at 20000g.
5. Separate supernatant fraction into another tube (mark it 'S').
6. Re-suspend pellet in equal volume of water or buffer or 2% SDS (mark tube with 'P').

For DNA-binding proteins for sonication take buffer:
1M NaCl, 50 mM tris-HCl pH 8.0 or use **Alternative way:**
Re-suspend debris pellet in 0.1 ml of 50 mM tris pH 8.0, 1M NaCl,
Spin down pellet as above.
Separate supernatant fraction in to a clean tube. Mark tube S1.
Re-suspend pellet in water or SDS as above (tube P).

SDS-PAGE analysis

1. Check protein concentration in supernatant fraction (tube S) and in tube S1 (if applicable)
2. To prepare a "Soluble proteins" sample take about 20 µg of total protein from tube S for a sample for gel, add water to make total volume 15µl if necessary. Take approx 10µg of protein from tube S1 if there is any noticeable protein concentration.
3. To prepare "Insoluble proteins" sample take the same volume from the tube P as you took from tube S for a sample for gel, add extra SDS (10% solution) so as to make the SDS concentration about 4%.
4. Preheat dry block to 100^oC or make bath with the boiling water.
5. Add 4x-Sample Buffer and 10x-Reducing agent (both from Invirogen) to the samples and boil them for 2 min. Apply on a gel. To make your gel more informative place pre-induction cells sample prepared the same way as over-expressed cells sample.

9. Analysis of expression for low expressed His tagged protein

1. Prepare CFE in BufferA+0.5M NaCl (0.5M NaCl 50mM trisHCl pH 8.0)
2. Using yellow tip with cut off end take 50µl of 50% Ni beads suspension in to 0.5 or 1.5ml eppendorf (depends on volume of CFE you are going to use).
3. Wash beads with above buffer :
    - Spin down beads at 1000g (preferably in swing out rotor) for 1min and remove liquid.
    - Add buffer to fill the tube, spin beads down
    - Remove liquid carefully , do not touch beads , leave a little bit of buffer above them
4. Add CFE to the tube to fill the tube to top.
5. Incubate on wheal in the cold cabinet for 0.5-1hour.
6. Spin down beads and take supernatant fraction in to the new tube (Unbound material)
7. Wash beads to remove traces of unbound material:
    - Fill tube with buffer. Realise buffer from the tip aggressively, so all beads are moved. Mix gently by reversing tube, do not vortex.
    - Spin down and remove supernatant fraction. Be careful do not take beads, leave a little bit buffer above them.
    - Repeat wash, but this time try to take out as much buffer as possible. Use gel loading tip to take the last portion of buffer from the beads. Still be very careful, do not allow any beads in to the tip.
8. Elute protein from the beads:
    - Add 50µl of 1M Imidazole in above buffer (1M Imidazole, 0.5M NaCl, 50mM tris pH 8.0)
    - Mix very gently with “cut off end” yellow tip. Some of the beads will be left in the tip, it is OK, ignore them.
    - Spin down beads. It is not necessary to take supernatant fraction out.
9. Analysis by SDS-PAGE
    - Check protein concentration in the eluate. Take 10µl for Bio-Rad assay.
    - Take 10-15µg of eluate for the SDS-PAGS sample. If protein concentration is low, take 25µl of the eluate.
    - Take 10-15µg of CFE and **equal volume** from Unbound material to make SDS-PAGE samples.
    - Run gel : Mark12, CFE, unbound, eluate

10. Bio-Rad protein assay protocol

Bradford Method

(Sveta's adaptation of the Bio-Rad micro assay procedure)

1. Into a plastic cuvette (1.6 ml volume) place 1-20 µl of protein solution, containing approximately 1-10 µg of protein

2. Add 0.8 ml of ultra pure water and 0.2 ml of Bio-Rad Dye Reagent Concentrate

3. Seal cuvette with parafilm and mix carefully by tipping upside down and right way up again.

4. Measure OD_{595} . It should be between 0.1 and 0.7.
   If readings are higher, dilute protein solution or take smaller volume, if they are lower, increase volume of protein solution taken for test and decrease the volume of water accordingly to keep total volume of test within 1 ml.

5. Calculate Protein concentration using formula:

$$\frac{OD_{595} \times 15}{\text{volume of protein (µl)}} = \text{protein concentration (mg/ml)}$$

Usually while monitoring protein concentration during purification we do not need high accuracy, but still be careful in aliquot handling and pipetting especially when you take small aliquots. I would not recommend you to take less than a 1µl sample. When taking 1µl, always check amount of liquid in the tip and remove any liquid attached to the outside surface of the tip. After pipetting sample out make sure that the tip is empty. Do two repetitions and if difference between them is more then 10%, repeat it again!

11. Protocol for accurate determination of concentration of pure protein

1. Place 1ml of 6M GuHCl, 20 mM NaP, pH 6.5 into a quartz cuvette and zero it at 280 nm on a spectrophotometer.

2. Add 10-50 µl of protein solution containing about 0.1- 0.3 mg of the target protein into the same cuvette, seal with parafilm and mix by tipping upside down. Take measurement at 280nm. For a reliable result the reading should be between 0.1 and 1.

3. Calculate concentration of protein using Abs 0.1% (1mg/ml) (this is given in the ProtParam data in Expasy data base for your protein). Multiply by dilution factor.

4. Using the same protein solution make a calibration plot for Bradford (Bio-Rad) assay and calculate accurate factor which you can then use for accurate determination of concentration for this protein by the Bradford method.

5. Place 1ml of appropriate buffer (for example Buffer A, PBS or Buffer A+0.1M-0.5M NaCl) into a quartz cuvette and zero it in the range 240-340nm. In the same cuvette add the same volume of the protein solution as in step 2 and take spectrum from 240 to 340nm. If there is no significant light scattering between 300 and 340nm (absorption is close to 0), calculate the extinction coefficient (at 280nm or at maximum) for the target protein under non-denaturing conditions (in the given buffer) (see **Appendix** for details).

12. Calibration plot for gel filtration column

1. Find suitable proteins to cover MW from 500kDa to 5kDa. Apart of proteins listed in the LMW and HMW kits (GE Healthcare) the following proteins may be suggested: Glutamate Dehydrogenase (330kDa), Catalase (250kda), , Amylase (200kDa), Alcohol Dehydrogenase (150kDa), Bovine albumin (66kDa), Trypsin inhibitor (20.1kDa), Cytochome C (12.3kDa), insulin (5.8kDa).
2. Chose set of 4 proteins with MW differs from each other 2-3 fold. Say, Catalase, Bovine albumin, Trypsin inhibitor, insulin. Or GluDH, AlhDH, Ovalbumin, Cytochome C and so on.
3. Balance about 10mg of each protein in to 1.5ml tubes and dissolve in 1ml of buffer 50mM tris pH 8.0, 0.1M NaCl.
4. Take optical density at 280nm for each protein. In order to do it make 0.8ml of 10 fold dilutions (0.72ml buffer+0.08ml protein solution) and take measurements in the 1ml quartz cuvette. Alternatively use NanoDrop or Nanophotometer.
5. Calculate volume of each protein corresponding to 1optical unit, take corresponding volume of each protein and mix it in the new tube. For example: 280nm density was: for protein 1- 6ou/ml, for protein 2 - 8ou/ml, for protein 3 - 10ou/ml, for protein 4 - 4.5ou/ml. To make a mix take 0.167ml of protein 1, 0.125ml of protein 2, 0.1ml of protein 3, 0.22ml protein 4.
6. Make volume of the mix to 1-2ml and apply on a column (1.6x60cm HiLoad Superdex). Run gel filtration at the same flow rate and in the same buffer as for an experimental protein. For smaller or bigger columns adjust amount of proteins accordingly (0.25ou/protein for 1x30cm Superdex columns or 2.5ou/protein for 2.6x60cm columns)
7. Carefully measure elution volume (Ve) of each peak. If you use a peristaltic pump and a pen-paper recorder it is important to measure the actual flow rate carefully in order to get accurate Ve values.
8. To make calibration plot you also have to know void volume Vo and total column volume Vt. For total volume take height of the column bed in cm, multiply by cross area of the column) For 1.6x60 Hi-LoadSuperdex200 column normally total column volume equals 120ml. To find Vo run separate run of Blue dextran. Apply 2-3 optical units at 280nm and take elution volume of the first peak as a Vo. For 1.6x60 Hi-LoadSuperdex200 this value typically is around 45ml. This value is rather stable; do not check it each time unless the column size was changed (as a result of a drying event or cleaning of the top of the column).
9. For full and more accurate calibration run 2 sets of different proteins.
10. Make plot K_{AV}=(Ve-Vo)/(Vt-Vo) versus logMW. The another way to plot results may be logMW versus Ve/Vo. Both give you a linear plot, but latter version is not used nowadays.

13. Ni-chelating cartridges: cleaning and recharging

To recharge Ni-chelating cartridge wash it using syringe:

1. with 5 volumes of ultra pure water
2. with 5 volumes of 0.2M EDTA
3. with 5volumes of ultra pure water
4. with 5 volumes of 0.1M $NiSO_4$ or $NiCl_2$ or 0.3 volumes of 1M Ni salt
5. with 5volumes of ultra pure water
6. with 5 volumes of the starting buffer.

Clean cartridge when flow rate have noticeably decreased.

- Try to wash column with at least 2 volumes of 0.2M EDTA and 2 volumes of ultra pure water
- Find the set of adapters and connectors to connect syringe to the bottom of the cartridge
- Fill syringe with 1M NaOH, connect it to the bottom of the cartridge carefully, do not allow air into the cartridge and wash it with at least 2 volumes of 1M NaOH. When cartridge is

blocked with the dirt it is not easy to push liquid trough it. Be patient, take your time, do not push too hard.

- Wash cartridge with at least 2 volumes of ultra pure water
- Apply syringe on the top of the cartridge and wash with at least 1 volume of 1M NaAcetate pH 4.0. At this point flow rate most likely to be significantly increased.
- Wash cartridge with 5 volumes of ultra pure water and charge with Ni.

14. SDS PAGE (Laemmli, Nature, 1970, 227, 680-685)

Sveta's easy protocol

Stock solutions:

1) 1.5 M tris-HCl, pH 8.8
2) 1.0 M tris-HCl, pH 6.8
3) Acrylamide 30% : (30g acrylamide, 0.8g bisAA per 100ml).
   Use ready made solution (better) or make from solid reagents
4) Acrylamide 20% (30% solution diluted 1.5 fold with H_2O)
5) Persulphate ammonium 10% (freshly made)
6) SDS 10%
7) TEMED

10 X Running Buffer: 144 g Glycine, 30g tris, 10g SDS.
For a run dilute 10 fold with distilled water.
pH is 8.3, do not adjust !!!

Sample buffer:
for 20 ml of 5x buffer:
6.25 ml 1M tris pH 6.8, 2g SDS, 5g sucrose, 1mg bromphenol blue, water to 18 ml. Before use, add 0.1 ml of 0.5M DTT to 0.9 ml of
buffer for reducing conditions.

It is better to use ready made 4x sample buffer and 10x reducing agent from Invitrogen (now Life technologies) .

Gel preparation:

component	separation gels (for 8ml)				stacking gel 5% (for 4ml)
	7.5%	10%	12.5%	15%	
1.5M tris pH 8.8	2ml	2ml	2ml	2ml	-
AA 30%	-	-	2ml	4ml	-
AA 20%	2ml	4ml	2ml	-	1ml
H_2O	4ml	2ml	2ml	2ml	2.5ml
SDS 10%	80µl/	80µl	80µl	80µl	40µl
TEMED	10-20µl	10-20µl	10-20µl	10-20µl	10-20µl
PSA 10%	40µl	40µl	40µl	40µl	20µl
1M tris pH 6.8					0.5ml

15. DTNB reaction

Reagents:
bufferN: 0.1M Na phosphate , 1mM EDTA pH 7.3
bufferG: 6M GuHCl, 0.1M Na Phosphate, 1mM EDTA pH 7.3
DTNB reagent: 10mM in bufferN
Reaction:

1. Take 0.9ml of bufferN or bufferG in cuvette and add 0.1ml of DTNB reagent. Mix and use the cuvette to blank spectrophotometer at 412nm.
2. To the cuvette add 10-50ul of the protein solution (0.5-1mM), mix, incubate for 1-2 minutes and take measurement at 412nm.
3. Calculations:
   $A_{412}/0.0136$ = concentration of TNB in µM

(concentration of TNB in µM) / (concentration of protein in µM)= SH groups/protein molecule

Make 2-3 protein concentrations

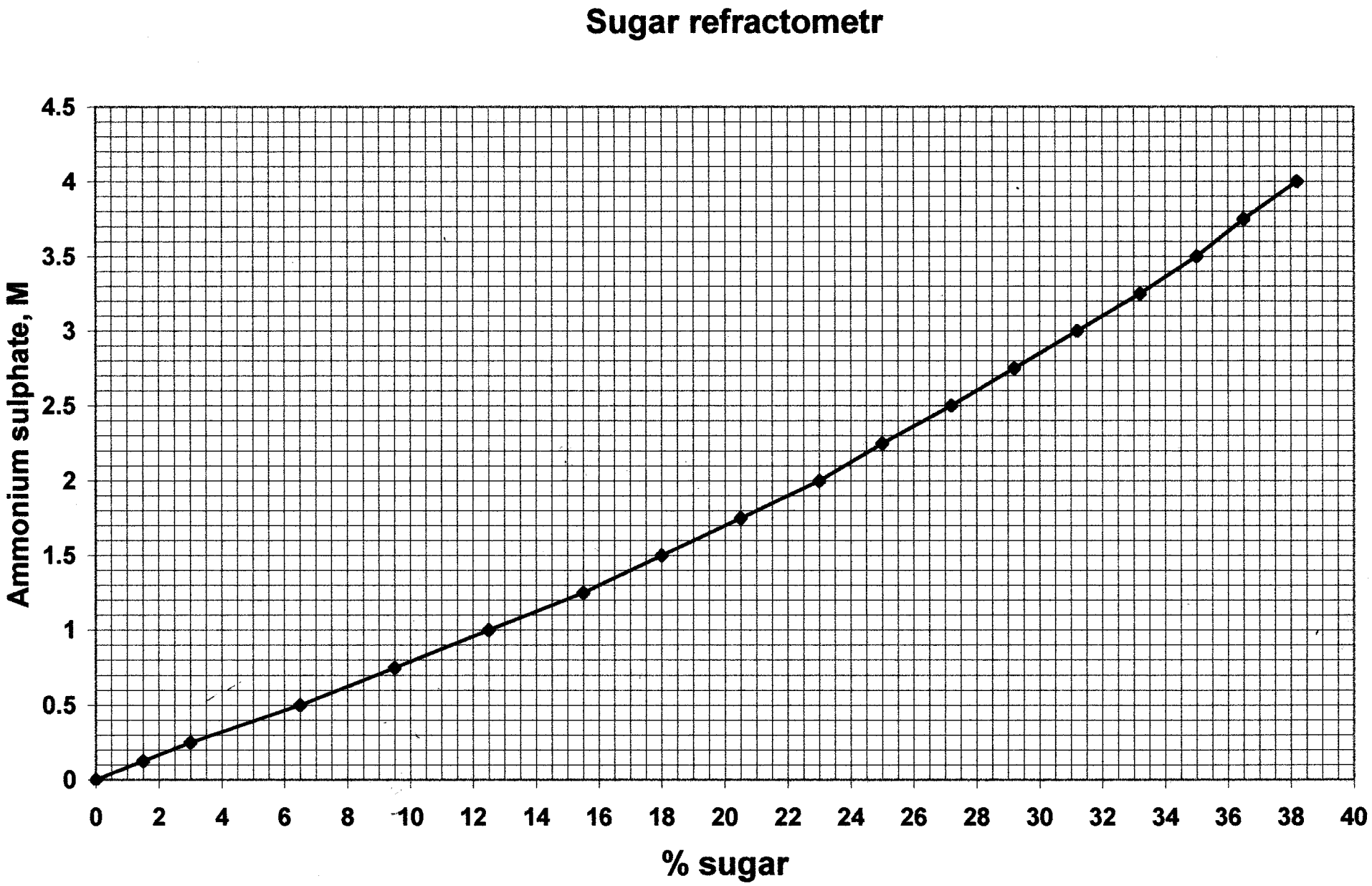
16. Refolding protocol

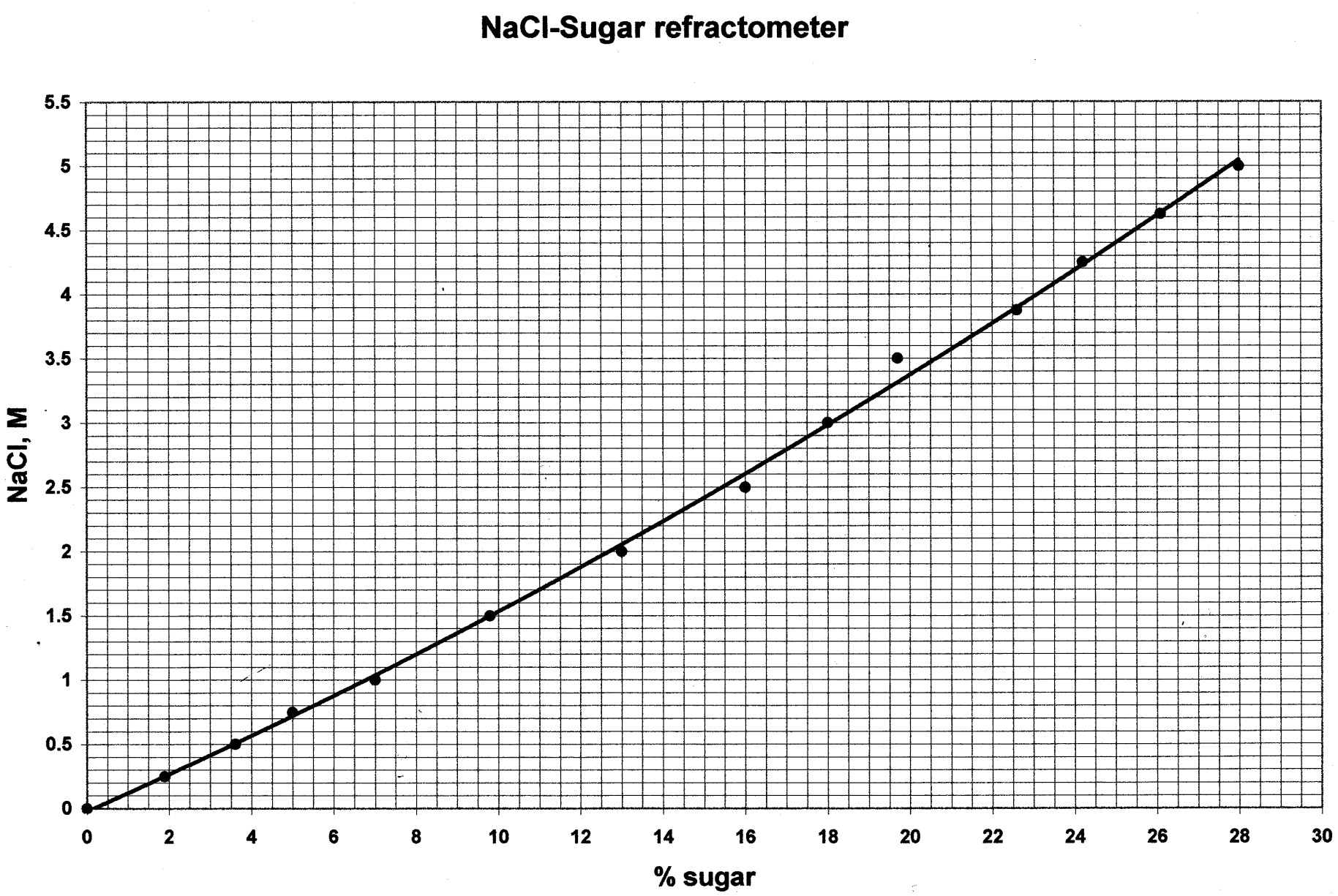
Buffers:
Buffer R : 40 mM tris-HCl pH 8.0, 1 mM EDTA, 1M NaCl.
Buffer U: 8M Urea, 50 mM DTT, 2 mM EDTA, 40 mM Tris-HCl pH 8.0.
Make about 20 ml of buffer U, take urea and DTT as a powder, EDTA and Tris as a stock solutions. Filtrate with 0.22µ filter, keep rest of buffer at -20°C.

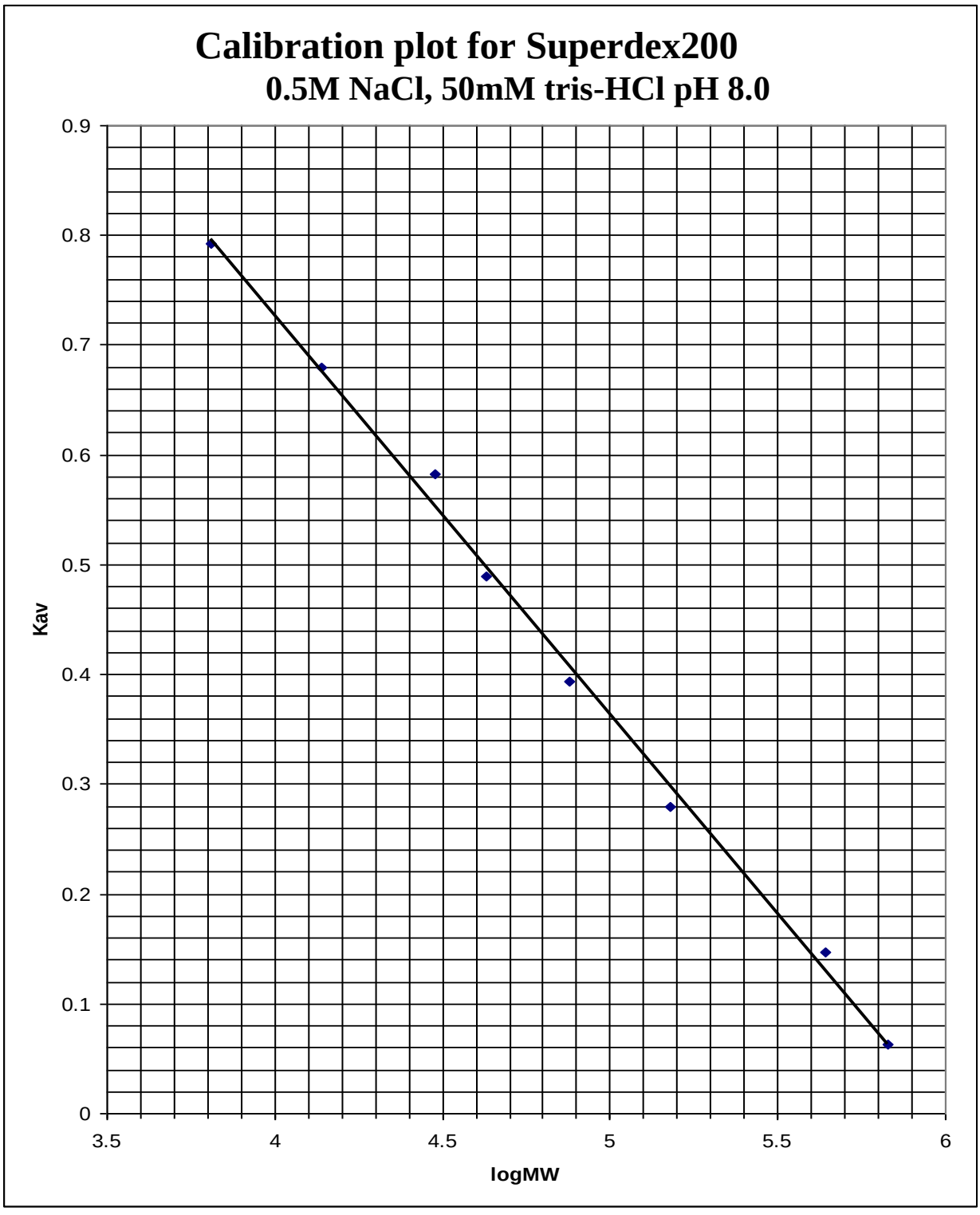
Procedure:

1. Resuspend cell pellets in about 10-20 volumes of Buffer R
2. Sonicate on ice 3x20sec at maximum, as usual.
4. Centrifugate 10000 g for 10 min., remove supernatant fraction (do not discard, keep and analyse on gel to be sure, that there was no any soluble target protein).
5. Resuspend pellets in about 5 volumes of Buffer R, for better result sonicate it briefly. Spin down at 10000 g for 5 min, discard supernatant. Repeat the same procedure once again.
6. Resuspend pellets in about 3 volumes of Buffer U. Place small magnetic bar in the tube and stir for 1h at room temperature or over night at 4^oC
7. Spin down at 40000 g -70000 g for 10 min. Check protein concentration. Calculate amount of protein.
8. Dilute supernatant 40 fold with buffer R. Spin down as above. Check protein concentration again. Calculate amount of protein in solution.
If protein could not be refolded by this method, you, probably, can recover just tiny fraction of protein in solution. In this case we need to think about another refolding conditions (you may try to add substrate or cofactor or other appropriate ligand in buffer R).
If you can recover significant amount of protein (more than 20%) move ahead.
9. Concentrate protein on Viva Spin concentrator (10 000 MWCO) some 50-100 fold. Check protein concentration and calculate amount of recovered protein. Once again you could have problem here.
If it is not much protein recovered, so method does not work (bad luck).
If you have some 50% or more, it is good.
10. Run gel to analyse procedure. Put on gel: whole cells, supernatant (soluble proteins), pellets (insoluble proteins) obtained after Urea extraction, Urea dissolved proteins, finally refolded protein. Try to take 10µg of protein per sample. If concentration is low, try to concentrate it to 1-2 mg/ml (if possible).
11. If TP is revealed apply sample on gel filtration column to make sure that protein peak appeared as a folded protein of a reasonable MW and not in the void volume as an aggregates (in case if TP is not properly folded)

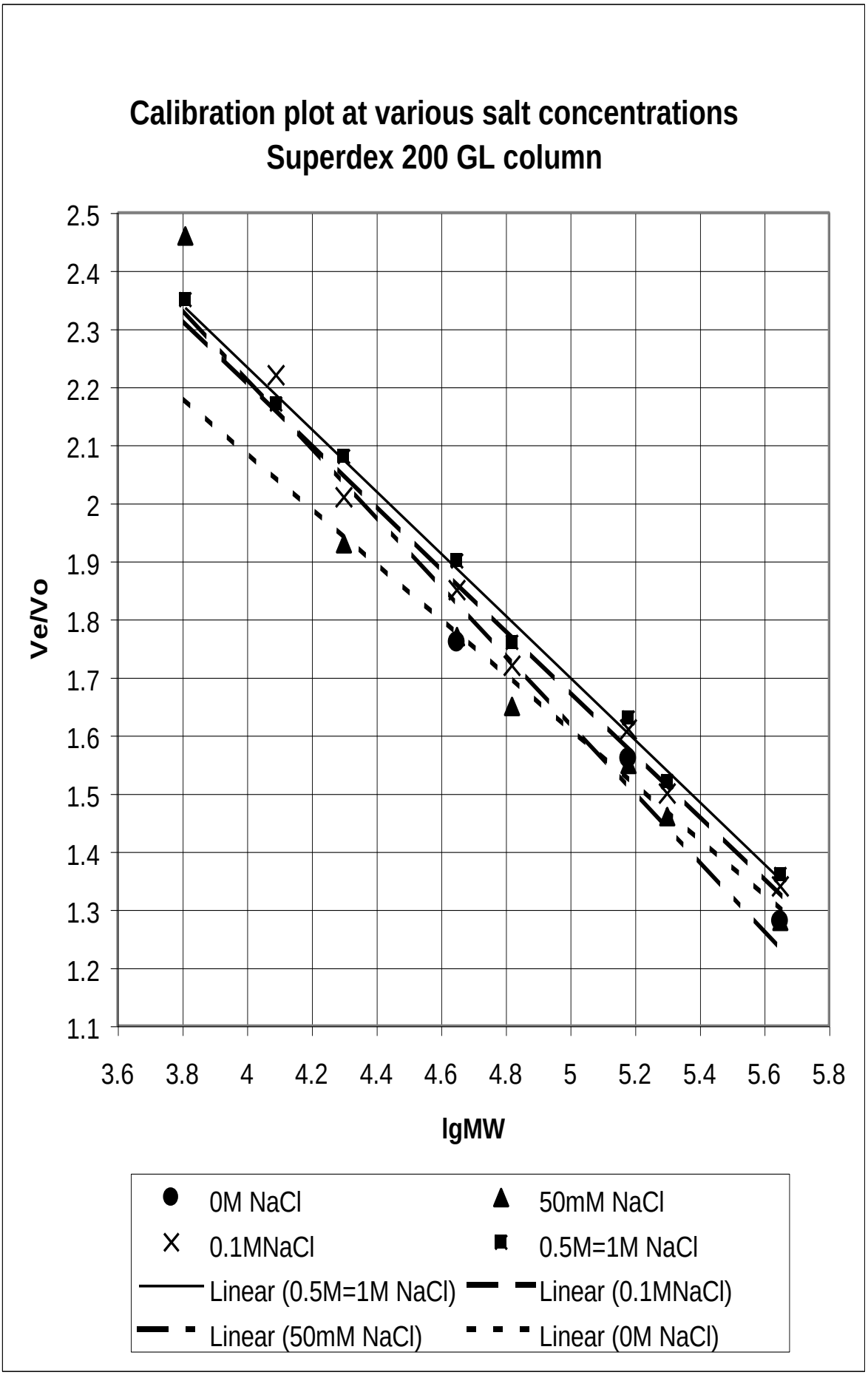
V. Charts and Tables


Sugar refractometr
Ammonium sulphate, M
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
% sugar


NaCl-Sugar refractometer
NaCl, M
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
% sugar



Kav=(Ve-Vo)/(Vt-Vo)=(Ve-45)/75
(for HiLoad 1.6x60cm column)


Calibration plot at various salt concentrations
Superdex 200 GL column
Ve/Vo
2.5
2.4
2.3
2.2
2.1
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
3.6
3.8
4
4.2
4.4
4.6
4.8
5
5.2
5.4
5.6
5.8
lgMW
0M NaCl
50mM NaCl
0.1MNaCl
0.5M=1M NaCl
Linear (0.5M=1M NaCl)
Linear (0.1MNaCl)
Linear (50mM NaCl)
Linear (0M NaCl)

Ammonium sulphate table

This table indicates the grams of solid ammonium sulphate (at 25°C) to be added to one liter of solution to produce a desired change in the percent saturation of ammonium sulphate. Saturated solution is 4.1M and requires 767 grams of salt per liter. This iiitable based on one published by Green and Hughes in Methods Enzymol. 1, 67-90 (1955).

Final concentration of ammonium sulphate
Per cent saturation

Initial concentration of ammonium sulphate
Per cent of saturation

	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	22	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	11	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

Properties of amino acid residues

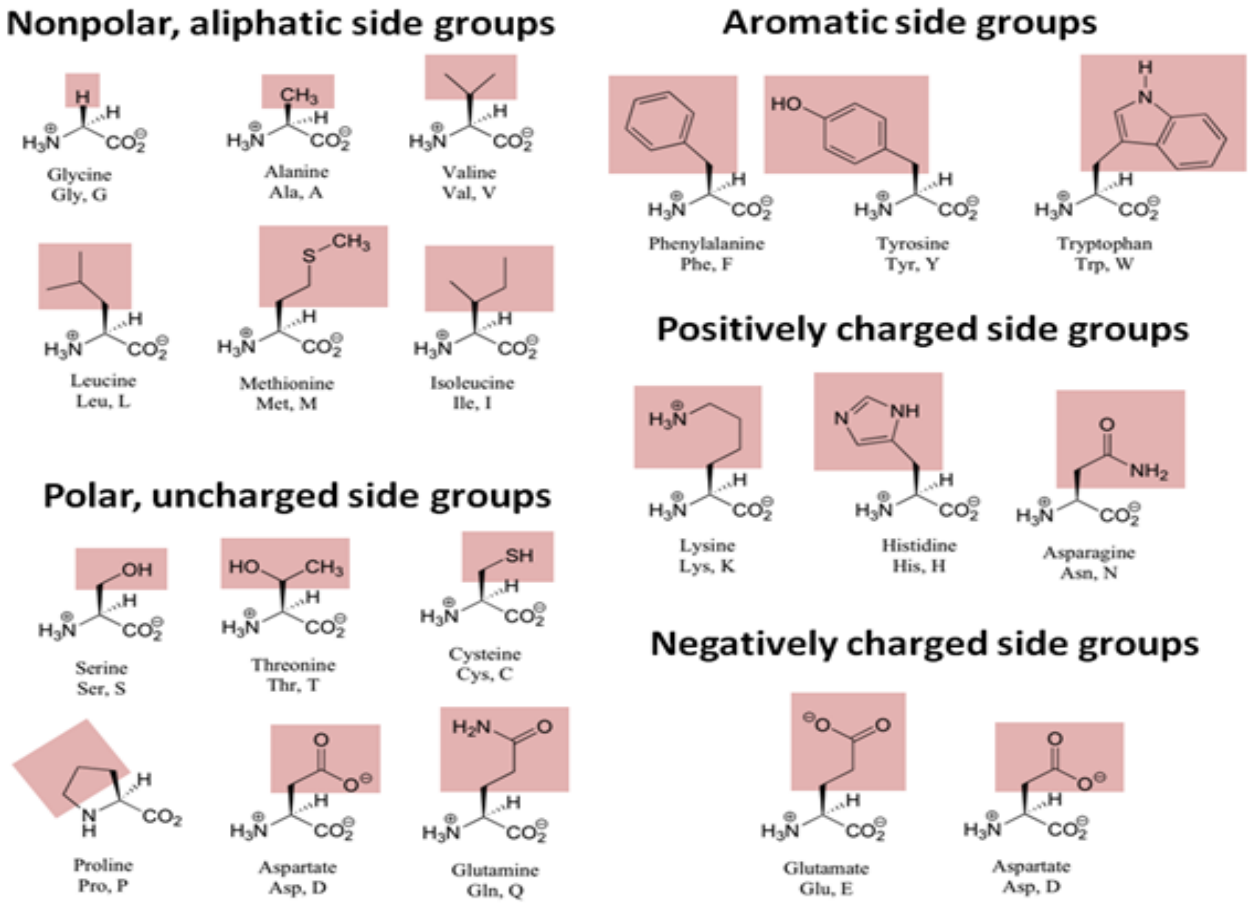


Table 1-1. Notations and Properties of the 20 Standard Amino Acid Residues of Proteins[a]

Amino acid or residue thereof	Three-letter symbol	One letter symbol	Mnemonic help for one-letter symbol	Relative abundance in *E. coli* proteins (19) (%)	M.W. of residue at pH7.0 (daltons)	pK value of side chain (19)	ΔG values for transfer of side chain from water to ethanol at 25°C (16) (kcal/mol)
Alanine	Ala	A	Alanine	13.0	71		−0.5
Glutamate	Glu	E	gluEtamic acid	10.8	128	4.3	
Glutamine	Gln	Q	Q-tamine		128		
Aspartate	Asp	D	asparDic acid	9.9	114	3.9	
Asparagine	Asn	N	asparagiNe		114		
Leucine	Leu	L	Leucine	7.8	113		−1.8
Glycine	Gly	G	Glycine	7.8	57		
Lysine	Lys	K	before L	7.0	129	10.5	
Serine	Ser	S	Serine	6.0	87		+0.3
Valine	Val	V	Valine	6.0	99		−1.5
Arginine	Arg	R	aRginine	5.3	157	12.5	
Threonine	Thr	T	Threonine	4.6	101		−0.4
Proline	Pro	P	Proline	4.6	97		
Isoleucine	Ile	I	Isoleucine	4.4	113		
Methionine	Met	M	Methionine	3.8	131		−1.3
Phenylalanine	Phe	F	Fenylalanine	3.3	147		−2.5
Tyrosine	Tyr	Y	tYrosine	2.2	163	10.1	−2.3
Cysteine	Cys	C	Cysteine	1.8	103		
Tryptophan	Trp	W	tWo rings	1.0	186		−3.4
Histidine	His	H	Histidine	0.7	137	6.0	−0.5
					Weighted mean 108.7		

[a]Further three-letter symbol: Asx, Glx = either acid or amide.
An amino acid residue, -HN-CHR-CO-, is the part of an amino acid (Figure 1-2a) occurring within a peptide chain; R denotes the side chain (Figure 1-1).
Further one-letter symbol: B = Asx, Z = Glx, X = undetermined or nonstandard amino acid residue.

VI. Appendix 1

1. Determination of the protein concentration

There are two widely used ways to determine protein concentration.

- Firstly there are a number of colorimetric methods based on the formation of reagent-protein coloured complexes. Most acknowledged are the Biuret method, Lowry method and Bradford method. The latter method is most simple and so most suitable for protein monitoring during purification.
- The second way is based on UV absorbance of the aromatic residues. Absorbance at 280nm is widely used. Also one can use absorbance at λ_{max} which can be revealed by taking a spectrum of the protein between 240nm and 340nm.

Bradford Method

This method is based on formation of Protein – Coomassie Brilliant Blue G-250 complex which has maximum absorbance at 595 nm.

Advantages: Rapid, Simple,
Sensitive (small amount of protein required for test),
Highly compatible with most reagents and substances

Disadvantage: Significant protein to protein variations

Applications: Monitoring of protein concentration during purification
Determination of relative concentration of pure proteins

See Protocol section for **Bio-Rad protein assay protocol**, the adaptation of Bio-Rad micro assay procedure, based on the Bradford method. Also read instruction enclosed with Bio-Rad reagent.

In the protocol mentioned above incubation with reagent (normally 5 min) is skipped to save more time during protein purification. The reason to do so is that about 90% of the coloured complex is formed within one minute while you mix the reagents. This is applicable to the majority of proteins, but some of them (1-2% from my experience) need more time to develop the full colouring. The most extreme case was one of the Fab fragments which required 1 hour incubation. Be aware of that when you start to work with the new TP.

Protein can be denatured to increase its reaction with the reagent.

Accurate determination of concentration of pure protein: UV absorbance at 280 nm

The method is based on UV absorbance of aromatic residues in proteins.

Tryptophan	ext. coefficient* at 279 nm	5579 M^{-1} cm^{-1}
Tyrosine	ext. coefficient* at 274 nm	1405 M^{-1} cm^{-1}
Phenylalanine	ext. coefficient* at 259 nm	195 M^{-1} cm^{-1}

*Extinction coefficients are given for solutions in water

For each individual protein Extinction coefficient and Abs 0.1% (1mg/ml) can be calculated from its amino acid composition.

Use **ProtParam Tool** program in **Expasy database** to do this.

Calculated extinction coefficient is given for fully unfolded protein in the presence of 6M Gu-HCl. The reason for this is that hydrophobic surrounding of aromatic residues involved in core of the folded protein leads to increasing of their extinction coefficient.

Advantages: High accuracy and Simplicity
Disadvantage: Requires relatively large amount of protein if use the standard cuvette

See **Protocols** section for: **Protocol for accurate determination of concentration of pure protein.**

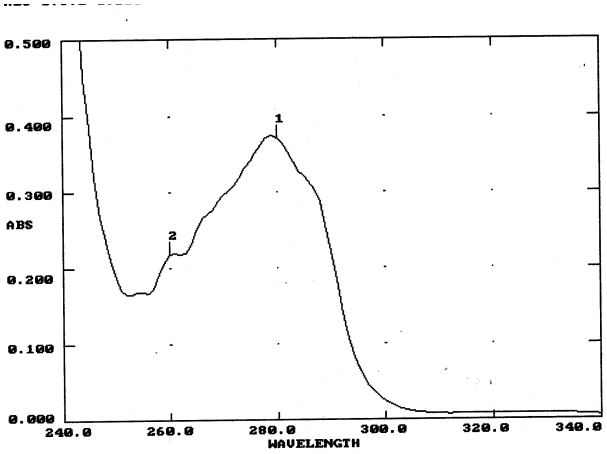
With the protein solution of known concentration (measured by above method) you could make a calibration plot for the Bradford method for the target protein. So afterwards you can use the Bradford method to measure concentration of the pure target protein accurately.

Also you can use the UV spectrum of the target protein solution in any appropriate non-denaturing buffer for the determination of the protein concentration. The best range of wavelength is from 240nm to 340nm. See typical spectra shown below.

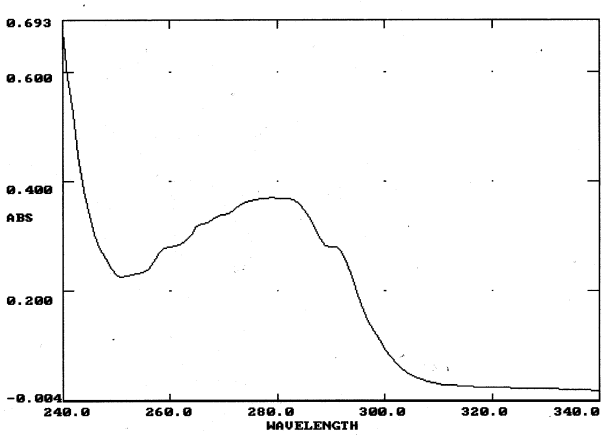
There are **two precautions** should be taken when you use UV absorbance to estimate the protein concentration

1. For many proteins **coefficients of extinction at denaturing and non-denaturing** conditions are fairly close, but for some of them the difference could be significant. So you should compare spectra taken from the target protein solutions prepared at the same concentration in both denaturing and non-denaturing conditions and calculate the extinction coefficient under non-denaturing conditions.
2. Also you should take into consideration that the result could be significantly over estimated due to the contribution of **light scattering** on the possible protein aggregates. UV absorbance between 300nm and 340nm is an indicator of the aggregates presence in the sample. The closer it is to 0, the lower is the light scattering contribution. If the spectrum shows low absorbance from 340nm to 300nm and a sharp increase in absorbance from 300nm to 280nm it can be used to accurately calculate TP concentrations. Otherwise it is better to use alternative ways (Bradford method or UV absorbance under denaturing conditions) because it is difficult to deduct the light scattering component from the total absorbance accurately.

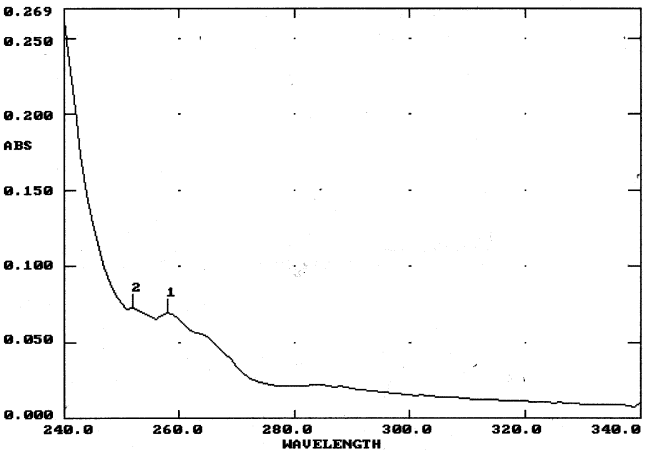
For example, looking at protein spectra below there is no doubt that the spectrum of the tryptophan-free protein is suitable for accurate calculations. But you can see that the spectrum of the tryptophan containing protein is not so irreproachable. It is still suitable for the calculations, but one should deduct Abs at 320nm from Abs at 280nm to get more accurate result.



UV spectrum of the tryptophan-free protein
The observed absorbance corresponds mainly to the tyrosine residues



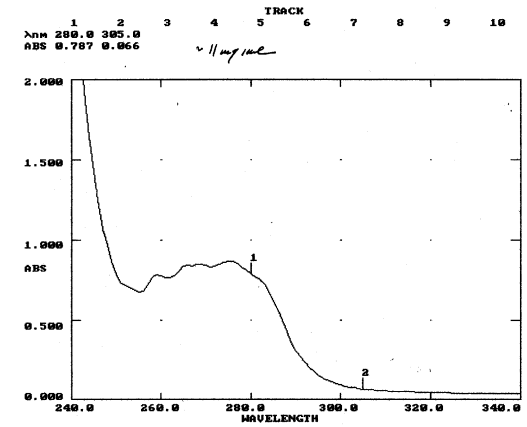
UV spectrum of the tryptophan containing protein
The observed absorbance corresponds to a combination of the tryptophan and the tyrosine residues in the protein



UV spectrum of the protein containing phenylalanine but no tyrosine or tryptophan
The extremely rare example of the protein with no Tyr or Trp, FtsZ from *S.aureus*
It is not possible to use absorbance at 280nm to determine its concentration

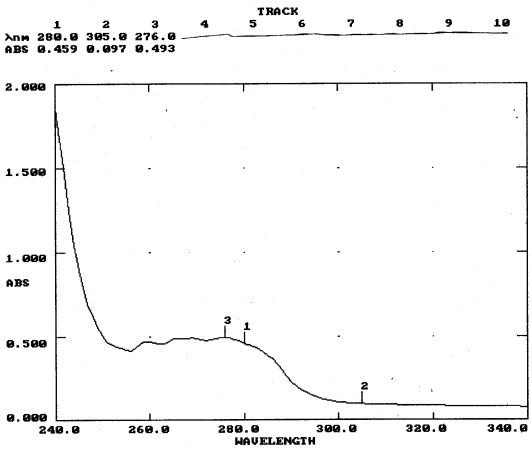
See example below for further explanation:
Spectra shown belong to FtsZ protein from Bacillus subtilis.

This protein has 2 tyrosines and 9 phenylalanines out of total 382 residues. This is why for this protein the value of Abs 0.1% (1mg/ml) at 280nm is as low as 0.063.
The shape of the spectrum is unusual because of the visible contribution from the phenylalanine absorbance which is normally hardly detectable. Also contribution from peptide bond absorbance at 230nm makes significant contribution to the shape of the spectrum almost eliminating the minimum at 250nm. As you can see without the deductions of underlined absorbance in Spectrum 1, for protein in 10mM tris-HCl pH 8.0 error is just 8%, well within acceptable for most of uses 10% level. For Spectrum 2 the error is high 21%. And if you calculate protein concentration from A280 in Spectrum 3, you overestimate it at least 100%.



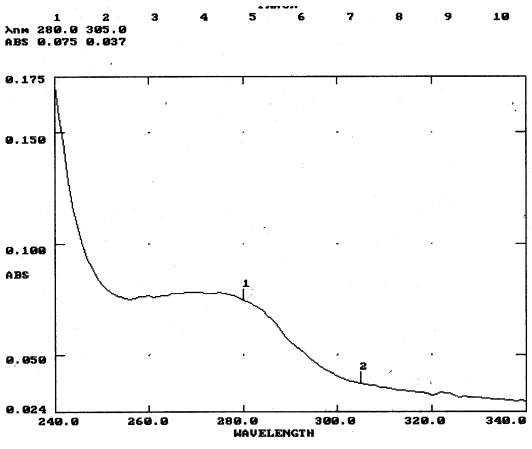
Spectrum 1.

This spectrum is taken from FtsZ in buffer 10mM tris-HCl pH 8.0
Light scattering is very low, however there is some absorbance at 305nm which should be deducted from absorbance at 280nm.
Abs_{280}-Abs_{305}=0.787-0.066=0.721
Conc=0.721/0.063=11.4mg/ml



Spectrum 2.

This is spectrum of FtsZ in buffer 50mM MES pH 6.5, 0.2M NaCl.
This spectrum is still suitable for calculations, light scattering is not too high, but base line obviously is not zeroed properly. So once again absorbance at 305nm should be deducted from absorbance at 280nm
Abs_{280}-Abs_{305}=0.469-0.097=0.372
Conc=0.372/0.063=5.9mg/ml



Spectrum 3

Spectrum of FtsZ in buffer 10mM MES, 2mM MgAc, 1mM EDTA
Significant light scattering on aggregates not only affects value of absorbance at 280nm but also a shape of the spectrum. This spectrum is unsuitable for calculations

2. How to concentrate proteins

The main ways of protein concentrating are as follow:

1. Precipitate protein from large volume and dissolve it in a small volume
2. Apply protein solution on a small column of appropriate matrix and wash protein out by a sharp step of appropriate eluent
3. Dialyse protein solution against concentrated (10-15%) solution of PEG 20000
4. Dry protein solution under steam of inert gas or air, under vacuum or freeze-dry
5. By Ultra filtration

Concentration by precipitation

Precipitants which are used for proteins:

Salts:

Ammonium Sulphate is mainly used for protein precipitation

Alcohols:

Ethanol, isopropanol, MPD

Polyols:

PEG of low MW (about 1000 Da)

Acetone

Acids:

Trichloracetic acid (TCA)

Using any kind of precipitation normally leads to at least 20% loss of the material (often more).

Precipitation by ammonium sulphate

This is a widely used method for concentration and storage of proteins. See **Protocols** section for '**Protocol of precipitation'** and **'Protocol for recovery of protein from the ammonium sulphate precipitate'**. Please notice that some proteins can be denatured by high concentration of ammonium sulphate and cannot be recovered from the precipitate. You should test this procedure on a small amount of TP first to be sure that it is appropriate.

Precipitation by PEG

Precipitation by PEG based on the same routine: keeping solution cool and stirring, add PEG_{1000} by small portions until it grows turbid. Stop stirring and leave sample overnight. To recover protein spin down pellet, dissolve in a small volume of buffer and dialyse intensively against large volume of buffer, 4-5 changes, each for 10-12 hours, because PEG is difficult to dialyse out.

Precipitation by acetone

Precipitation by acetone could be useful if you need to prepare samples of protein under denaturing conditions (for example, for gel analysis). Very few proteins could be recovered from acetone precipitate natively.

To the protein sample add 10 volumes of cold (from the freezer) acetone. Spin down at 0-4ºC for 20 min at 5000g or higher speed. Remove acetone from the tube and evaporate out the rest of it under an air stream. Dissolve pellets in small volume of SDS, urea or Gu-HCl solutions.

This method could not be applied for samples with high salt concentration because salt is precipitated with the acetone as well.

Precipitation by acids

To precipitate proteins by TCA, add 50% solution into protein sample to make an acid concentration of about 10%. Collect pellets by centrifugation. To dissolve precipitate, use a basic solution of denaturing agents. This method is applied for preparation of denatured samples and has no restrictions from the high salt concentration.

Mild acidic precipitation could be achieved by dialysing protein solution against Na acetate pH 5.0. Proteins with pI close to 5 precipitate and could be collected by centrifugation and dissolved in appropriate buffer of pH 7-8. As usual the method is suitable for some proteins but not all of them. It is not rare for proteins to denature when precipitated under acidic conditions.

All the above methods except the ammonium sulphate precipitation are rarely used today.

Concentration by chromatography

This method is not one in frequent use, but sometimes you may want to use it.

It is most useful for concentration of very diluted protein, especially if the sample has a high ammonium sulphate concentration.

Make a small column of appropriate hydrophobic resin (Phenyl-Toyopeal, as a rule). Take 1 ml of resin for 20mg of protein. Wash it with ammonium sulphate solution of appropriate concentration (2.5M is fine for most proteins). Make sure that ammonium sulphate concentration in the sample is high enough to allow protein to bind to column (2.5M-3M of ammonium sulphate). Apply sample on a column. To collect concentrated protein wash column with appropriate buffer (i.e. 50mM tris-HCl, pH 8.0). Collect 10 fractions equal to 1/3 – 1/2 volume of the column. Check protein concentration in the fractions and combine fractions with the desirable level of protein concentration. Yield of recovery could be between 50 and 80%.

A similar procedure can be applied for concentration of a dilute protein sample in low salt buffer solutions. Choose appropriate ion-exchange resin. Elute protein with 1M NaCl in appropriate buffer.

His-tagged protein could be concentrated this way too, but because affinity of His-tag to Ni column vary from one protein to another, for many proteins it is not possible to obtain a high concentration. For some proteins it is not possible to reach a concentration higher than 3-4 mg/ml using this method.

Concentration by PEG 20000

Make 10-15% solution of PEG 20000 in appropriate buffer. Place on stirrer and make sure the viscous solution is mixing properly. Put protein solution into a dialysis tube and leave to dialyse. Check volume frequently, do not allow sample to become over concentrated. When desirable volume is achieved, dialyse sample intensively against fresh buffer to remove some low MW contaminations picked up from the PEG solution. Recovery is up to 80%. Be careful about the integrity of the dialysis tube.

Concentration by evaporation

Volume of solution can be reduced (so protein concentration can be increased) by evaporation of water. Water can be evaporated by a few techniques. To reduce volume of the small samples (less than 0.2 ml) the simplest way is to use stream of air or better inert gas (nitrogen, argon, etc). Place the protein solution into the eppendorf tube and mount it on the stand. Connect tubing with thin opening (such as a gilson tip) to the air or gas tap

and mount it above the eppendorf tube. Make stream go through tubing, open tap slowly, to make the solution in the tube move slightly. Check volume in the tube every 5-10 min. It takes about 30-40 minutes to reduce volume by 0.1 ml or so. Remember that the salt concentration increases too so you may need to perform dialysis against required buffer afterwards.
Another way to reduce volume by evaporation is to use a vacuum to let water evaporate quickly. Normally such evaporation is performed in a vacuum centrifuge called SpeedVac. Depending on vacuum, it takes a few hours to evaporate about 1ml of water.
Another way to use evaporation is to dry the protein solution completely. For some proteins (most stable ones) it is a good way of storage. It may be appropriate for some proteins to be desalted by dialysis or gel filtration prior to drying. Alternatively, protein can be dried in the presence of buffer. Drying (also known as lyophilisation) is performed in the machine called a vacuum-freeze dryer. Place protein solution into the round, pear-shaped or conical bottom flask. Volume of solution should be about 20% of the flask's volume. Solution should be distributed on the inner surface of the flask and frozen by rotating flask in the bath with dry ice or liquid nitrogen. The frozen flask should be immediately mounted on the working dryer.
Small volumes of protein (0.5-1ml) can also be dried on the machine. Put solution into an eppendorf tube, close the lid, make a few holes in the lid, freeze sample in the liquid nitrogen, place it in to the flask and mount flask to the dryer.

Concentration by ultra filtration

This is the most mild and widely used method. It is based on filtration of the protein solution through a membrane with pores small enough to retain molecules of protein and allow buffer to pass through. Because pores are very small, considerable force is required to allow buffer to pass.
Mainly two types of devices are in use.
The first type is a pressure stirred unit. Compressed nitrogen is used to apply pressure for filtration. Stirring prevents proteins from clogging the pores. So called Amicon units of different sizes were common in the labs but not anymore.
The second type is a spin unit where the force of centrifugation is used. This method is more convenient and simple to use and so practically expelled pressure units out of the labs. There are a number of designs from different manufactures. We use Viva Spin concentrators (Sartorius).
The most useful are Viva Spin 20 (for 20ml sample) and Viva Spin 6 (for 6ml sample).
Spin down at 4500xg-5000g.
Time required for concentration varies from protein to protein.
Viva Spin units can be reused several times, but remember to wash them with plenty of water immediately after use.
Use a 200µl tip to collect sample from the Viva Spin unit.
Viva Spin 20 with a diafiltration cap could also be used for buffer exchange.
We also use 0.5ml VivaSpin units for concentrating of small volumes. Apply 12000g for a few minutes. Use gel loading tip to take concentrated sample out of unit.
Please note that during centrifugation protein ***concentration becomes very high at the bottom of the concentrator*** *which could cause* ***protein precipitation****. For some proteins it is useful to do concentration in short (5-10min) shots with the mixing of the sample after each shot.*

3. How to store proteins

The safest way to use proteins is to use them fresh, in a few hours or days after purification. Nevertheless the majority of proteins can be successfully stored using three main methods:

- Frozen at -20^{o}C or better -70^{o}C
- Precipitated with ammonium sulphate at 4^{o}C or even room temperature
- Vacuum dried

Freezing

Protein solutions could be frozen and kept at freezing temperatures for a very long time.
The best way to freeze protein is to place it in liquid nitrogen. But for many proteins it is OK just to put them in the freezer.
For concentrated protein solutions (more than 10mg/ml) it is not necessary to add anti-freezing agent such as glycerol, but for dilute solutions it is better to add glycerol to a 10-40% concentration.

Precipitation

Follow the '**Protocol for precipitation of proteins using ammonium sulphate'.**
Samples can be stored at 4^{o}C for years. Make sure that the container is properly sealed.
To recover protein for use, refer to '**Recovery of protein from the ammonium sulphate precipitate'** protocol.
This is a good and fairly universal method for protein storage, but remember, some proteins do not survive this procedure.

Vacuum drying

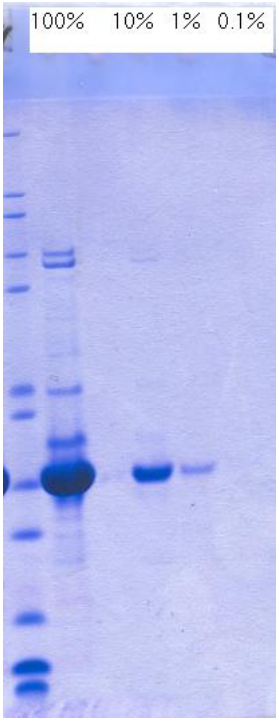
Proteins can be dried as described above (***Concentration by evaporation***).
Seal container with the dried protein properly and keep it at room temperature, at 4^{o}C or in the freezer.

If you keep sample below room temperature and you do not want to use the whole sample at once ***remember to warm sample to room temperature before you open the container*** *to prevent moisture condensation on the cold powder.*

The same rule is applied to any reagent kept below room temperature.

4. How to estimate purity of a protein preparation

It is difficult to estimate purity of a protein preparation accurately. Generally speaking to do this we have to measure accurately total protein concentration and concentration of a target protein in the preparation. Is it possible? Quantitive N-terminus analysis? Some of contaminations can have the same N-terminal amino acid...
Any way it is just SDS-PAGE analysis we typically use for this task and we only can roughly estimate the purity of a protein preparation.
We run gel, stain it with appropriate stain and hopefully have got clear gel with sharp protein bands. If we have scanning instrument we can have a scan and calculate proportion of light absorption of target protein band from the total of all peaks.
If we have not got a scanner we can use more rough method below.



On gel applied is protein called EctC:
100% 20µg
10% 2 µg
1% 0.2 µg
0.1% 0.02 µg

Comparing band of contaminating proteins from
100% line with EctC bands on 10%, 1% and 0.1% lines we can estimate purity of the prep to be around **85%** :
four main contaminating bands are 1-3%
(say about 10% in total)
20 or so small bands are in range 0.1-0.5%
(say about 5% in total)


Prepare your solutions right!


D11a
Keep door shut
Sterile Room
No Gloves

VII. Appendix 2

1. Prepare your solutions right!

1. Calculate appropriate amounts of reagents

2. Prepare a BEAKER of appropriate volume with a magnetic bar in it

3. Weight out reagents using the weight boats or directly in to a beaker (for stock solutions)

4. Place all reagents in the beaker and add 80-90% of Milli Q water volume. Leave on a stirrer to dissolve

5. If preparing buffers adjust pH using acid or alkali solutions (1M, 5M or concentrated depending on buffer concentration)

6. Transfer solution in to a volumetric flask (for stock solutions) or a cylinder. Rinse beaker with small volumes of water 2-3 times and add to the flask. Adjust volume.

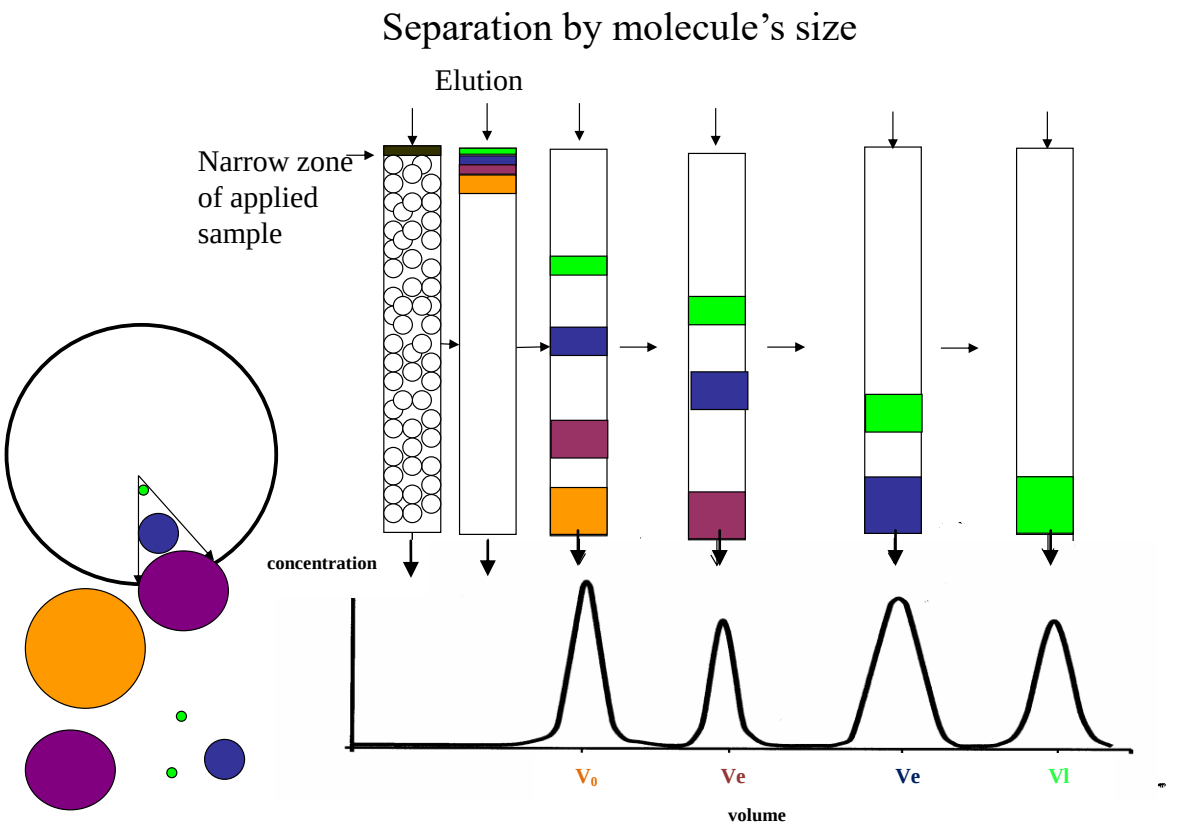
7. To mix solution properly just pour it in to the clean beaker.

8. Filter solution trough 0.2 micron filter

9. Transfer solution into the bottle. Mark bottle clearly including date and initials

9. Carefully clean the filter device, especially the porous glass part

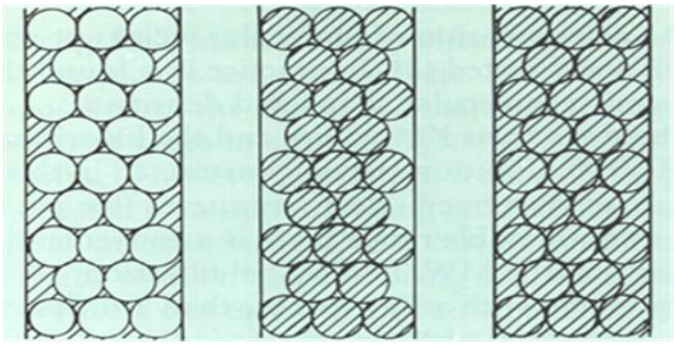
2. Gel Filtration



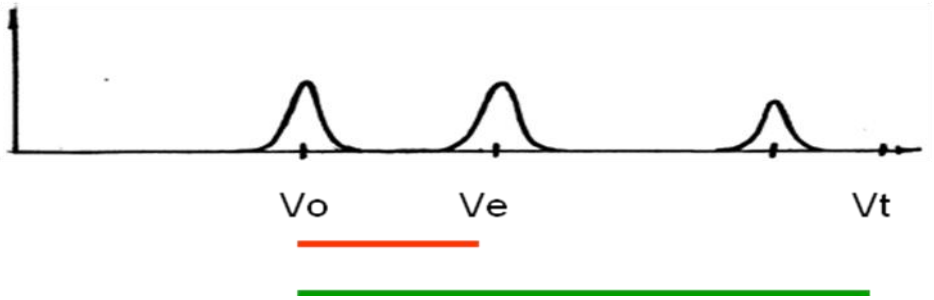
In gel filtration molecules in solution are **separated by their size** while they are pass trough column packed with gel matrix filled with liquid. This matrix consists of **porous beads**.
The pores in the beads are comparable in size to the molecules to be separated. Very small molecules can penetrate the beads completely, while the bigger molecules can only enter larger pores, and there could be molecules which are too big to enter pores at all. For simplicity of understanding we can present the bead with large conical pore which represents all possible pore sizes, as shown on the carton. By penetrating pores deeper smaller molecules stays in matrix longer, or in more accurate terms they need larger volume of liquid to be washed out of column.
Zonal separation. The sample is applied on a column as a narrow zone, 1-5% of column volume. During elution zone of each component become wider due to **diffusion**. Separation power depends on a beads size: the **smaller are the beads**, the lower is diffusion, peaks are sharper and **separation is better**.
Depending on pores size there are different gel filtration media for separation molecules of different size range.
Practical use of gel filtration is not just for **protein purification**, but also for **analysis of the oligomeric state** of proteins and protein complexes. **Column can be calibrated** using proteins of a known size, which for globular proteins is proportional to molecular weight.
In practice the calibration plot is produced as **elution volume versus molecular weight**. The **linear relation** in gel filtration was found between elution **volume and logMW.**

The calibration plot however is usually presented as a **Kav versus LogMW**. This way the calibration plot became **independent of the column parameters**.

Kav is proportion of inner beads volume available for the particular protein.

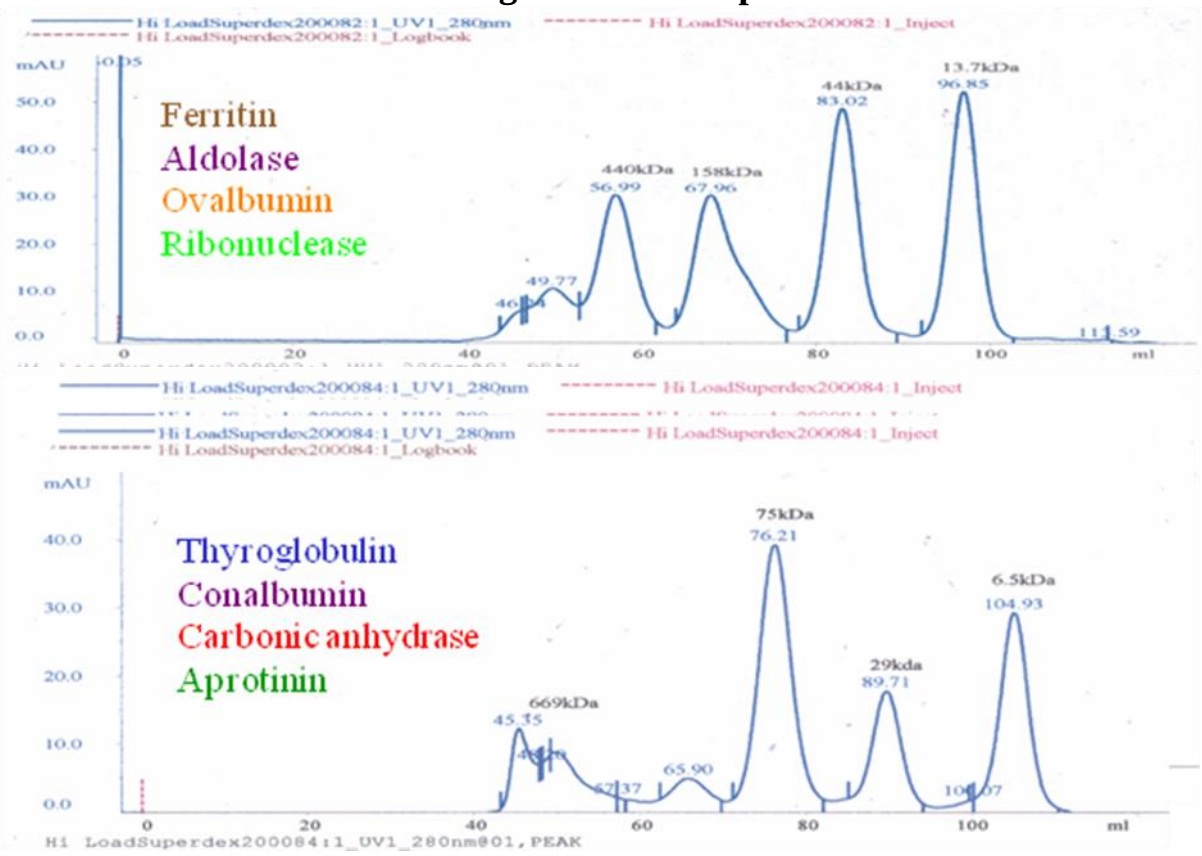


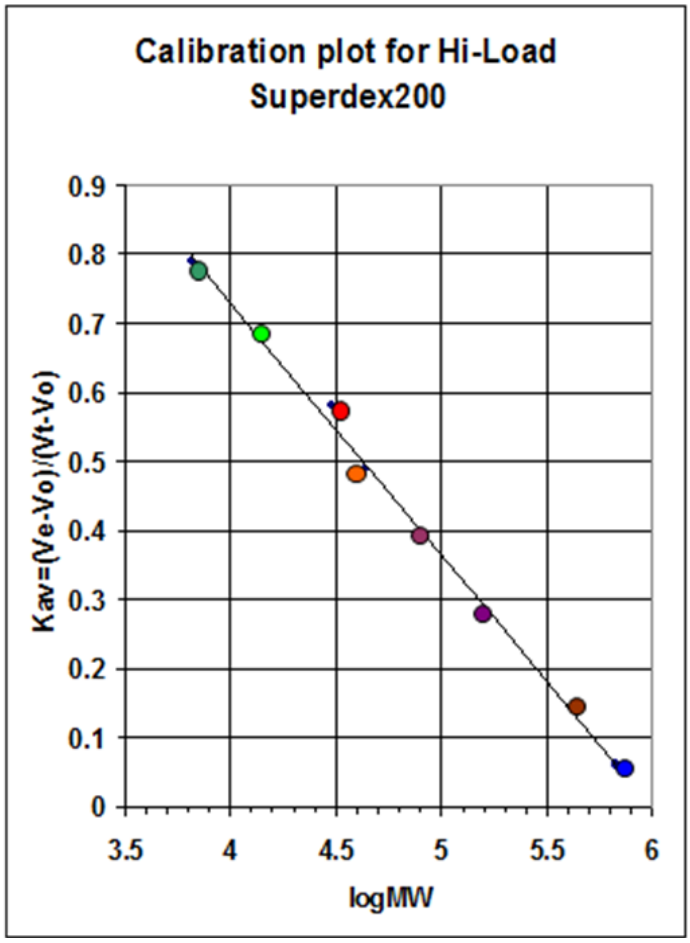
Kav=(Ve-Vo)/(Vt-Vo)



Vo – void volume
Ve – elution volume
Vt – total column volume

Obtaining a calibration plot





Here is presented the calibration plot obtained for the gel filtration media Superdex 200.

Mixtures of a standard proteins (Low and High MW kits from GE Healthcare) were run on a 1.6x60 cm HiLoad Superdex200 column (GE Healthcare).

Kav was determined for the each peak and plotted versus logMW.

Main reasons for "abnormal" mobility of some proteins during gel filtration

1. **Compactness**. Calibration plot we use for calculations is done for MW, but gel-filtration separates molecules by their sizes and not by their masses.
Proteins of the same MW can have more compact fold and can have less compact fold, so two protein of the same MW can differ from each other in **size.**
The standard proteins have average fold, typical for most of proteins.
Among other proteins, as for everything, we have Gaussian distribution, so some of the proteins have more compact fold then the standards, some can have less compact fold.
2. **Shape.** Most of the proteins are globular, and so are standard proteins.
But some of the proteins may be very asymmetrical. Very elongated molecules behave as larger ones, and elutes from the gel filtration column early in comparing with globular molecules of the same MW.
3**." Water coat"**. Also, having different surfaces, proteins have different amount of tightly bound water molecules on their surfaces, which also contributes to mobility of their molecules in solution.
4**. Interaction with matrix**. Proteins can have some affinity to the gel filtration matrix and so, being bound-unbound to the matrix during elution, such proteins elute from the column later, demonstrating lower apparent MW then the real one.
5. **Equilibrium**. Some of the proteins do not form stable oligomers, but exists in state of fast equilibrium monomer-dimer or dimer-tetramer, etc. In these cases the apparent MW on gel filtration occurs to fall between the two values.

3. Time-saving tips in protein purification

- Make proper stock solution which will last for a long time so you can prepare any buffer solution for your purification in seconds
- When preparing cell free extract by sonication divide cell suspension between 2-4 portions and put pieces of ice into them so you can save time on the cooling
- To remove cell debris apply only 10-15 min centrifugation, the longer spins is not practical because there is no need to remove small particles as they pass the column safely causing no harm
- After you apply sample on the column **do not** wash out unbound material with the starting buffer. The only exception is if the target protein (TP) is going to be eluted from the column very close to the start of the salt gradient. With the beginning of the salt gradient the unbound material is removed from the column much more effectively than with the starting buffer
- If expression level of TP is higher than 10%, in most cases the highest protein peak on the chromatogram is TP. So you can find it by checking protein concentrations in fractions by the fast Bradford method and save time on gel analysis
- If you need to analyse fractions by gel and you have not got pre-cast gels, then start to prepare gel immediately after you have started the chromatography
- Do not wait until all gradient is applied on a column. It is useful to start to analyse protein concentration and activity (if applied) in the fractions after about one third of the gradient have been applied on a column. This is normally the position of TP elution if gradient was properly optimised
- If you use a gel to find TP after first chromatography, to reveal result quickly stain gel for 5-10 min with fresh stain and distain it briefly for a few minutes so as to reveal just the major bands. As a rule, TP can be identified successfully this way. Combine appropriate fractions and move on for the next purification step leaving gel to be properly stained-distained later.

4. Golden Rules in protein purification

- **DO NOT DISCARD ANYTHING (pellets, supernatant fractions or fractions obtained during any chromatography) until purification is complete and analysed by SDS-PAGE.**
  Always collect flow through column fraction.

- **Keep small samples of protein from each stage of the purification procedure for the final SDS-PAGE analysis**

- **Keep your attention to the purification, do not get distracted by other activities**

- **Keep chromatography system clean, always wash it with water, 20% ethanol or 1-2mM EDTA solution after use**

- **Have a basic set of columns pre-packed and keep them in good order. Always wash ion-exchange columns with 2M NaCl and hydrophobic columns with water after use**

- **Keep your pipettors in good order, never allow liquid inside the pipettor. It is best to have your own set, clean them trough once a year and check their calibration**

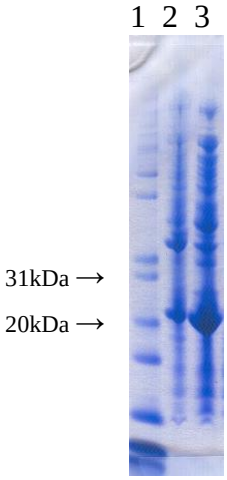
VIII. Show cases

1. BPSL 1549 (BLF1)

Two step purification and a lesson to learn

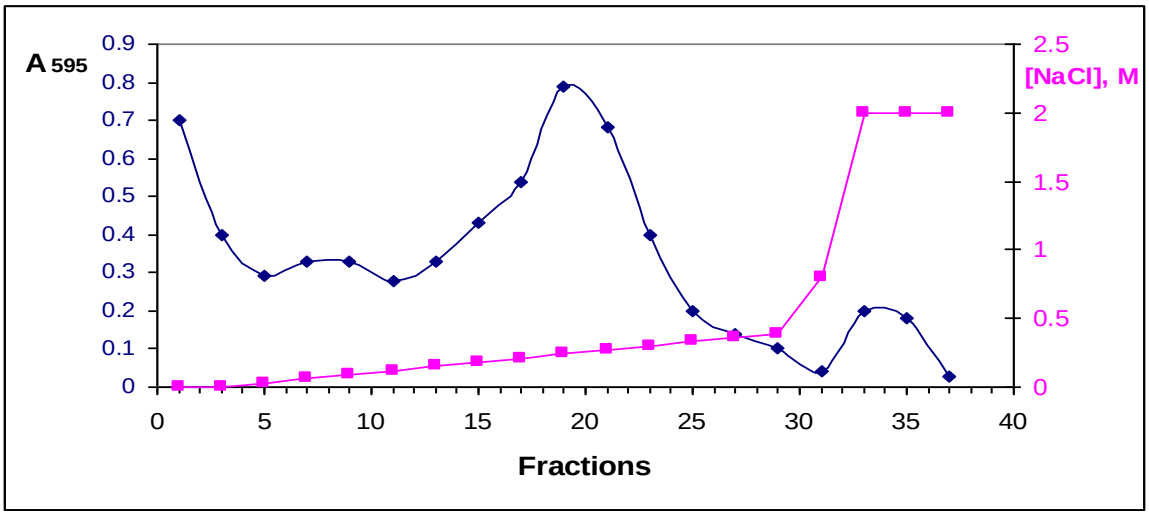
BPSL 1549 (Burkholderia Lethal Factor 1) was the most interesting project done in our crystallographic group so far. Apart of its biological role (Science 334, 821-824, 2011) it also shows some unusual features as a protein.
ProtParam results revealed just one unusual feature:
MW 23342Da
pI 5.14
Abs 0.1% (1mg/ml)= 2.4 It is very high and rather unusual.

BPSL 1549, back then being referred as a putative protein of unknown function, was highly expressed in TUNER cells, where chloramphenicol-acyl-transpherase (CAT) (23kDa) normally also being expressed to a significant level.
Because of that analysing level and solubility of expression (see gel picture below) we could not be sure was there the target protein expressed or was it CAT.

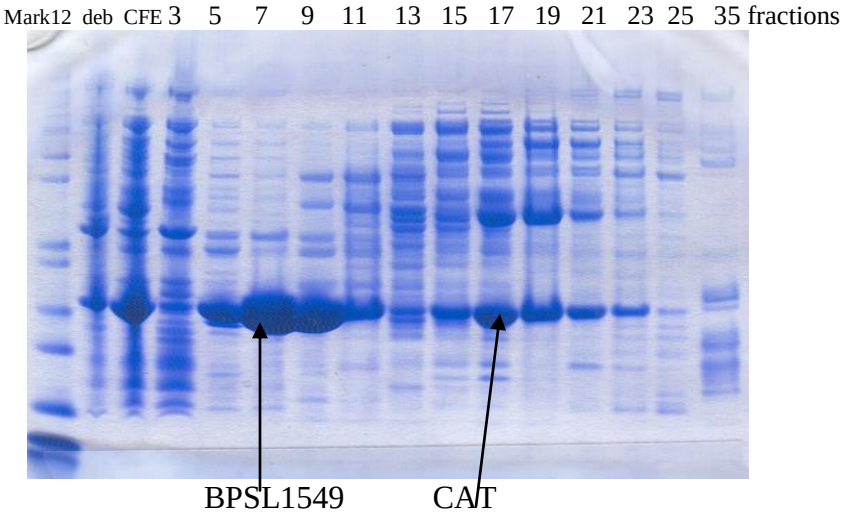


1. Mark12 MW standard
2. Cell debris
3. Cell free extract CFE)

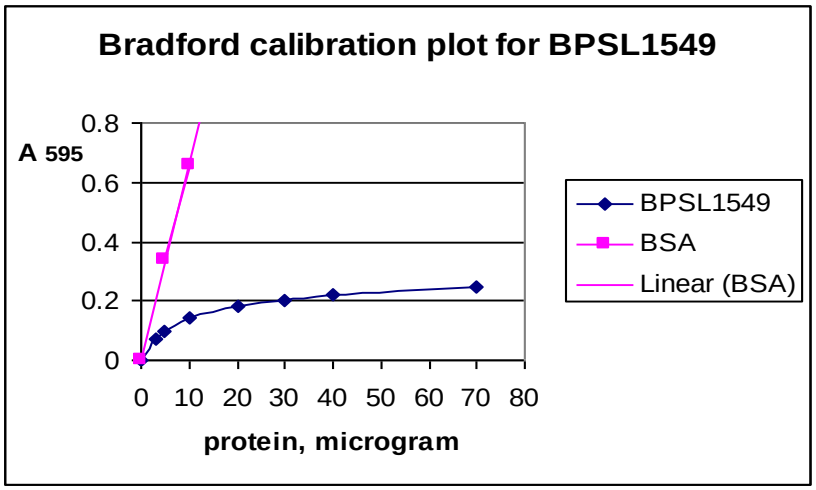
The standard approach for an acidic protein was applied for the purification.
Cell free extract was prepared in Buffer A and applied on a 25ml DEAE-Sepharose FF column. 300ml gradient from 0 to 0.5M NaCl was applied for elution. 7.5ml fractions were collected. Because of the very high level of expression I have relayed on a protein concentration estimated by method of Bradford to find the elution position of the target protein (see the chart below)



The main protein peak was found eluted with about 0.24M NaCl . It was position where CAT elutes from the column, but it also did not contradict with the target protein pI 5.14.
Two fractions with the highest protein concentration were combined, concentrated using Viva Spin concentrator and applied on a gel filtration column, Hi Load Superdex200.
Peak was eluted at 75ml, suggesting MW about 70kDa, the position where CAT elutes from the Superdex200 column.
To confirm that it was CAT, the sample was supplied with 1.5M ammonium sulphate and applied on 8ml Phenyl Toyopearl 650S column. Elution was performed by the 80ml reverse gradient of ammonium sulphate concentration from 1.5M to 0 in Buffer A. 4ml fractions were collected and analysed by method of Bradford. Protein peak was found at a very end of the gradient, where CAT elutes from the Phenyl Toyopearl column.
It looked like that target protein was not expressed, but if so, the one thing was odd – the level of expression of the 23kDa protein was much higher than the normal level of CAT expression under the same standard growth conditions.
At that point SDS PAGE analysis was employed (NuPage 4-12% BT Novex gel, see picture below) and revealed massive protein peak in the beginning of the gradient, at about 80mM NaCl (fr. 5-11) .



Fractions 7 and 8 were combined, concentrated to 2ml and applied on a Superdex200 column. Peak was eluted at 90ml, which suggested a monomeric state of the BPSL1549 in solution.
So, BPSL1549 does not react with the Bradford reagent properly. It does react with Coomassi only when denatured by SDS and exhibits a good bands in the gel, but it does show very low reaction with Coomassi under normal assay conditions (see Bradford calibration plot for BPSL1549 below).



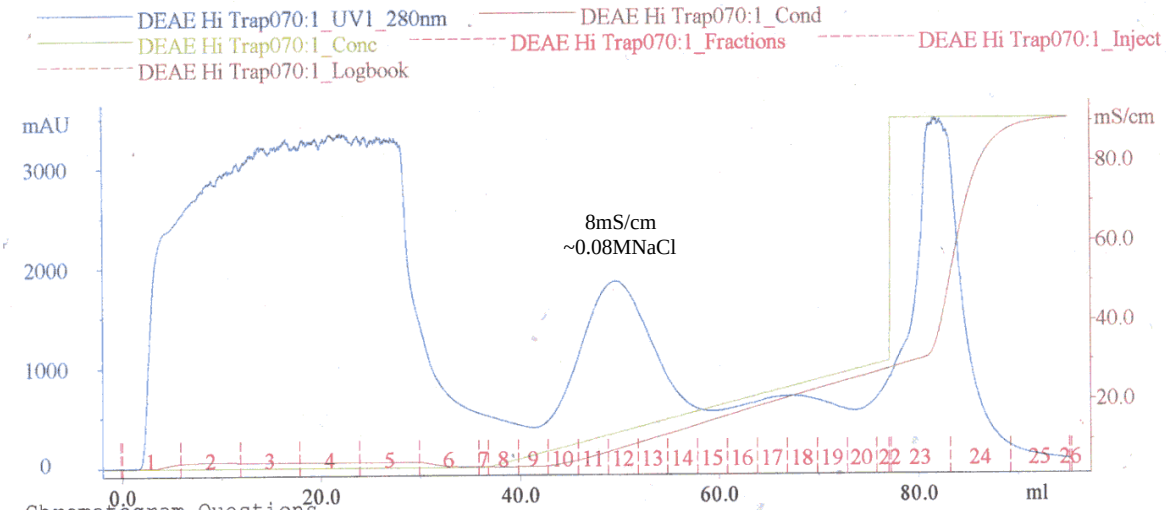
It was a good lesson which showed me that 20 years experience is not 100% relevant and one day the convinient short cut may fail dramatically. Since then when I develop the purification protocol for a new protein I run the SDS PAGE to analyse an elution profile on a first chromatographic step.

Apart of the trouble with the Bradford's method, this purification is a good example of two step purification protocol. High level of expression and early elution from DEAE-Sepharose makes it possible to obtain 95%+ purity by combination of AEC and SEC. See below the refined purification procedure with the use of AKTA system.

Typical purification of BPSL1549

About 1g of the cell paste was defrosted, suspended in about 27ml of buffer A and disrupted by sonication. Debris was removed by centrifugation (10 min at 70000g). CFE was obtained in 27ml, protein concentration by Bradford =2.7mg/ml, total 73mg (total protein here mainly corresponds to *E.coli* proteins).

CFE was applied on a 5ml Hi-Trap DEAE FF cartridge. 40ml (8CV) of the gradient 0 to 0.3M NaCl was applied for elution. Elution was performed at flow rate 4ml/min, 3ml fractions were collected (see chromatogram below).

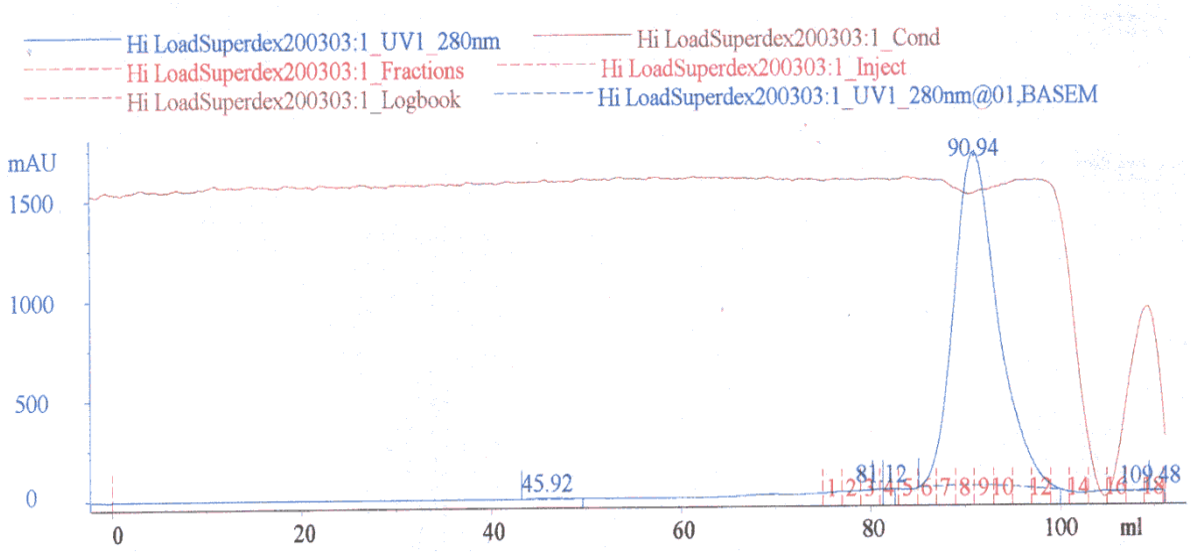


In fractions from 10 to 14 there is a big peak of absorbance at 280nm . This peak corresponds to BPSL1549 due to unusualy high extinction coefficient, which makes it easy to identify fractions to be combined, namely, 11-13.

Volume of combined fractions was 9ml, concentration was mesuared by absorbance at 280 nm and was equal 3.3mg/ml, total protein about 30mg.

Sample was concentrated to 2ml using Viva Spin concentrator and applied on a 16x60 Hi-Load Superdex200 column. Gel filtration was performed at flow rate 1.5ml/min in Buffer A suplimented with 0.5M NaCl ("standard GF buffer").

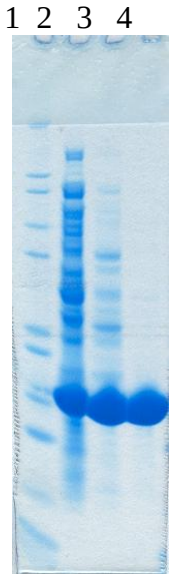
2ml fractions were collected after 74 ml of elution (see chromatogram below)



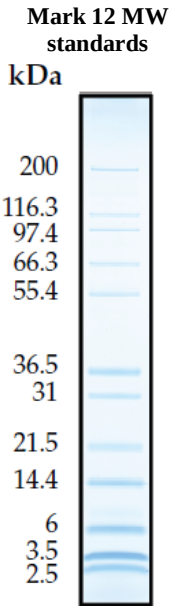
Peak of A_{280} was observed in fractions 7 to 11.
Fractions 8-10 were combined, volume 6ml, concentartion 2.6mg/ml (by A_{280}), total yield 15mg.

Progress of BPSL1549 purification

SDS PAGE analysis on NuPage 4-12%BT Novex gel



1. Mark 12
2. CFE
3. DEAE fr 11-13
4. Gel filtration fr 8-10



Purity estimated by SDS PAGE was not less then 95%.
Yeild 15mg from 1g of cells

2. It is easy with coloured proteins!

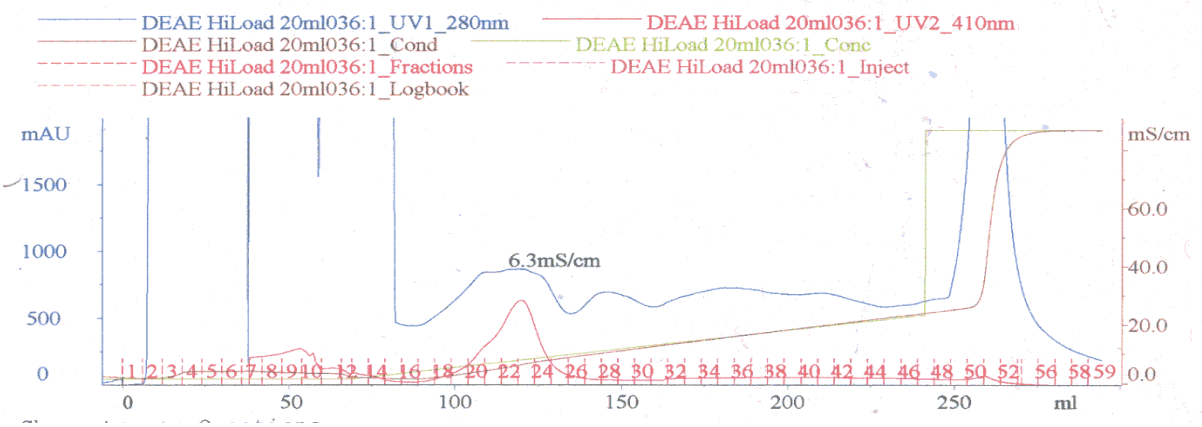
Proteins containing chromophores are easy to purify because no time is required for identification of fractions with the target protein. Typically coloured fractions are combined after each chromatography.
Represented below are purification of two haemoglobins, Cgb from *Campylobacter jejuni* and Hmp from *Escherichia coli* (in collaboration with Prof. R. Pool group)

Single-domain haemoglobin Cgb from *Campylobacter jejuni*

MW 16081Da
pI 6.14
$A_{280\ 1mg/ml}$ 0.9

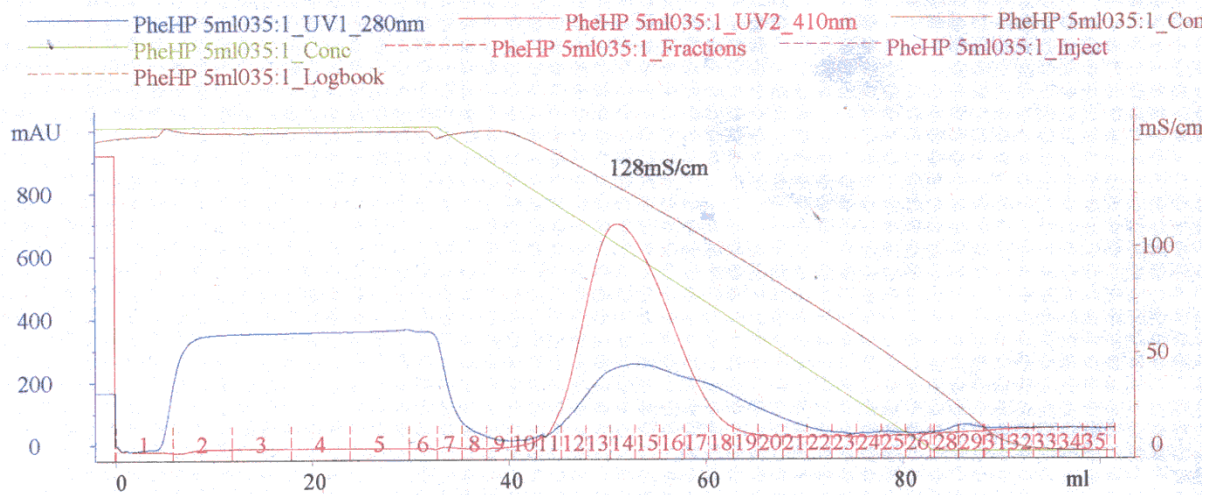
Cgb was expressed in *E.coli* BL 21 strain and though the overall level of expression was relatively high it was mainly insoluble. Only 10-15% of the expressed Cgb was presented in the cell free extract, so that Cgb represented not more then 1-2% of total protein in the CFE. Generally speaking it is unlikely to succeed with 3step purification protocol with so low expression level of the target protein, but lucky combination of the properties of Cgb made it possible.
Firstly, the protein has pI 6.14 and so it have relatively low affinity to anion exchanger. pH for the first (DEAE-Sepharose) chromatography had to be increased to 9 and salt concentration had to low down to 3mS/cm in conductivity terms in order to bind Cgb to the column. So it was eluted from the column at very beginning of NaCl gradient, where not too many *E.coli* proteins appeared.
Secondly, Cgb was found to be relatively hydrophilic protein so powerful HIC was applicable.
Thirdly, Cgb is a monomer of 16kDa and so gel filtration worked well to separate main contaminations remained after HIC.
Below is presented optimized and refined procedure for Cgb purification.

Cell free extract (CFE) was prepared in bufferA(50mM tris buffer pH 9.0) using cells from 2l culture (about 7g). Cells were disrupted by sonication (3x20sec at 16micron amplitude) and cell debris was removed by 10 min centrifugation at 72000g.
CFE: V=34ml, C=16mg/ml, total protein 540mg. Conductivity was checked in CFE and it was diluted with water to 3mS/cm (total volume of the sample became 50ml).
CFE was applied on a 25ml DEAE-Sepharose column at flow rate 5ml/min on AKTA purifier system. 5ml fractions were collected. Elution was performed with 250ml of NaCl concentration gradient from 0 to 0.3M in 50mM tris-HCl pH 9.0 (see chromatogram below).

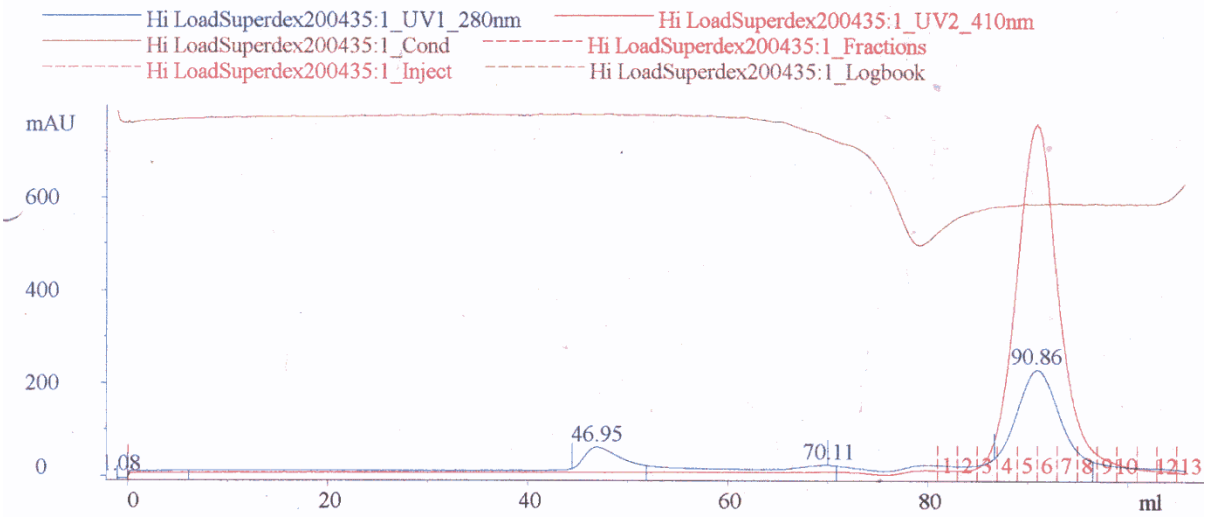


Red fractions 21-24 were combined: V=20ml, C=0.9mg/ml, 18mg

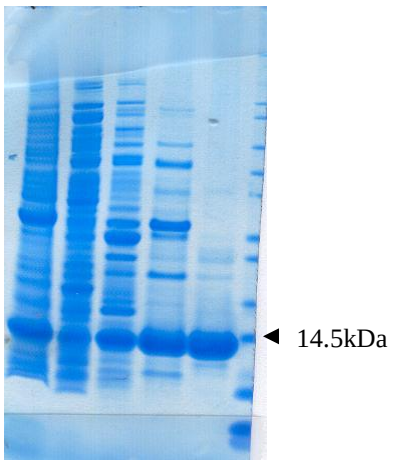
Sample was supplemented with 9ml of 4M ammonium sulphate (to 1.25M) and applied on a 5ml HiTrap-Phenyl-HP column at flow rate 4ml/min. Elution was done by reverse concentration of ammonium sulphate from 1.2M to 0 in bufferA. 2.5ml fractions were collected.



Red fractions 13-15 were combined: V=7.5ml, C=0.6mg/ml, 4.5mg. Red fraction 16 had not been taken on grounds of having high A_{280} and so being more contaminated than other fractions. Volume was reduced to 2ml using VivaSpin device with MWCO 5000 and sample was applied on a 1.6x60cmHiLoad Superdex200 column. Gel filtration was performed at flow rate 1.5ml/min and 2ml fractions were collected:

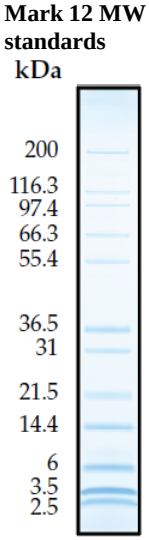


Red fractions 5+6 were combined: V=4ml, C=0.5mg/ml, 2mg.
Progress of purification was analysed by SDS-PAGE (NuPage 4-12%BT Novex gel with MES running buffer):



1. Cell debris
2. CFE
3. After AEC
4. After HIC
5. Final prep
6. Mark12 MW

Cgb was purified to purity of about 90%
Yield was 1mg from 1 l culture
0.4% of total CFE protein.



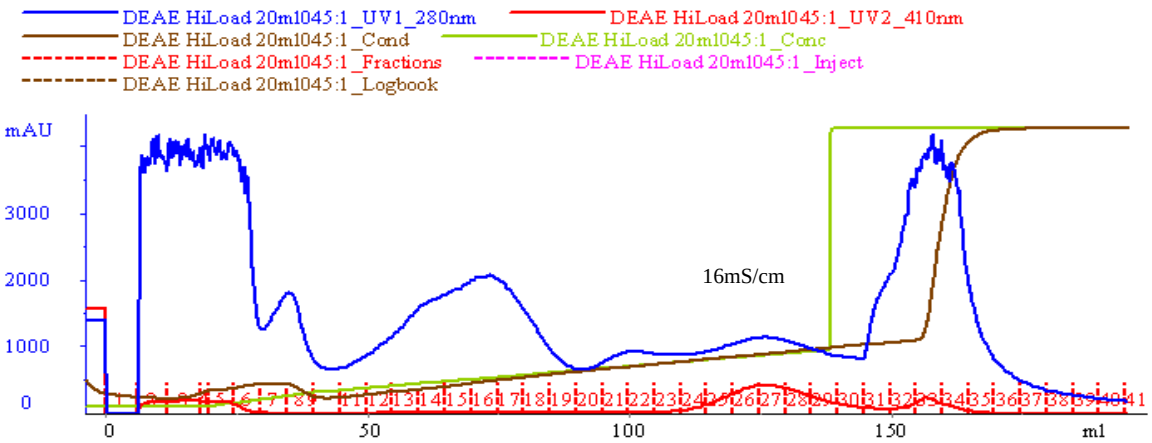
Flavohemoprotein HMP from *Escherichia coli*

MW 43837Da
pI 5.5
$A_{280\ 1mg/ml}$ 1.16

HMP contains two chromophores, Flavin and Hem, so colour of the protein has a bit of orange shade, which does not make a significant contribution to the shape of a visible spectrum of the protein any way.
Level of expression of HMP typically is low, hardly exceed 3%, so we would not expect to purify it to 90%+ purity in 3 step procedure, but this case is an exception and it is possible with application of standard AEC-HIC-SEC protocol. However it was suspected that protein is losing some of flavin during HIC step and it was replaced with second AEC using different matrix (DEAE Toyopearl or Resource Q) and pH. Purity of the protein is lower in this case (about 80%) and preps have one significant contamination (a few %) of 20kDa protein.

Typical purification using AKTA purifier was as follows:
About 7g cell paste was defrosted and suspended in 20ml of buffer A (tris-HCl pH 9.0).
Sonication: 3 cyclesx20sec.
Centrifugation: 15 minutes at 72000g.
CFE: V=23ml, C=20mg/ml, 460mg
AEC was performed on AKTA purifier system at flow rate 5ml/min. Sample was applied on a 1.6x10cm Hi Prep DEAE Sepharose column (GE Healthcare). Elution was done by 160ml of gradient of NaCl concentration from 0 to 0.5M in buffer A. 5ml fractions were collected. 1M NaCl wash was applied when red fractions were eluted:

```
No 1:   Sample Volume and Type
        HMP 450mg
No 2:   Column
        DEAE Hi Load
No 3:   Eluent A
        tris pH 9.0
No 4:   Eluent B
        A+1MNaCl
No 5:   Remarks
        0-5%B 1CV, 5-30%B 8CV  2.6.14
```



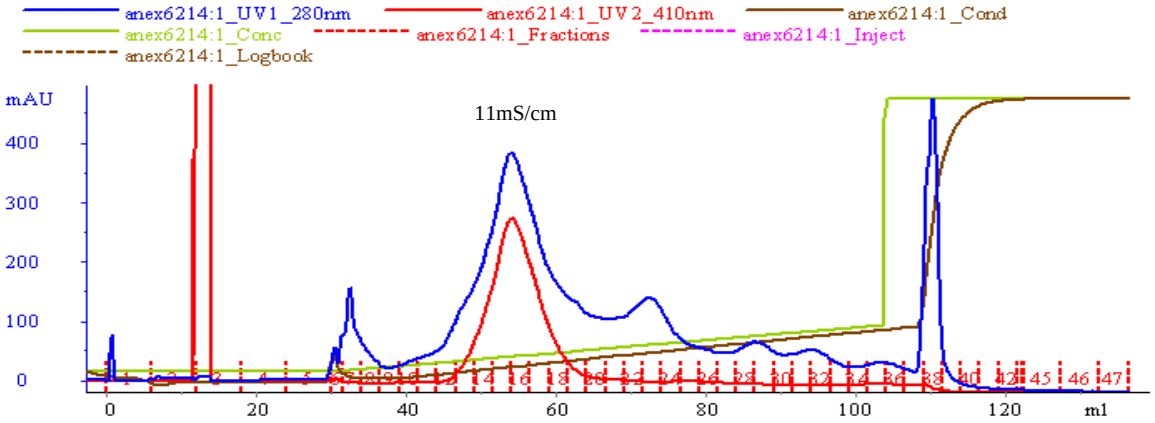
Spectra were measured for red fractions and ratio A_{412}/A_{276} was calculated:

Fr	A_{412}/A_{276} (R)
25	0.18
26	0.32
27	0.36
28	0.32
29	0.25

Fractions with highest ratio, 26-28, were combined: V=15ml, C=4.35mg/ml, 65mg, R=0.33

Sample was diluted with water to conductivity 6mS/cm and applied on a 6ml Resource Q column.
Flow rate 5ml/min, fractions 2.5ml. Gradient: 90 ml 50-250mM NaCl in 50mM MES pH 6.5.

```
No 1:   Sample Volume and Type
        HMP 45mg
No 2:   Column
        ResQ 6ml
No 3:   Eluent A
        MES
No 4:   Eluent B
        A+1MNaCl
No 5:   Remarks
        5-25% 15CV
```

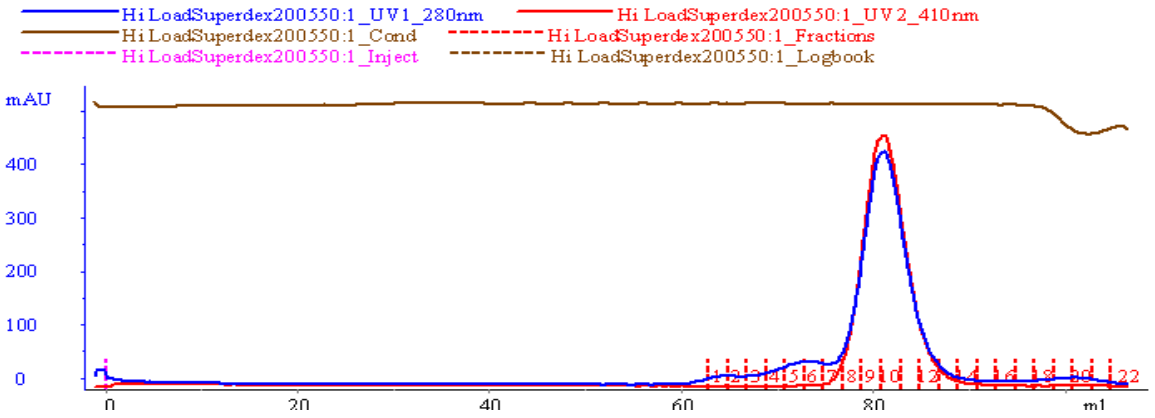


Spectra were taken from red fractions and peaks ratio was calculated

Fr	A_{405}/A_{280}
14	0.66
15	0.77
16	0.86
17	0.82
19	0.64

Fractions 15-17 were combined: V=7.5ml, C=1.25mg/ml, 9.3mg R=0.82
Volume was reduced (using VivaSpin) to 1.25ml, C=7mg/ml, 8.5mg and was applied on a 1.6x60cmSuperdex200 column equilibrated in 0.5M NaCl, 50mM tris-HCl pH 8.0 buffer.
Flow rate1.5ml/min, fractions 2ml.

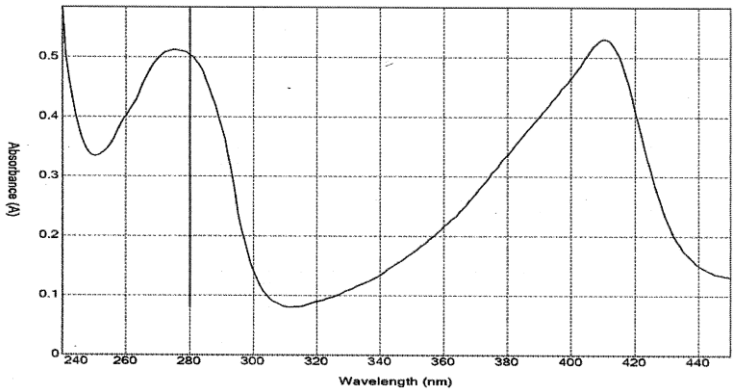
```
No 1:   Sample Volume and Type
        HMP 8.5mg
No 2:   Column
        Superdex200 10
No 3:   Eluent A
        tris 0.5M NaCl
No 4:   Eluent B

No 5:   Remarks
        2.6.14
```



Fractions 9-11were combined: V=6ml, C=0.75mg/ml, 4.5mg R=1.05

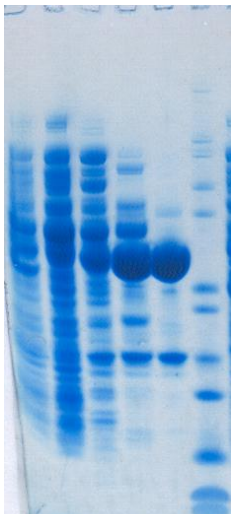
Peak nm	Absorbance A
275	0.513
280	0.504
401	0.473
410	0.531

Cursor nm	Absorbance A
280	0.504

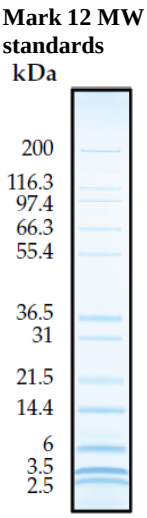


Purification progress was analysed by SDS-PAGE (NuPage 4-12% BT Novex gel)

1 2 3 4 5 6

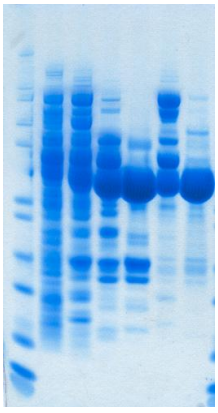


1. Cell debris
2. CFE
3.After DEAE
4. After Resource Q
5 Final prep
6. Mark 12



On a different occasion I had compare two protocols, AEC-AEC-SEC and AEC-HIC-SEC. Second AEC was replaced with HIC. HMP is relatively hydrophobic protein, so early I used Ethyl-Toyoperl and later a standard 5ml Phenyl-HP cartridge, from which HMP eluted in very end of reverse gradient of ammonium sulphate concentration, with 0.1M. As you can see on gel below, purity after this step is poorer then after second AEC(compare line 4 and 6) , but most of contaminations were successfully separated by gel filtration.
Yield was equal in both cases, 0.8% from total CFE protein.

1 2 3 4 5 6 7

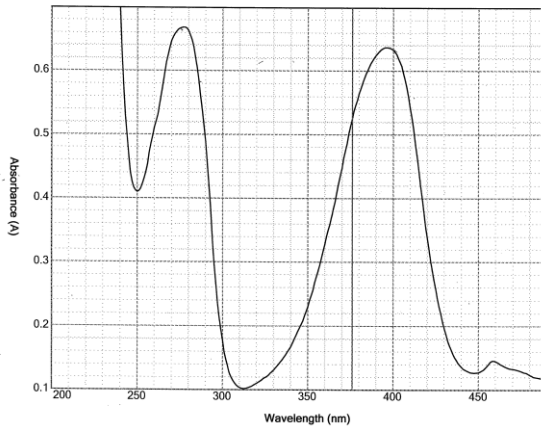


1. Mark12
2. CFE
3. after DEAE-Sepharose pH 9.0
4. after Resource Q pH 6.3
5. after gel filtration (prep 1)
6. after Phenyl-HP (part of the sample from DEAE-Sepharose was applied on a hydrophobic column)
7. after gel filtration (prep2)

Peak nm	Absorbance A
277	0.668
396	0.637
459	0.146

Cursor nm	Absorbance A
376	0.525

R=0.95

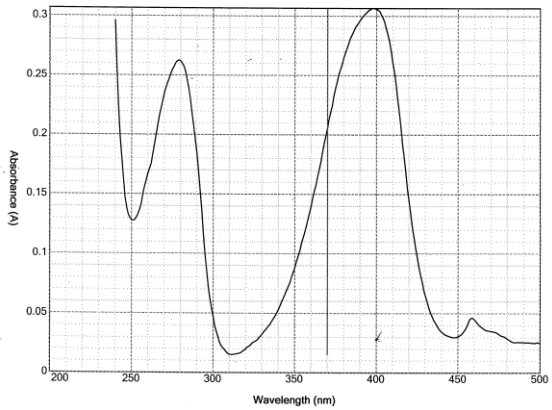


Prep 1(AEC-AEC-SEC)

Peak nm	Absorbance A
279	0.263
398	0.307
459	0.046

Cursor nm	Absorbance A
370	0.205

R=1.2



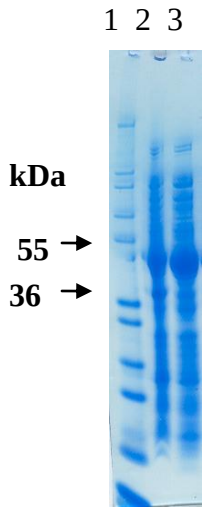
Prep 2 (AEC-HIC-SEC)

3. Almost typical three step purification

Glutamate dehydrogenase from *Clostridium symbiosum*

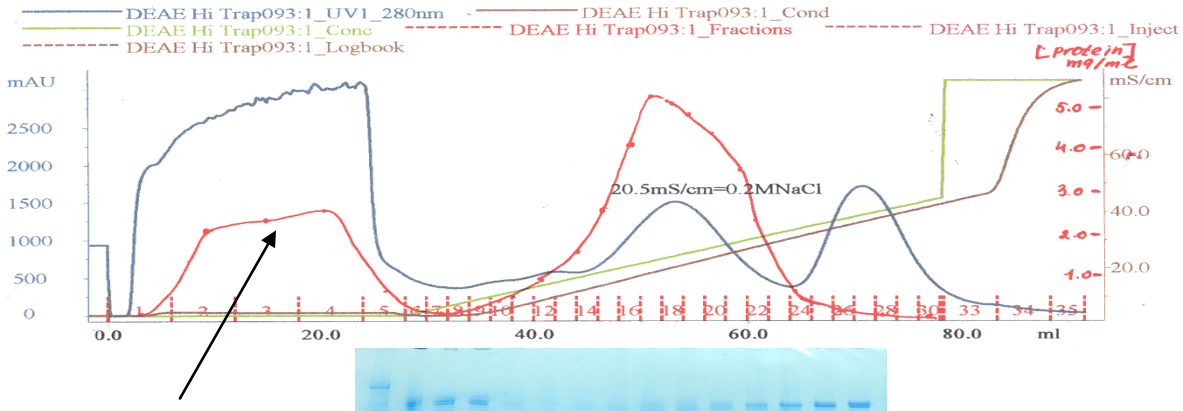
ProtParam results for the target protein:
MW 49296 Da
pI 5.1
Abs 0.1% (1mg/ml) 1.195

GluDH was over expressed in BL21 (DE) cells. Analysis of over expression revealed a very high level of soluble expression. Typically for such a high level of expression a significant band of GluDH was also found in the cell debris.

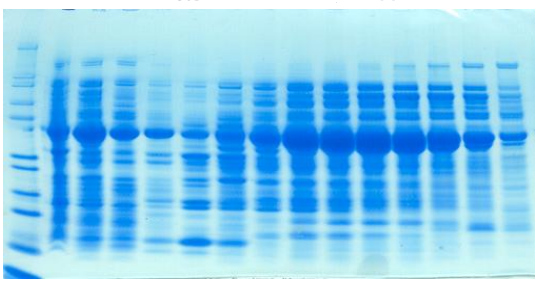


1. Mark 12 MW standards
2. Cell debris (pellet)
3. Cell free extract

Cell free extract was prepared in Buffer A (50mM tris-HCl pH 8.0) from about 2g of defrosted cells as described in the Protocol number 2.
CFE: V=23ml, protein concentration (by Bradford, Protocol number 9) = 5.5mg/ml,
total protein 125mg.
CFE was applied on a 5ml Hi Trap DEAE FF cartridge washed with 5ml of BufferA and proteins were eluted by 50ml (10CV) gradient 0-0.5M NaCl in Buffer A at flow rate 4ml/min. 2ml fractions were collected. Protein concentration was measured in fractions by method of Bradford and fractions with protein were analysed by SDS-PAGE (Novex Nu-Page BT 4-12% gel). (see chart and gel picture below).

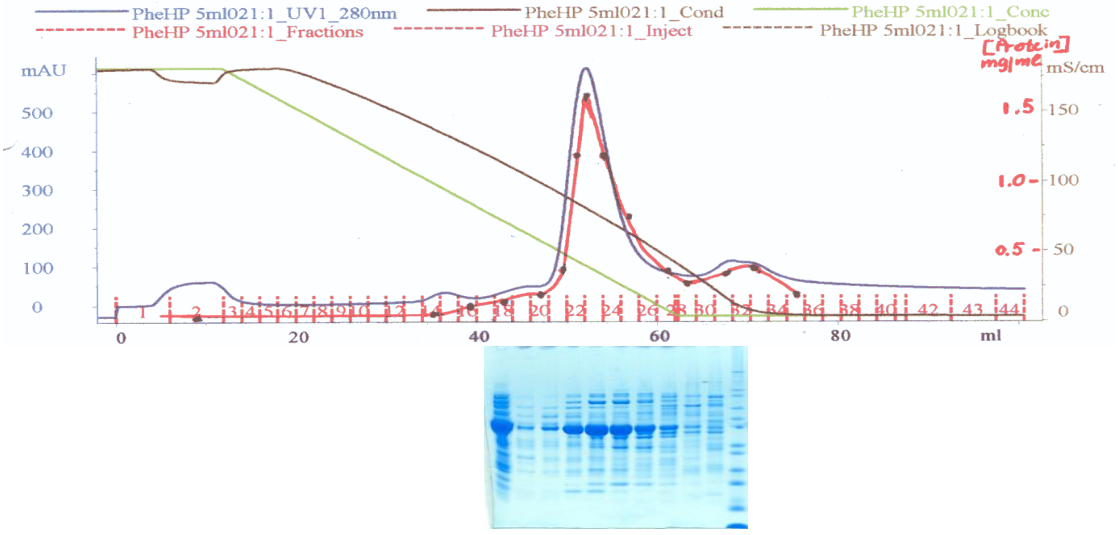


Unbound material



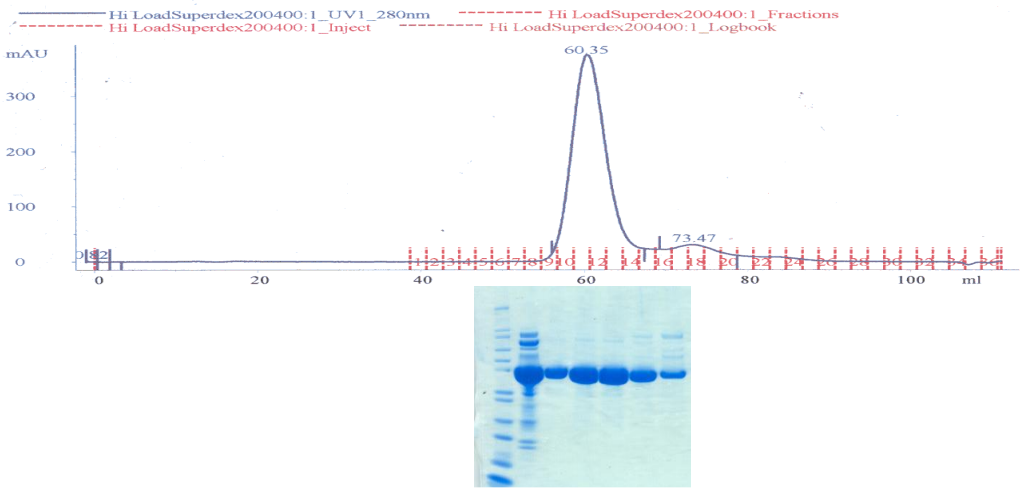
CP CFE 4 10 12 14 16 17 18 19 20 21 22 24 fractions

GluDH were eluted as a broad peak due to a very high expression, also some of the GluDH was found in the unbound material, which also is not unusual for heavily expressed proteins. Column volume has to be doubled in this case (two 5ml cartridges screwed together) to improve protein binding and yield.
Fractions 17-22 were combined, volume 12ml, C=4.5mg/ml, total 54mg.
The sample was supplemented with 7.2ml of 4M ammonium sulphate to bring ammonium sulphate in the sample to 1.5M and applied on a 5ml Phenyl-HP cartridge. Elution was done by 50ml (10CV) of the reverse gradient of concentration of (NH4)2SO4 from 1.5M to 0 in Buffer A at flow rate 5ml/min. 2ml fractions were collected.
On this stage the peak is very obvious, A_{280} corresponds to protein, fractions to combine are easy to identify as 22-24. SDS-PAGE is not essential here and was done later just for demonstration.



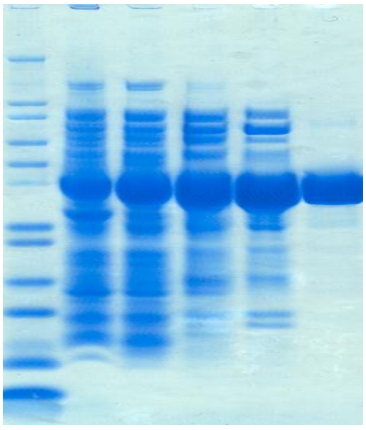
Phe-HP 20 21 22 23 24 25 26 28 31 Fractions

GluDH was eluted from column with 0.65M ammonium sulphate, which was found by measuring the refraction in fraction 23. This means that optimal gradient for this protein is from 1-0.9M to 0 of ammonium sulphate while length of gradient could be reduced to 40ml (8 CV).
Fractions 22-24 were combined, volume 6ml, protein concentration 2.7mg/ml, total protein 16mg.
The volume of the sample was reduced to 2ml using Viva Spin concentrator 30000 MWCO and sample was applied on a gel filtration column, 1.6x60cm HiLoad Superdex200.
Gel filtration was performed in buffer 0.5M NaCl, 50mM tris pH 8.0 at flow rate 1.5ml/min. 2ml fractions were collected after void volume. GluDH was eluted at 60.3ml with apparent MW 290kDa, which indicates that GluDH is a hexamer. Once again, there are two obvious fractions to harvest, 11 and 12.
Fr 11+12: V= 4ml, C=1.7mg/ml, 7mg totally.



GFl 10 11 12 13 14 fractions

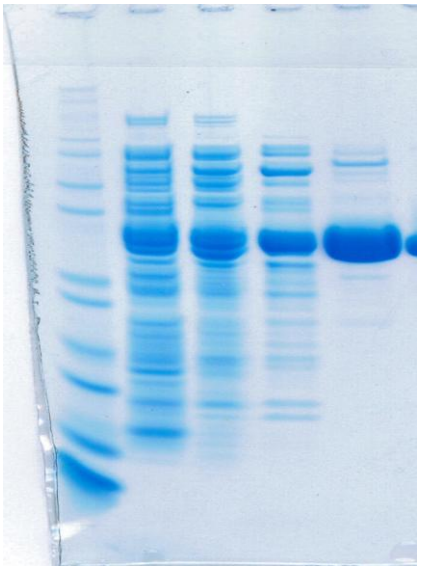
SDS PAGE (Novex BT 4-12% gel) was performed to analyse progress of the purification. In this case, despite a very high expression, two purification steps were not enough to reach acceptable purity. This is because on both AEC and HIC GluDH elutes with the majority of other proteins. In this case gel filtration works very well, due to hexameric state of the GluDH molecule.



CP CFE AEC HIC GF

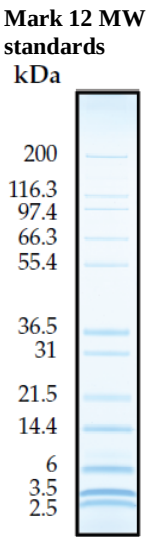
	Total protein	Purity
CFE	130mg	~15%
AEC	54mg	~40%
HIC	16mg	~60%
GF	7mg	~90-95%

The protocol works very well with much lower expression level:



CFE AEC HIC GF

CFE	50mg	~3%
AEC	11mg	~6%
HIC	6.7mg	~25%
GF	0.6mg	~80-85%



4. Monitoring purification by enzyme activity

Glutamate dehydrogenase from *Clostridium symbiosium*

Here I would like to show how purification can be performed based on enzymatic activity of a target protein. GDH is a good example because activity assay is very simple and quick.

Activity assay:

1mM NAD, 20mM Na glutamate, 50mM K Phosphate pH 7.0. NAD reduction to NADH can be monitored by absorbance at 340nm.

Mixture composition:

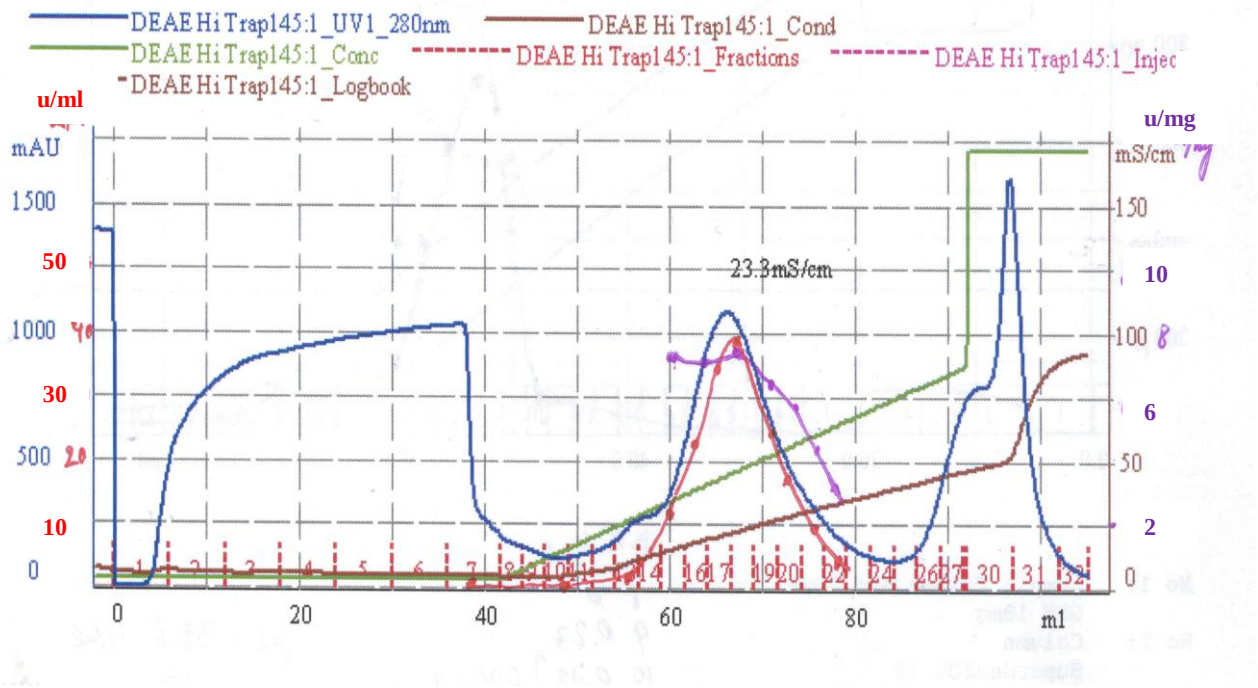
0.96ml KPhosphate buffer
20µl 1M Na Glu
10µl 0.1M NAD
1-20µl GDH solution

Absorbance at 340nm is monitored for 1-2 minutes. Change in absorbance is used to calculate NADH production using extinction coefficient $6220 M^{-1} cm^{-1}$.

Purification procedure

CFE was prepared in 0.1M K Phosphate pH 7.0 on this occasion. C=9.5mg/ml, V=9ml, total protein 85mg. Specific activity in CFE was found to be 8.0u/mg. Total activity 680u.

CFE was diluted fourfold to reduce ionic strength and was applied on a 5ml DEAE FF cartridge. Chromatography was performed on AKTA purifier system at flow rate 4ml/min. Elution was performed with 50ml gradient of NaCl concentration from 0 to 0.5M in 20mM K Phosphate pH 7.0 buffer. 2.5ml fractions were collected.

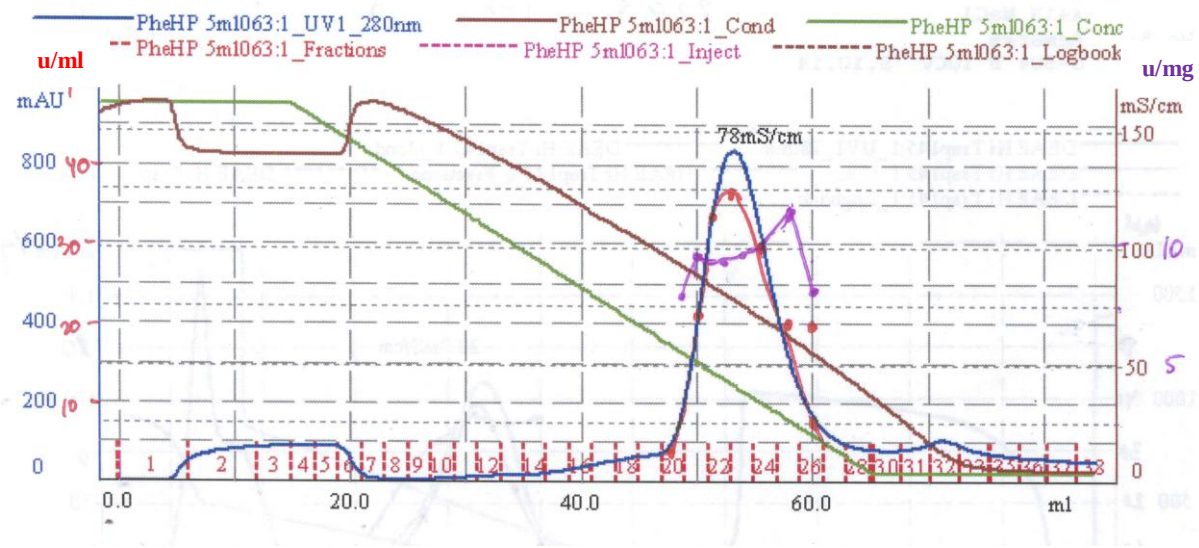


Activity was measured in every third fraction to the point when activity was found and then activity and protein concentration were measured in each fraction as shown below:

Fraction	Activity (u/ml)	Protein conc. (mg/ml)	Specific activity (u/mg)
6	0.8		
10	0.3		
13	0.3		
15	12.1	1.65	7.3
16	22.0	3.1	7.1
17	34.1	4.7	7.2
18	39.3	5.2	7.6
19	25.8	4.1	6.3
20	18.4	3.1	5.9
21	10.1	2.4	4.2
22	4.5	1.5	3

Fractions 15-19 with highest specific activity were combined: V=12.5ml, C=3.45mg/ml, 43mg, 7.1u/mg, 305u

Sample was supplemented with 1M ammonium sulphate and applied on a 5ml Phenyl-HP cartridge. Elution was performed by 50ml of reverse gradient of ammonium sulphate concentration from 1.2 to 0M in 20mM K Phosphate buffer. 2 ml fractions were collected.

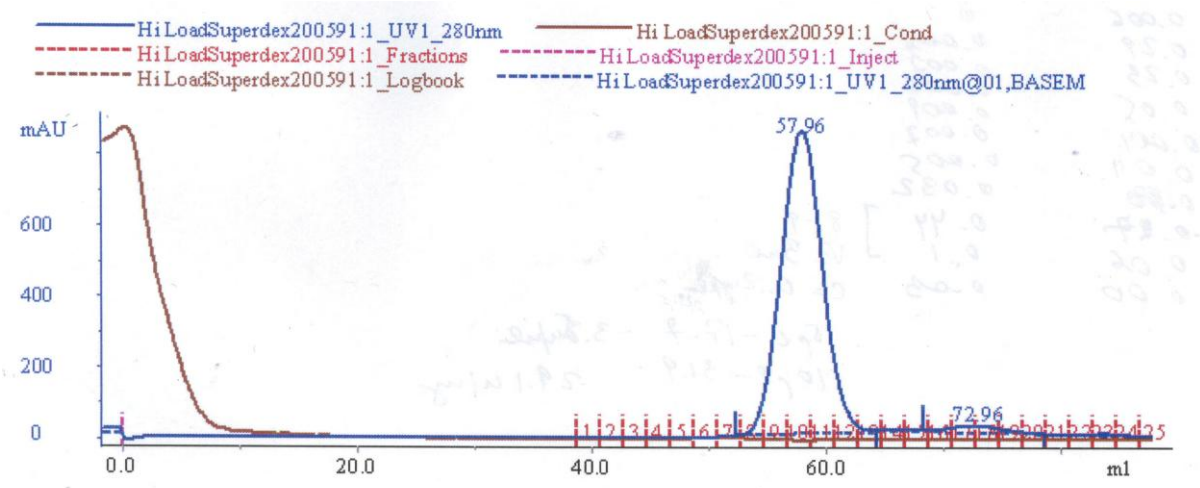


Activity and protein concentration were measured across the UV peak:

Fraction	Activity	Protein conc.	Specific activity
20	4.0	0.4	8.0
21	18.0	1.9	9.5
22	34.3	3.7	9.3
23	35.7	3.8	9.4
24	29.9	3.0	10.1
25	20.9	1.7	11.8
26	7.7	1	7.7

Fractions 21-25 were combined: V=10ml, C=2.55mg/ml, 25.5mg, 10.5u/mg, 268u

Volume of the sample was reduced to 1ml using VivaSpin and applied on a 1.6x60cm HiLoad Superdex200 gel filtration column.
Gel filtration was run at flow rate 1.5ml/min in 0.1M K Phosphate pH 7.0 buffer. 2ml fractions were collected.



Peak fractions 10+11 were combined: V=4ml, C=2.4mg/ml, 9.8mg. Specific activity 21.7u/mg.

Summary:

	Protein conc. mg/ml	Total protein mg	Activity u/ml	Specific Activity u/mg	Total Activity u	Purification fold	Yield %
CFE	9.5	85	34.8	8.0	680		
DEAE	3.45	43	30.8	8.9	390	1.1	57
Phenyl-HP	2.55	25.5	27.9	10.9	278	1.2	41
GF	2.4	9.8	72	21.7	212	2.7	31

5. Power and failure of dye pseudo affinity chromatography

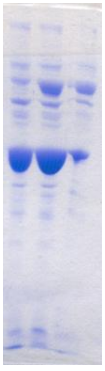
Phenylalanine Dehydrogenase from *Nocardia sp.*

Purification of PheDH from *Nocardia sp.* is interesting example of the protein purification from the native source. PheDH was naturally over expressed in the *Nocardia* cells by growing culture in the presence of phenylalanine. Half a kilo of the cell paste was sent to us from Proton Down alongside with the simple one step purification protocol which employed pseudo affinity chromatography on a Procion Red HE-3B Sepharose4B. A pot of the matrix was also included. This type of chromatography often is used for purification of dehydrogenases because of similarity in molecular structures of dye and NAD, one of the dehydrogenase substrates. Purification protocol suggested that CFE to be applied on a Red column in 50mM tris buffer pH 8.5, unbound material to be removed by washing the column with the starting buffer and then PheDH to be eluted bio specifically with 1mM NADH in the same buffer.

According to the protocol CFE was prepared from 50g of cell paste in 50mM tris buffer pH 8.5 by the standard procedure (Protocol 2).
CFE: V=180ml, C=15mg/ml, total protein 2700mg.
Monitoring of the target protein in this case was done by activity. There is a fast and easy assay based on monitoring of NADH produced in reaction of oxidative deamination of phenylalanine by measuring absorbance at 340nm.
The specific activity in the CFE was found to be 0.1U/mg, suggesting level of expression of PheDH was about 2% of total protein.
Red Sepharose chromatography: Sample of 80ml of CFE (1200mg total protein, 120U activity) was applied on a 30ml Red Sepharose column at flow rate 3ml/min. Column was washed with the starting buffer (50mM tris-HCl pH 8.5).
Unbound fraction: V= 150ml, 4.5mg/ml, total 675mg, 66U activity. It was clear that something is not right: just 50% of total protein was found instead of expected 90% or so and also specific activity in this fraction was the same as in the CFE. It looked like half of the protein was bound to the column non-specifically.

Active fractions

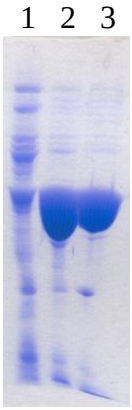
1 2 3



Elution was performed with 1mM NADH. 8ml fractions were collected and checked for activity. Activity was found in three fractions and was just 8U in total. Specific activity was 1.8U/mg. SDS-PAGE (10% Laemmly gel) analysis reveal purity not better then 60-65%:
So, 80% of bound PheDH was stacked on the column irreversibly, yield of the chromatography was 6.6% and a lot of protein was bound to the column non-specifically.
Further purification was attempted employing **gel filtration** on a 1.6x60cm Hi-Load Superdex200 which resulted in 2mg prep with specific activity 2.9U/mg. SDS-PAGE analysis suggested about 75% purity. (See below, Preparation 1).

So, Dye chromatography failed and most likely particularly batch of the home made matrix was to blame. Commercial Red Sepharose CL-6B was ordered from Pharmacia (now GE Healthcare) for comparison.
What may be wrong with the matrix? The fact that half of material was bound to the column non-specifically most likely is due to that “aggressive sites” in the matrix which was a common knowledge among biochemists on early days of protein purification, when cellulose based matrixes were in use. Sepharose normally is much better in respect with non-specific binding, but it was subjected to some drastic conditions (like NaOH) during the dye binding which could lead to such an “aggressive sites”. The method to fight a problem is to saturate these sites with the protein. In hope that saturation was achieved during the first chromatography I give the column the second chance. But on a way I decided to try IEC as well using the material that was not bound to the column on the first run.

Anion exchange chromatography. Flow through fraction from Red column (675mg of total protein, 66U of activity) was applied on a 25ml DEAE-Sepharose column; elution was performed by 300ml NaCl gradient from 0 to 0.4M NaCl in 50mM tris-HCl buffer pH 8.5. 8ml fractions were collected and checked for activity. Active fractions containing 140mg of total protein, 49U activity, 0.35U/mg. Purification fold is 3.5 which is reasonable, but not very high.
Red Sepharose. Sample obtained after DEAE chromatography was concentrated to 4.5ml and applied on a 30ml Red-Sepharose column . Column was washed with 50mM tris-HCl buffer pH 8.5 and PheDH eluted with 1mM NADH. This time activity was bound to the column and successfully eluted, given 7.9mg of total protein and 24U activity. SDS-PAGE analysis revealed purity on this stage being about 80-85%:

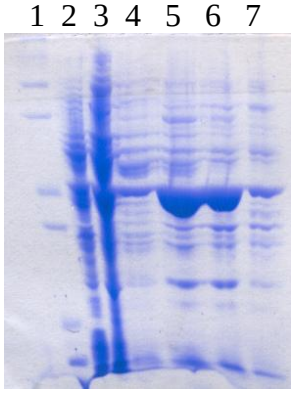


1. Sample applied on a Red-Sepharose
2-3. Active fractions

Activity assay: 0.2M Glycine buffer pH 10.8
3mM NAD^+
5mM Phenylalanine

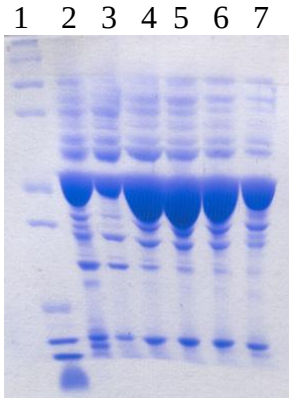
Still PheDH yield on this stage was 50% which was an improvement from 6.6%, but what we expect from affinity chromatography is 70-90%, so seems some of protein still was stacked to the matrix.
For further purification sample was concentrated and subjected to gel filtration. Combined active fractions contained 4.8mg of protein with specific activity 3.4U/mg. Purity estimated by SDS-PAGE was about 90%. (See gel below, Preparation 2)

The remaining 100ml of the CFE (1500mg of total protein, 150U activity) was subjected to 3 step purification protocol.
AEC: chromatography was done on a 25ml DEAE-Sepharose column, gradient 300ml from 0 to 0.4M NaCl, 8ml fractions were collected and checked for activity. Active fractions were combined, in total 122U of activity, 380mg of total protein, specific activity 0.32U/mg.
AS cut:$AmSO_4$ was added to the sample to 1.4M. Significant pellet was removed by centrifugation. No activity was found in the pellet. In the supernatant fraction there was 270mg of total protein with specific activity 0.4U/mg, 108U.
HIC: Sample was applied on a 25ml Butyl-Toyopearl column and eluted by 300ml of gradient of ammonium sulphate from 1.4 to 0 in buffer A. Activity was found in fractions eluted with about 0.3M ammonium sulphate. Two fractions with the highest specific activity were combined, 15mg total protein, purity by SDS-PAGE about 40%:



1. Mark12 MW standard
2. CFE
3. DEAE-Sepharose
4-7. Butyl-Toyopearl fractions with PheDH activity

SEC:Sample was concentrated and applied on a gel filtration column equilibrated in 50mM tris-HCl pH 8.5. 2ml fractions were collected and analysed for activity and protein concentration. Three fractions with the highest activity were combined, yielded in 5mg of the protein with specific activity 2.5U/mg, about 60% purity estimated by SDS-PAGE:



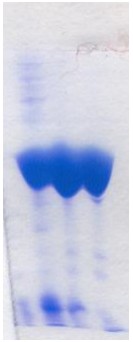
1. Mark 12
2. Sample applied on a gel filtration column

3-7. Fractions across activity peak

With a target protein still not pure and with a little option left I have attempted to employ Red column on this stage. Sample obtained after gel filtration was applied on a 15ml Red –Sepharose (commercial, from Pharmacia) column and this time activity was bound to the column and after elution with 1mM NADH 3.8mg of PheDH was obtained with specific activity 3.6U/mg and apparent purity on SDS-PAGE better than 90%. (Preparation 3). Still very low yield, but at least purity was OK.

Comparison of the three preparations:

Preparations 1 2 3



	Prep	Specific Activity	Purity by SDS-PAGE	Yield
1	RShm+GF	2.9U/mg	75%	4.8%
2	UnboundRShm+IEC+RShm	3.3U/mg	85%	24%
3	IEC+HIC+GF+RSc	3.6U/mg	90%+	9.5%

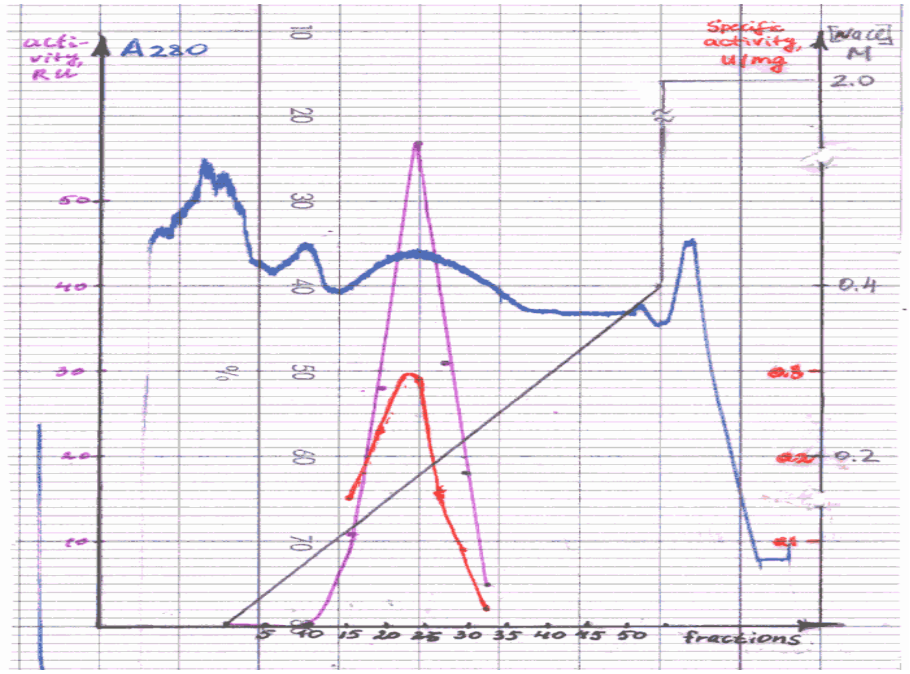
RShm=Red Sepharose home made
RSc=Red Sepharose commercial

Apparently method 2 was the best, but the disadvantage of this one was that we still need to use big Red-Sepharose column and spend about 60ml of expensive NADH for elution.
Later I have found way to improve ammonium sulphate cut and even without Red chromatography about 80-85% purity of the PheDH preparations could be achieved with "3step protocol". Purity was good enough for crystallisation. However, later we had attempted to obtain amino acid sequence of the protein by Edman degradation. For that purpose high purity of the protein was essential and Red chromatography was back, but on a smaller column, with less NADH to be spent.
The typical purification procedure of PheDH from *Nocardia* is presented below.

Purification of PheDH Nocardia 1.8.2002

CFE was prepared from 30g of cell paste in Buffer A.
AEC: Sample (total 2400mg of protein, 220U activity) was applied on a 60ml column with DEAE-Sepharose FF at flow rate 10ml/min. Elution was performed with 500ml of NaCl

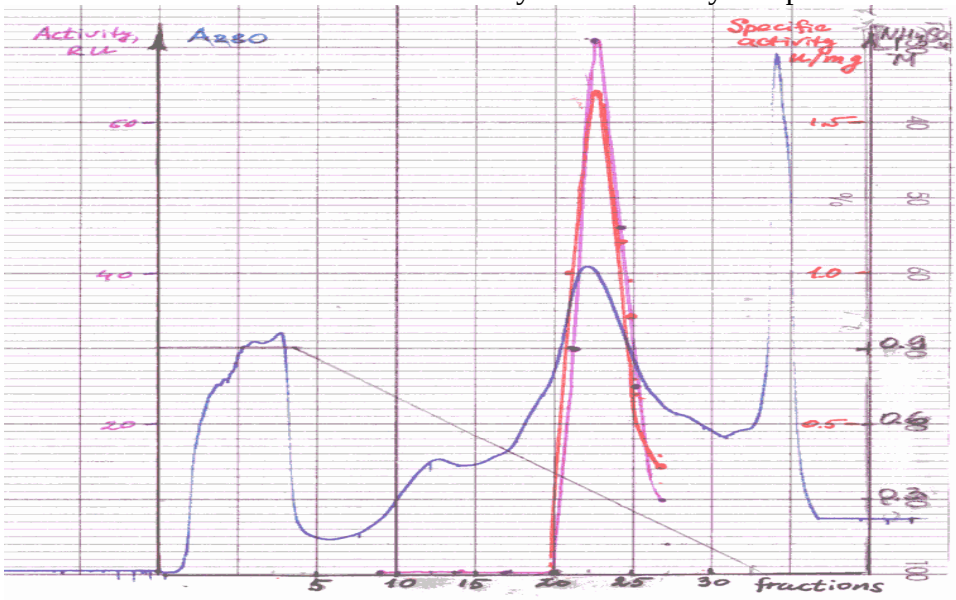
concentration gradient 0-0.4M. 8ml fractions were collected and analysed for activity and protein concentration (by Bradford):



Fractions 16-28 were combined, V=92ml, total protein 662mg, 170U activity.

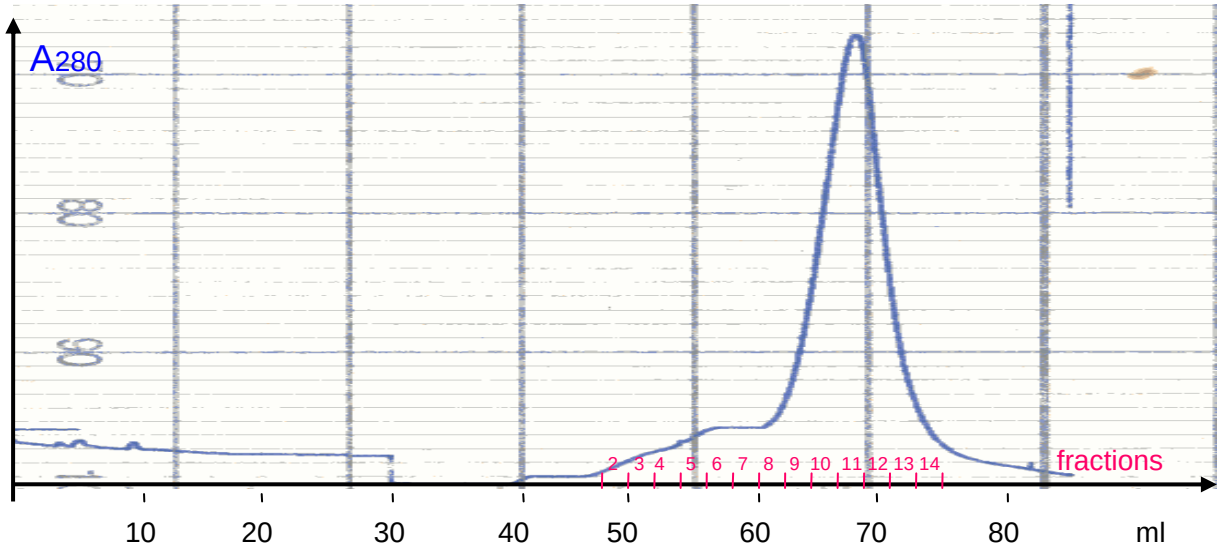
AS cut: 73ml of 4M ammonium sulphate were added to bring AS concentration to 1.8M. Pellet was spun down and dissolved in Buffer A. 4M AS solution was added to the sample to bring AS concentration to 0.9M. Pellet was spun down.

HIC: Supernatant fraction containing 227mg of total protein and 152U of activity was applied on a 25ml column with Butyl-Toypearl 650S, equilibrated in BufferA+0.9M AS at flow rate 3ml/min. Elution was performed with 250ml of reverse gradient of AS in Buffer A from 0.9M to 0. 7ml fractions were collected and analysed for activity and protein concentration:

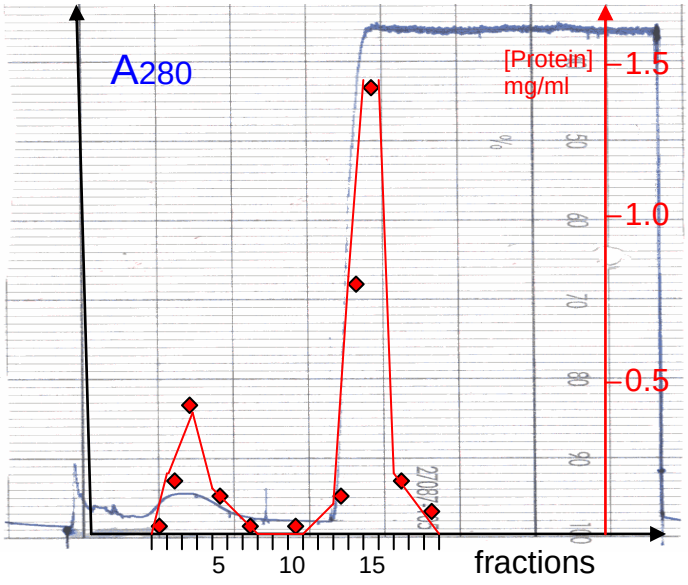


Fractions 22-24 were combined, total 37mg and 81U of activity and volume was reduced to 1.2 ml using Viva Spin concentrator 30000MWCO.

SEC: Sample was applied on a 16x60 HiLoad Superdex200 column equilibrated in 50mM tris-HCl buffer pH 8.5. Gel filtration was done at flow rate 1.45ml/min. 2ml fractions were collected (see chart below).



Three peak fractions (10-12) were combined (18mg of total protein) and applied on a 10ml column with Red-Sepharose (Pharmacia) at flow rate 1ml/min. Column was washed with 15ml of buffer 50mM tris-HCl pH 8.5 and then eluted with 1mM NADH in the same buffer:

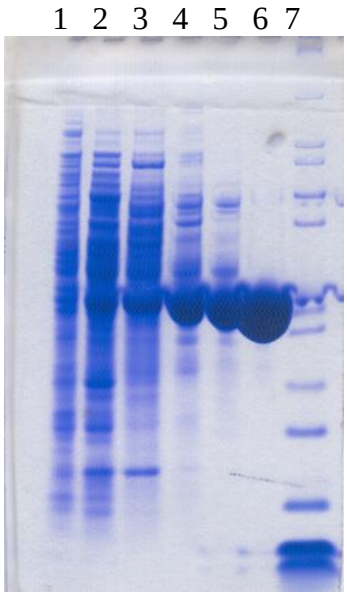


Fractions 14 and 15 with the highest protein concentration were combined (10mg in total).

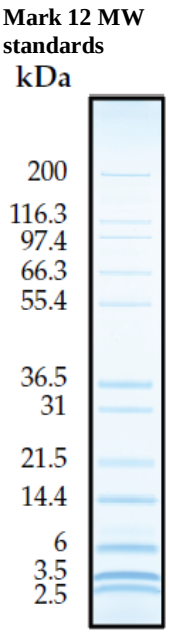
Purification process was analysed by checking specific activity on each purification step (see table below) and also by SDS-PAGE (4-12% BT Novex gel)

Progress of purification of PheDH from *Nocardia*

	Protein concentration mg/ml	Activity U/ml	Specific activity U/mg	Total protein mg	Total Activity U	Yield %
CFE	28	2.75	0.09	2400	220	100
DEAE	5.6	1.45	0.26	660	171	75
AS cut	3.7	2.05	0.55	277	152	69
BTP	2.85	6.3	2.2	37	81	37
GF	2.4	7.1	3.0	18	54	25
Red Sepharose	1.5	6.4	4.4	10	44	20



1. CFE
2. DEAE-Sepharose
3. AS cut
4. Butyl-Toyopearl
5. Gel filtration
6. Red Sepharose
7. Mark12 MW standard



Lessons learned about Dye chromatography

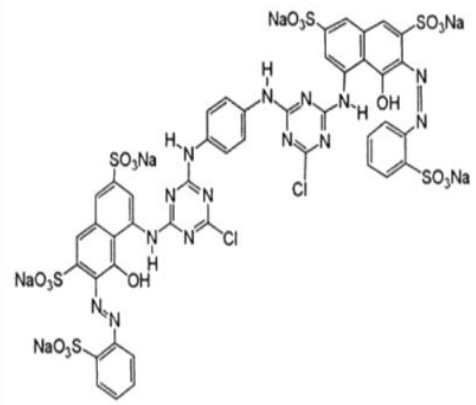
Condition for binding should be optimised.
In case of PheDH from *Nocardia*:

- Buffer – 50mM tris-HCl pH 8.5, no salt
- Amount – 1.5mg of PheDH/ml of matrix maximum
- Sample volume – do not exceed column volume
- Bio-specific elution is preferable. Better to use higher concentration of ligand. 3mM NADH elutes about 10% more enzyme than 1mM.

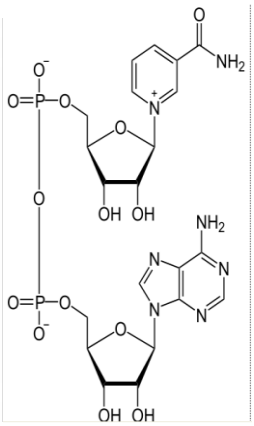
Glutamate dehydrogenase from *Clostridium symbiosum* again
Dye chromatography versus three step purification protocol

Unlike majority of NAD-binding proteins Glutamate dehydrogenase from *Clostridium symbiosum* does not bind to commercial Red Sepharose which has Procion HE-3 dye cross linked.

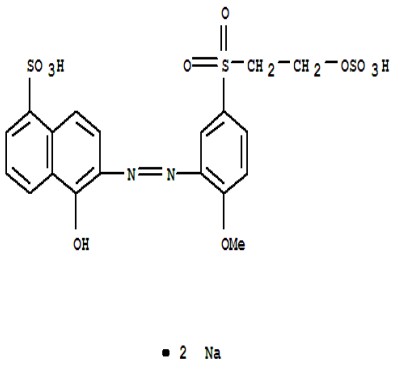
However it can be purified using different red dye, Remazol red (Syed et all., 1991, BAA, v.1115, p. 123)



Procion HE-3



NAD



Remazol red

Purification of GDH from C. symbiosum using Remazol red pseudo affinity chromatography

Remazol-Sepharose was prepared as follows:

200ml of Sepharose 6B were washed with water to remove ethanol, suspended in 700ml of water. 1.9g of Remazol red was dissolved in 200 ml of water and added to Sepharose. The mixture was incubated for 30 minutes with occasional stirring. 100ml of 20% NaCl was added and incubated for next 30 minutes. Then 5ml of 5M NaON was added and left for 4 days with occasional stirring. The matrix was placed in the column and washed with water, 6M Urea, 1M NaCl and 10 volumes of water. Matrix was taken from the column and used to make columns of an appropriate size. We use 2ml columns packed with Remazol-Sepharose in practical classes for undergraduate students in GDH purification experiment.

We found that maximal capicity of our matrix is about 0.5mg of GDH/ml. However capicity seems was declining with every use of the column.

Biospecific elution which was succesfully applied for purification of PheDH from Nocardia (see chapter above) was proved to be not cost effective as it required at least 5mM of NADH to complete elution while resulting prep was not of that " 100%" purity as we obtained for PheDH. So elution was performed with 1M NaCl as in original Syed at al. paper.

Analytical purification on 2ml Remazol-Sepharose column went very well.

0.25ml of CFE (C=8mg/ml, Specific activity 11u/mg (2mg, 22U)) prepared in 0.1M KP pH 7.0 buffer (buffer K) was applied on a 2ml Remazol-Sepharose column (made in PD-10 column).

1ml fractions were collected.

Column was washed with 6ml of buffer K and then GDH eluted with 1M NaCl in buffer K.

Activity was checked in fractions. Some of the activity was found in unbound material and wash. Majority of activity was found in elution fraction.

The balance of activity and total protein was found to be:

	Total activity	Total protein	Specific activity
Unbound and wash:	2u	1.4mg	1.4u/mg
Elution:	19u	0.55mg	34.5u/mg

So, 1.95mg (97%) of total protein and 21u (95%) of activity was recovered.

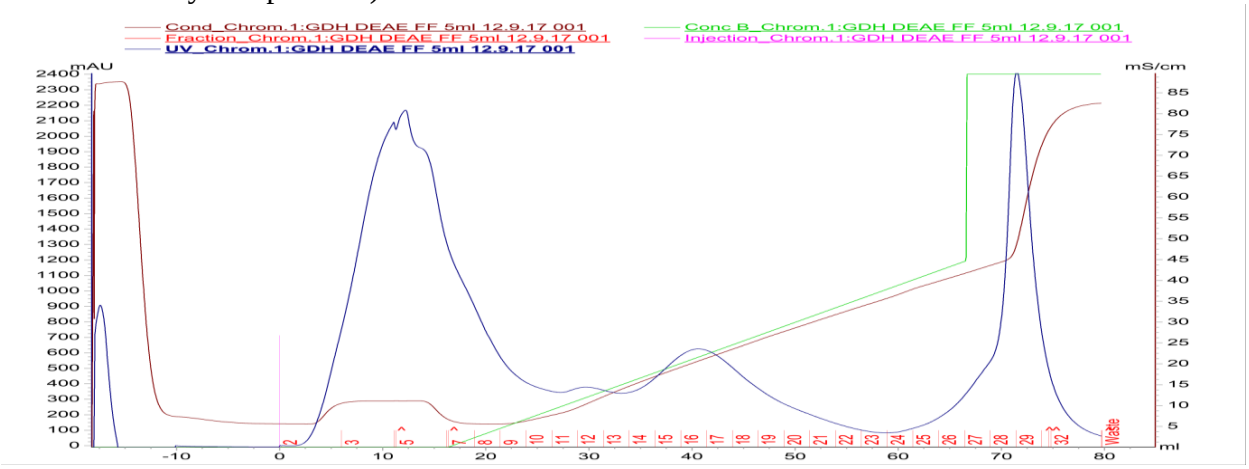
Column seems was overloaded with GDH, so, capicity of matrix was about 0.25-0.3mg GDH per ml.

Preparative purification:
Three step protocol (see Show cases, cases 3 and 4) versus Remazol-Sepharose

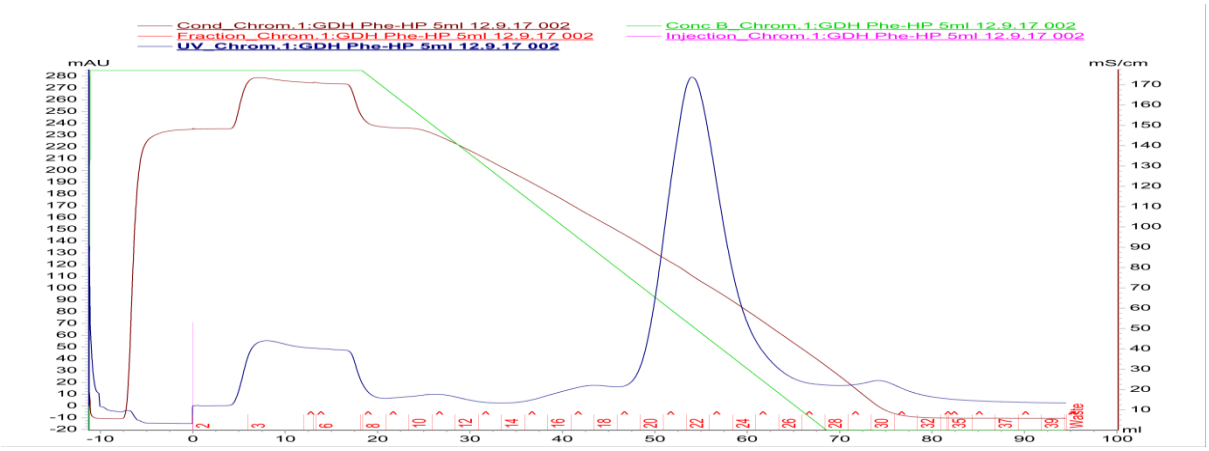
CFE prepared in KP pH 7.0 buffer was defrosted and filtred through 0.45µ filter:
V=12ml, C=6.7mg/ml
CFE was divided in two portions, 6ml (40mg of total protein) each.

Three step protocol

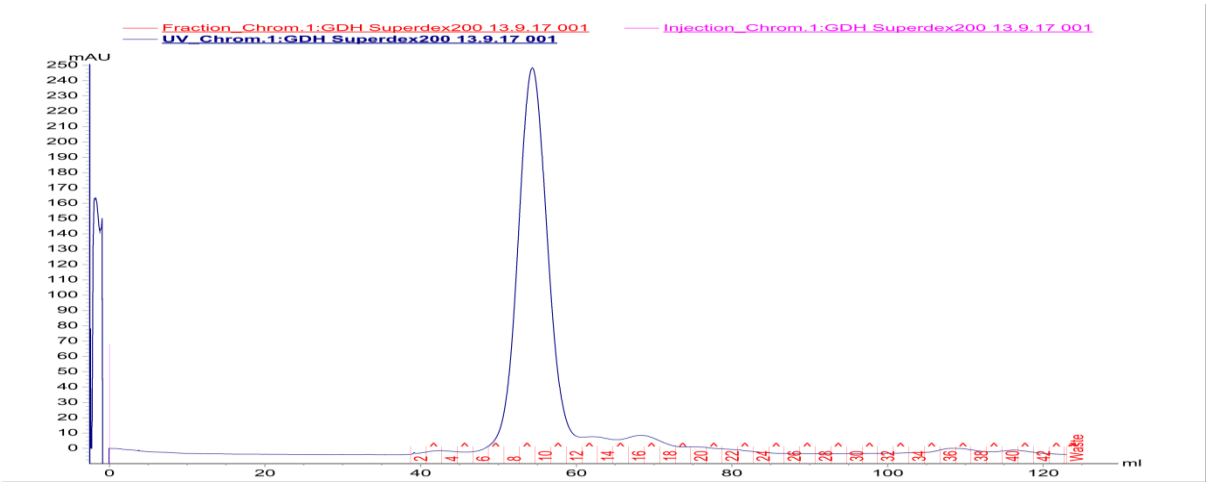
Anion exchage chromatography: 40mg of CFE was diluted three fold and applied on a 5ml DEAE FF cartridge in buffer 20mM KP pH 7.0. Elution was performed by 50ml of gradient of NaCl concentration from 0 to 0.5M. 2.5ml fractions were collected and analysed for activity (see case 4 for assay composition).



Active fractions 14-19 were combined, V=15ml, C=1.3mg/ml, 19.5mg, suplimented with 1M ammonium sulphate and applied on a 5ml Phenyl-HP cartridge.
Hydrophobic chromatography: Elution was done by 50ml of reverse gradient of AS concentration from 1-0M in 20mM KP pH 7.0. 2.5ml fracttions were collected.



Based on elution profile fractions 21-24 were combined, V=10ml, C=0.67mg/ml, 6.7mg
Volume was reduced using Viva Spin with MWCO 50000 to 1ml, C=5.4mg/ml, 5.4mg.
Gel filtration : Sample was applied on a gel filtration column (1.6x60 HiLoad Superdex200) equilibrated in 0.5M NaCl, 50mM tris pH 8.0 (standard GF buffer).
Flow rate 1.5ml/min.2ml fractions were collected.



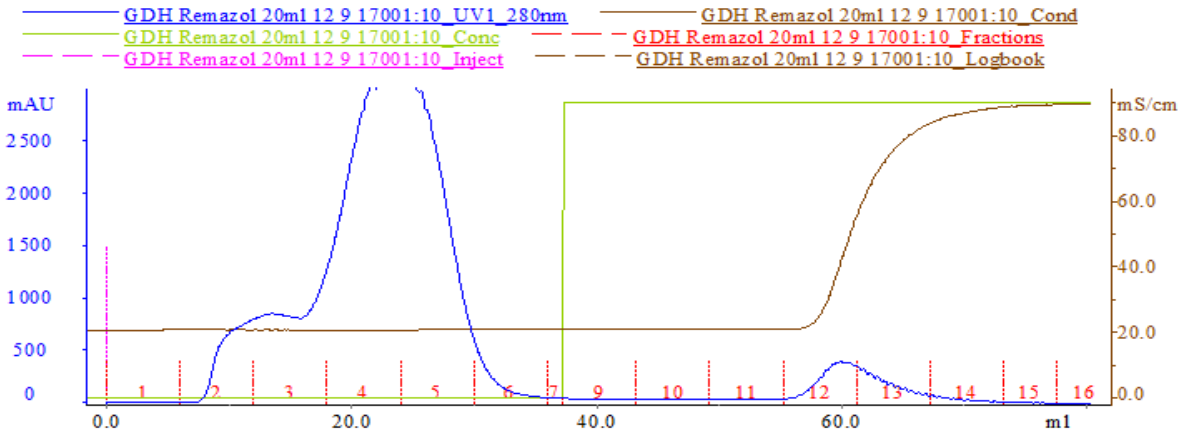
Fractions 8-10 were combined: V=6ml, C=0.56mg/ml, 3.4mg (**Final prep $_{3sp}$)**

Remazol-Sepharose purification

20ml column was made for purification. Preliminary experiments have shown that new unused matrix have capicity about 0.5mg GDH per ml of matrix, so expected output from this column could be up to 10mg.

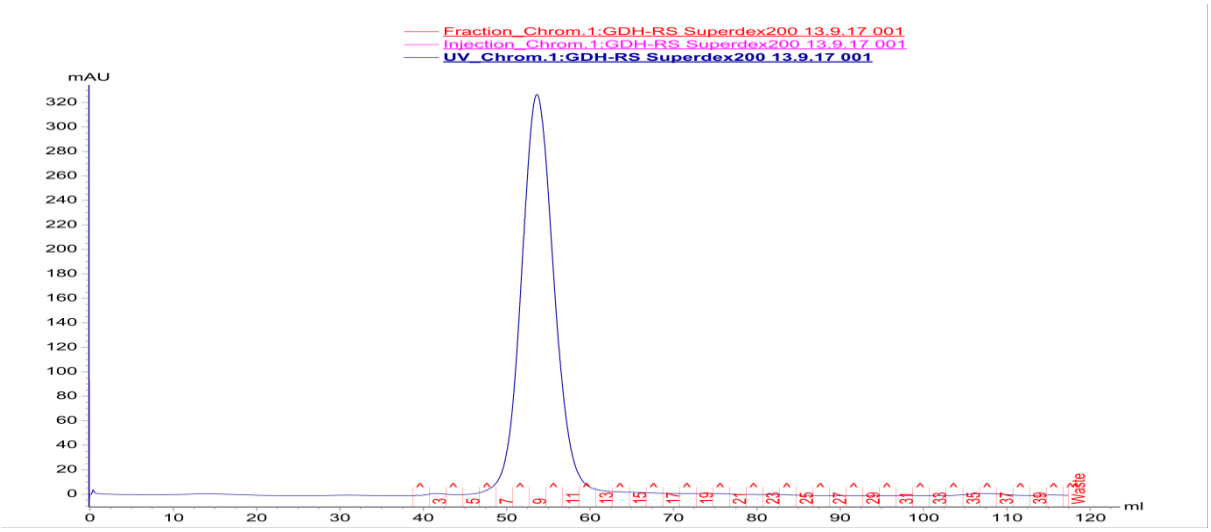
Dye chromatography: CFE (6ml, 40mg of total protein) was applied on a 20ml Remazol-Sepharose column eqilibrated with 0.1M KP pH 7.0 at flow rate 3ml/min. Column was washed with 30ml of the starting buffer and then GDH eluted with 1M NaCl in the same buffer. 5ml fractions were collected.

```
No 1:   Sample Volume and Type
        GDH  40mg

No 2:   Column
        Remazol-Separose 20ml

No 3:   Eluent A
        0.1M KP pH 7.0

No 4:   Eluent B
        A+1MNaCl

No 5:   Remarks
        step elution  12.9.17
```



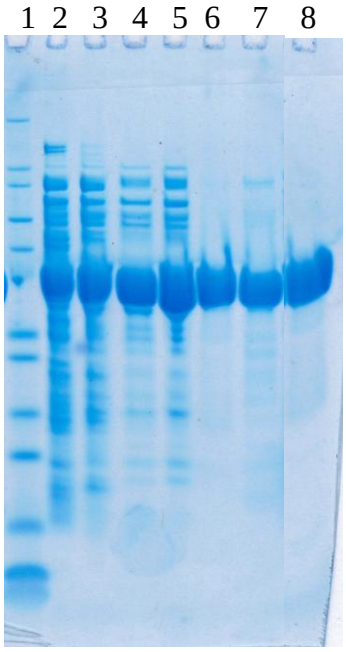
Fractions were analysed for activity. No activity was found in fractions 2-11. Fractions 12-14 with all activity were combined: V=15ml, C=0.56mg/ml, 8.4mg

Gel filtration: Sample was reduced in volume to 1ml (C=7.5mg/ml) and additional gel filtration step was applied. All conditions as above.

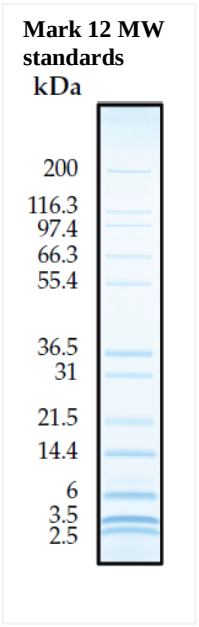


Fractions 8-10 were combined: V=6ml, C= 0.9mg/ml, 5.4mg **(Final prep$_{RS}$)**

SDS-PAGE analysis: 4-12% NuPageBT Novex gel



1. Mark12
2. CFE
3. After DEAE column
4. After Phenyl-HP column
5. GF loading
6. Final prep $_{3sp}$
7. Fr 12-14 Remazol Sepharose
8. Final prep $_{RS}$



When compare line 6 (3 step protocol) with line 7 (Remazol-Sepharose prep) we can see that purity of the first one is noticeably higher. After additional GF step for the RS prep there still are a number of small contaminating bands, but purity now looks a little bit higher than for 3 step prep.

Activity analysis

Despite simplicity of performing the activity assay there is a significant problem when we want to measure activity accurately to obtain reliable figures for specific activity to compare different enzyme preparations. The problem is that kinetics of the reaction in this case is not linear and so it is practically impossible to measure initial velocity. It is also seems that NAD concentration is not saturating under commonly used activity assay protocol (1mM NAD, 20mM NaGlu, 0.1M KP pH 7.0). I found that saturated concentration is 10mM NAD.

To achieve more or less linear kinetics under the standard assay conditions the concentration of enzyme has to be as low as it practically possible.
I found that the best way to compare activity of different samples is to dilute them in order to get similar and relatively low activity around 5u/ml. Kinetics becomes almost linear in this range.
In my experiments I used spectrophotometer which could be programmed to make calculations of activity. I have prepared a number of dilutions of the enzyme sample to get readings around 5u/ml and then I used dilution factor to calculate activity in the original sample. Data obtained this way are presented in the table below.

	V ml	C mg/ml	Total mg	Act u/ml	Sp. Act u/mg	Total act u	Yield %	Purity by SDS-PAGE
CFE	6	6.7	40	67	10	400		
DEAE	15	1.3	19.5	16.1	12.4	242		
Phenyl-HP	10	0.67	6.7	16	23.8	160		
GF load	1	5.4	5.4	145	26	140		
Final $Prep_{3sp}$	6	0.56	**3.4**	16.6	**30**	100	**25**	**~90%**
Fr12-14 RS	15	0.56	**8.4**	17.4	**31**	260	**65**	**~80%**
Final $prep_{RS}$	6	0.9	**5.4**	35.4	**39.4**	212	**53**	**~95%**

Verdict
Yes, yield with one step purification is significantly higher, while purity is lower. The strange observation is that Specific activity of the two preps is the same, while purity estimated by SDS-PAGE is higher with 3step protocol. The reason probably is that not all molecules of GDH are active and so have not got affinity to Remazol.
Still with the preparative red column the yield was not as high as for small analytical run. However analytical experiment result was too good to be true.
So, if you have ready remazol column of appropriate size, it is better to go for Remazol protocol. In case if you only need to purify this protein once or twice, the amount of time and effort to make the Dye column is not worthily.
Another issue is the Specific activity. In my hands under the same conditions as reported in foundation Syed at al. paper Specific activity for clostridium GDH purified by Remazol Sepharose falls in range 30-35u/mg, while they reported figure just 18.3u/mg. This is because real initial velocity is really difficult to determine for this GDH. The lesson I took from this experience was do not take Specific activity reported in the papers too seriously.
Also here rises the question:
Can Specific activity be good measure for purity as it was taken in the past? (For sure, prep on line 6 does not look to be 25% less pure then the prep on line 8)

6. DNA binding proteins

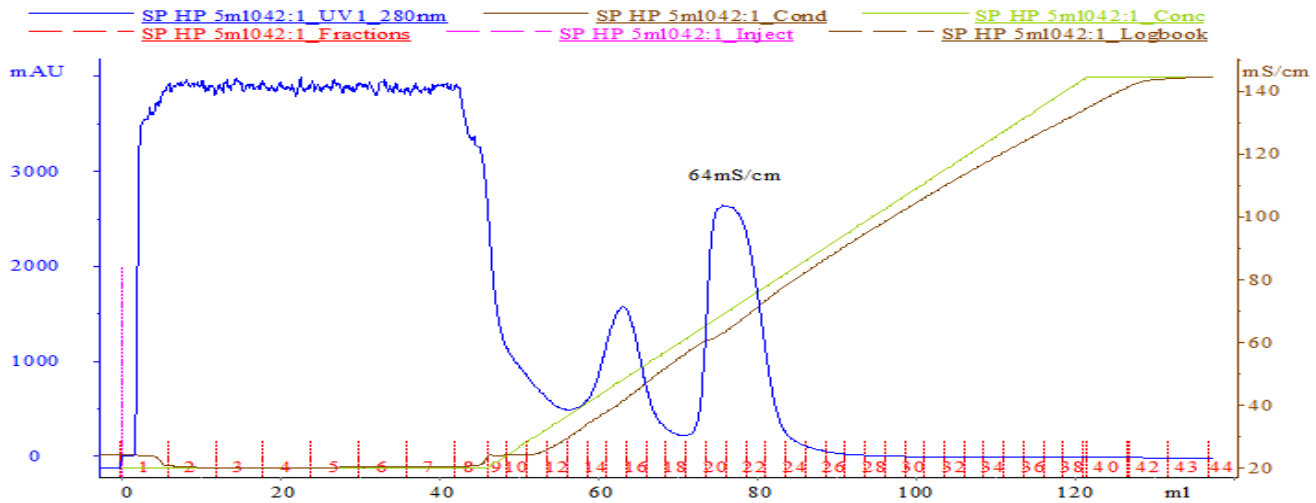
Typical Heparin purification: RuvC from phage bIL67

Ruv C proteins are crossover junction endodeoxyribonucleases (nucleases that resolves Holliday junction intermediates in genetic recombination).

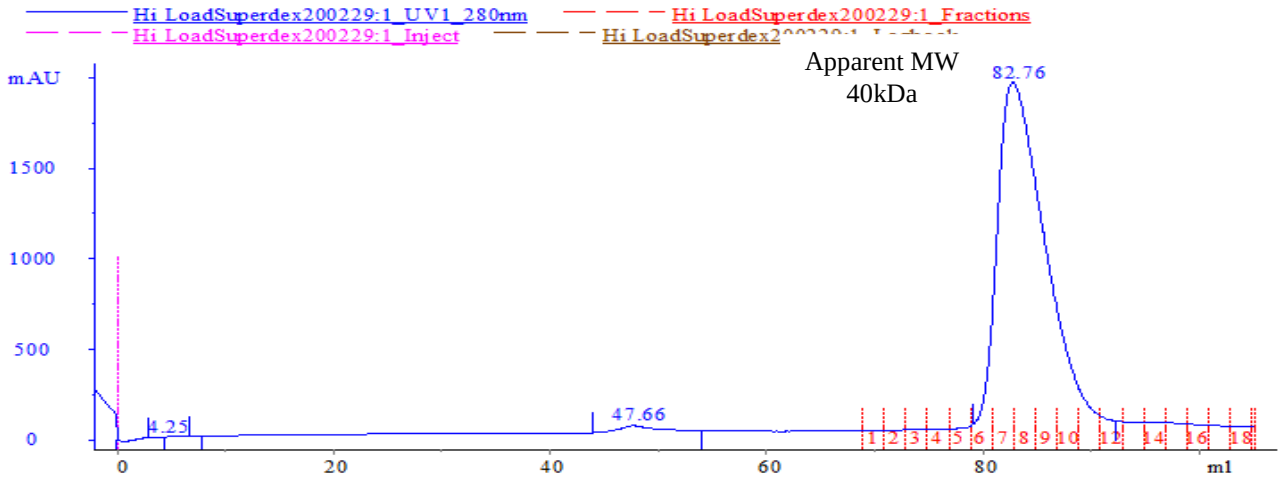
RuvC from phage bIL67:
MW 18097 (dimer)
pI 7.64

Large scale purification

CFE was prepared in buffer 50mM tris pH 8.0, 0.25M NaCl from 7.5g of cell paste. Extra sonication cycle was applied and centrifugation time was extended in order to obtain CFE at higher than usual concentration.
CFE: V=42ml, C=12.5mg/ml, 525mg
Heparin Chromatography. CFE was applied on 5ml Heparin HP cartridge. AKTA purifier system was used for purification.
Flow rate 5mg/ml. Fractions 2.5ml. Gradient: 75ml (15CV) 0.25-2M NaCl in 50mM tris pH 8.0

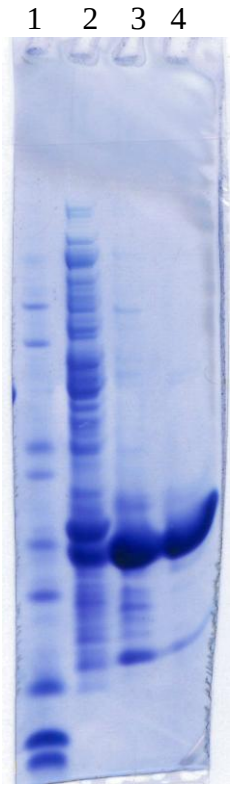


Fractions were analysed for protein concentration and fractions with highest concentration 20+21 were combined: V=5ml, C=10mg/ml, 50 mg
Volume was reduced to 2ml using VivaSpin MWCO 10000: V=2ml, C=23mg/ml, 46mg
Gel filtration. Sample was applied on a 1.6x60cm HiLoad Superdex200 column equilibrated in 0.5M NaCl 50mM tris pH 8.0 buffer. Flow rate 1.5ml/min. Fractions 2ml
.

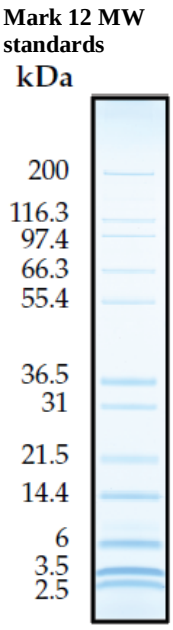


Fractions 7-9 were combined: V=6ml, C=6mg/ml, 36mg

Purification progress was analysed by SDS-PAGE using NuPage 4-12% BT Novex gel:



1. Mark12
2. CFE
3. GF loaded
4. Final prep



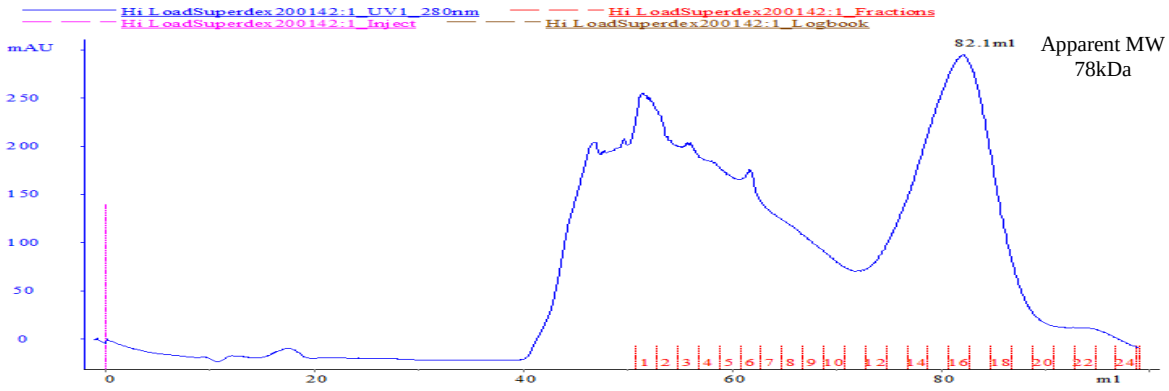
Summary:

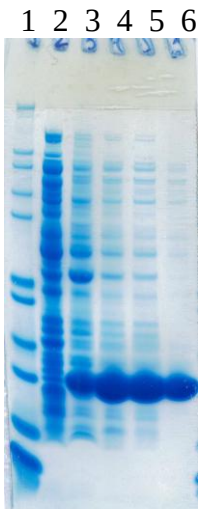
This is an example of typical purification of DNA-binding protein. Often Heparin +GF protocol works satisfactory allowing to produce 80-90% pure protein suitable for structural studies.
Good expression level and relatively high affinity to Heparin allow to purify RuvC endonuclease to purity of about 90% .

Playing with solubility: LrpC from *Bacillus subtilis*

LrpC is HTH-type transcriptional regulator.
MW 16450, forms a tetramer
pI 7.6
LrpC is one of relatively rare proteins which have very sharp dependency of its solubility upon the salt concentration. Solubility of LrpC in low ionic strength buffer (without salt) is practically zero, while in the presence of 1M NaCl it is over 50mg/ml. This property was used to develop optimal purification protocol.

LrpC was over expressed in *E.coli* cells to a good level (10% or higher).
Cells were disrupted in 50mM tris-HCl pH 8.0 buffer and pellet was collected by centrifugation as usual (Protocol 2).
CFE contained 700mg of total protein and had practically no LrpC (see resulting gel).
Extraction of LrpC from cell debris. Pellet was suspended in 7ml of buffer with 1M NaCl, 50mM tris-HCl pH 8.5. Sample was stirred for 1hour at 4°C. Insoluble material was spun down at 70000g for 10 minutes. Supernatant fraction contained 90mg of total protein (V=9ml, C=10mg/ml)
Precipitation of LrpC with ammonium sulphate. 14ml of 4M AS was added to bring AS concentration to 2.5M. LrpC precipitated and was collected by centrifugation as above. Pellet was dissolved in 7ml of 50mM tris pH 8.0 and clarified by centrifugation as above.
V=8ml, C=11mg/ml, 88mg (about 0.3M AS).
Gel filtration. Sample was divided to 3 portions and gel filtration was performed on 1.6x60cm HiLoad Superdex200 column equilibrated in buffer 50mM tris-HCl pH 8.0, 1M NaCl
2ml fractions were collected in the same set of tubes for each of 3 runs.
Fractions 14-19 were combined: V=35ml, C=2mg/ml, 70mg. Purity estimated by SDS-PAGE was better than 90% (see gel below).





Purification progress was analysed by SDS-PAGE, using NuPage 4-12% BT Novex gel:

1. Mark12
2. CFE obtained in 50mM tris-HCl pH 8.0
3. Cell pellet suspended in 1M NaCl
4. 1M NaCl extract (after centrifugation)
5. GF load (2.5MAS pellet dissolved and clarified)
6. Final prep

Heparin *and* solubility:
FrmR from *Escherichia coli*

FrmR is transcriptional repressor, sensor for formaldehyde.
MW 10318 Da, forms tetramer
pI 5.84

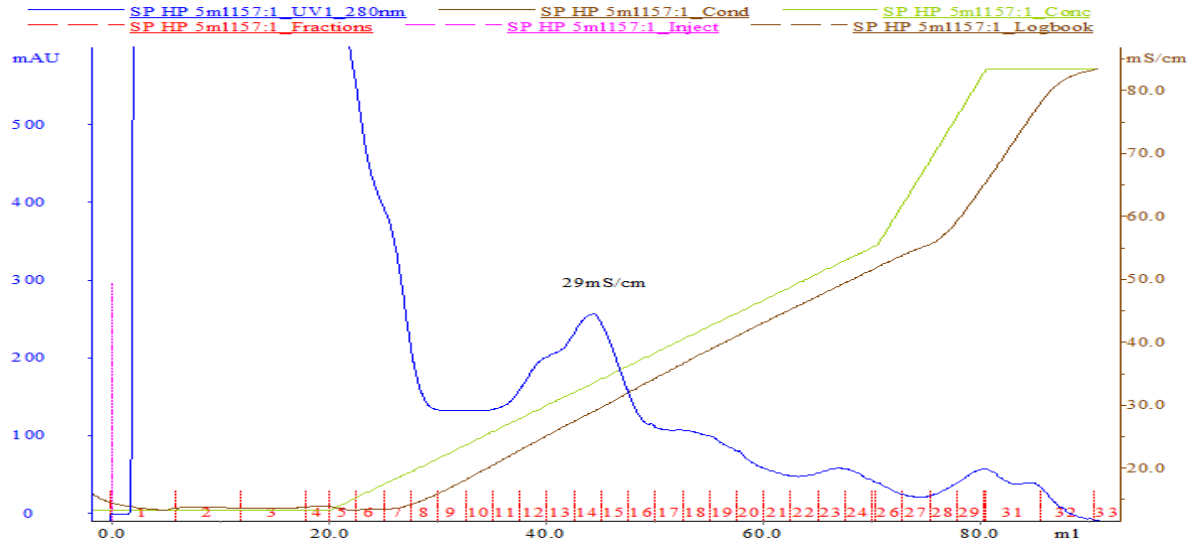
FrmR comes to me with the purification protocol which included Heparin chromatography, ammonium sulphate precipitation and gel filtration. I was told that protein needs 0.3M of NaCl to stay in solution and 10mM DTT and EDTA to keep it active. I quickly found that FrmR has low affinity to Heparin column at 0.3M NaCl and in order to have it bound to the Heparin-HP cartridge I had to decrease NaCl concentration to 0.1M. No FrmR was precipitated. In fact 0.3M NaCl appeared to be eluting concentration. Later I decided to simplify my life by exclusion of DTT and EDTA from gel filtration buffer and started to run gel filtration in our standard buffer 0.5M NaCl 50mM tris-HCl (so I saved 2 hours on equilibrating - re-equilibrating the column). It did not affect activity. So I decided to try to remove DTT and EDTA from the purification what so ever. Once again, as for a number of proteins before, DTNB reaction proved that all 4 cysteins in the FrmR preparation were reduced despite absence of DTT during purification. The protein was active and we have good publication on the structure-function of this protein.
However, preparations of FrmR purified by Heparin–GF two step protocol contained significant amount of 40kDa contamination (see gel below)

1. Mark 12
2. Cell Debris
3. CFE
4. After Heparin column
5. Final preparation

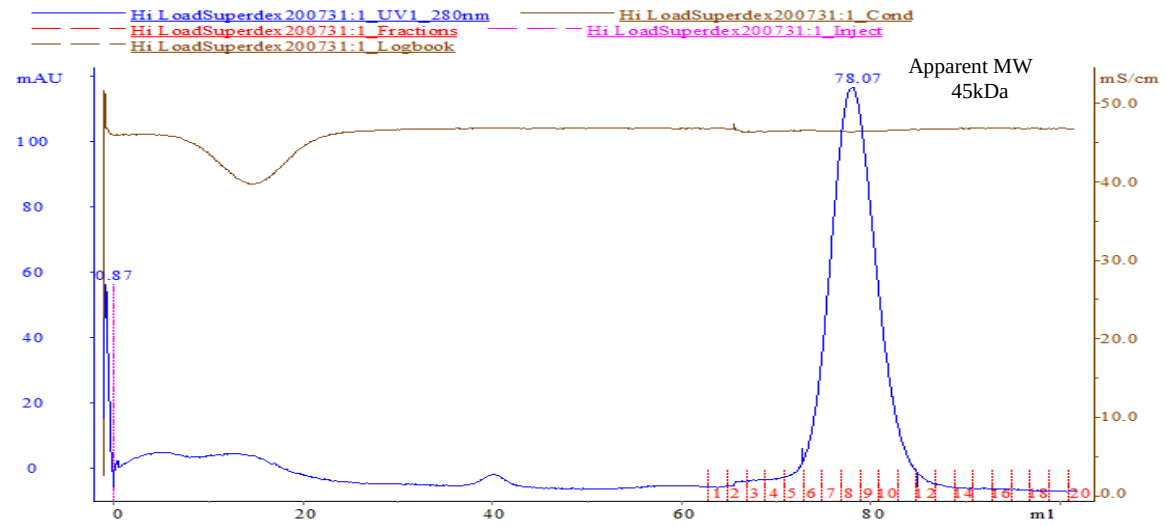
To improve purity I decided to try to use low solubility of the protein at low salt conditions. Dialysis in 50mM tris-HCl pH 8.0 buffer was performed on a sample obtained after Heparin chromatography and majority of FrmR went out of solution in form of micro crystals. However, it appeared that for successful crystallisation FrmR protein concentration has to be at least 3mg/ml, otherwise significant amount of FrmR was left in solution. Also crystallisation was found to be temperature depended, so dialysis was performed at 4^{o}C. Crystals were collected by centrifugation and dissolved in 50mM tris buffer pH 8.0 containing 1M NaCl. Solubility under these conditions appeared to be more than 40mg/ml. For the best result sample was gel filtered. Purity of the resulting preps was typically better than 90-95%. To prevent crystallisation or precipitation final preparations obtained at high concentration in the presence of 0.5M NaCl had to be kept at room temperature.
Typical purification is presented below.

FrmR purification 30.3.16

Cells obtained from 1l culture were used to prepare CFE in buffer 0.1M NaCl, 50mM tris-HCl pH 8.0 by a standard protocol (**Protocols**, 2).
CFE: V=15ml, C=7.5mg/ml, total protein 110mg
Heparin chromatography: CFE was applied on a 5ml Heparin-HP cartridge equilibrated in the same buffer. Chromatography was performed on AKTA purifier system at flow rate 5ml/min. Elution was achieved by 50 ml of gradient of NaCl concentration from 0.1 to 0.7M in 50mM tris-HCl pH 8.0 buffer. 2.5ml fractions were collected.

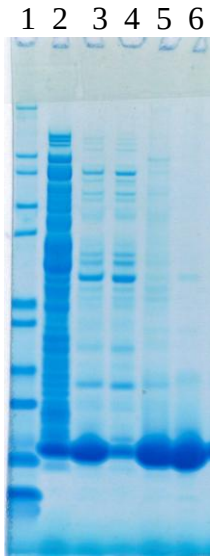


Fractions were analysed for protein by method of Bradford and peak fractions 13-15 were combined: V= 7.5ml, C=3.3mg/ml, 25mg.
Crystallisation: Protein concentration in this case was high enough and so sample was subjected to dialysis without additional concentrating.
Sample was dialysed against 0.5l of 50mM tris-HCl pH 8.0 (Buffer A) over night in the cold cabinet (at 4^{o}C).
Micro crystals were collected by centrifugation (5 minutes 21000g) and dissolved in about 1ml of 1M NaCl in Buffer A: V=1.6ml, C= 8.55mg/ml, 13mg
Gel filtration: Sample was applied on a 16x60HiLoad Superdex200 column equilibrated in Buffer A +0.5M NaCl. Gel filtration was run at flow rate 1.5ml/min. 2ml fraction were collected.



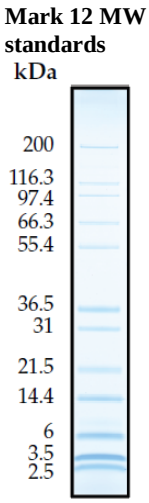
Fractions 7-9 were combined: V=6ml, C=2mg/ml, 12mg
FrmR was concentrated using VivaSpin with MWCO 30000:
V=0.43ml, C=18.5mg/ml, 7.8mg

SDS-PAGE analysis:
NuPage 4-12% BT Novex gel with MES running buffer:



1. Mark12
2. CFE
3. After Heparin column
4. Supernatant fraction after dialysis
5. GF loading (micro crystals dissolved in 1M NaCl)
6. Final preparation

Purity about 90-95%
Yield: 12mg from 1 litre of culture
11% of total protein
This is surprisingly high yield, as expression level on the gel does not looks higher than 5%



Real power of Heparin: Human Flap endonuclease

Over the years we worked with a number of Flap endonucleases from different organisms in collaboration with Dr Sayers_from Medical school. Typically for crystallisation they were purified using Heparin-GF or Heparin-IEC protocol. Most of them were over expressed in E.coli to a reasonable level (at least 2-3%, so you can see the band in CFE sample on SDS-PAGE). Human FEN was different as expression level was low and practically insoluble, so it was impossible to identify certain band corresponding to FEN in CFE on SDS-PAGE gel.
hFEN
MW 42593Da
pI 8.8

This protein is also interesting as it came to me with the most ridiculous purification protocol I have ever seen.
Cells were suspended in tris buffer pH 8, 2mM EDTA, 0.2M NaCl and 5% glycerol.
Lysozyme (0.2mg/ml**!!!!**) was added and cells incubated for 20 minutes on ice
PMSF (23µg/ml) was added and cells were incubated 40 minutes at room temperature
Na-deoxycholate (0.5mg/ml) was added and incubated for 20minutes at room temperature
DTT (1mM) was added and incubated for 5 minutes
Sonication: 20% amplitude, 5sec bursts for 30 sec (*not sure what that means**???***), repeated three times
Centrifugation: 25000rpm at 10^{o}C for 20 minutes
Ammonium sulphate was added to 0.5M
5% PEI (Polyethylenimin) precipitation was performed with mixing for 10 minutes
Pellets were removed by centrifugation at 25000rpm for 15 minutes
Supernatant was dialysed in buffer 20mM KP pH 6.0, 1mMDTT, 2mM EDTA, 5% glycerol at 4^{o}C over night
Heparin chromatography (0.1-1M NaCl gradient in above buffer)
Fractions with FEN were pooled and a 55% AS precipitation was performed for 2h at 4^{o}C
Pellets were removed by centrifugation and supernatant fraction was dialysed in 20mM tris buffer pH 8, 1mM DTT, 2mM EDTA, 5% glycerol (*I guess overnight*)
The sample was run trough Q column and unbound fraction was collected.
Sample was dialysed again in KP buffer pH 6.0, 50mM NaCl
Sample was applied on a combination of HiTrap SP and Heparin columns
No details how proteins were eluted. !!!!
It seems, the purification takes four days. It includes many manipulations of unclear purpose and looks like mixture of ritual actions and sporadic desperate moves...

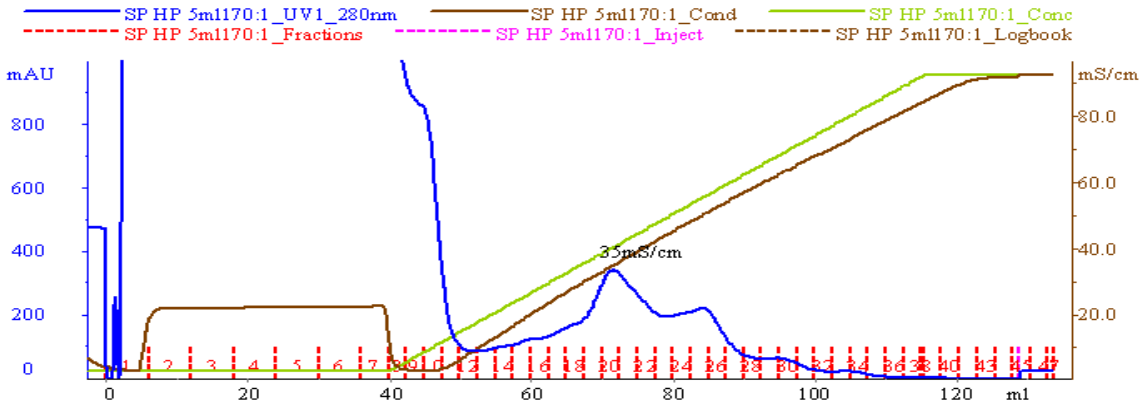
We purified it by Heparin-Cation Exchange Chromatography to about 85% purity, good enough for successful crystallisation.

hFEN purification

6g of cells were defrosted and CFE was prepared in about 40ml of buffer 0.2M NaCl 50mM tris-HCl pH 8.0. The standard protocol was amended due to high concentration of cell suspension: sonication was performed in three cycles instead of two and centrifugation was run at 70000g for 15 minutes instead of ten.
CFE: V=35ml, C=11mg/ml 380mg

Heparin Chromatography. CFE was applied on a 5ml Heparin-HP column. Flow rate 4ml/min. 2.5ml fractions were collected. Elution was performed by 75ml gradient of 0-1M NaCl concentration in 50mM tris-HCl pH 8.0 buffer.

```
No 1:   Sample Volume and Type
        hFEN  16.8.16
No 2:   Column
        Heparin-HP 5ml
No 3:   Eluent A
        50mM Tris pH 8.0
No 4:   Eluent B
        A+1MNaCl
No 5:   Remarks
        0-100% B 15CV   16.8.16
```

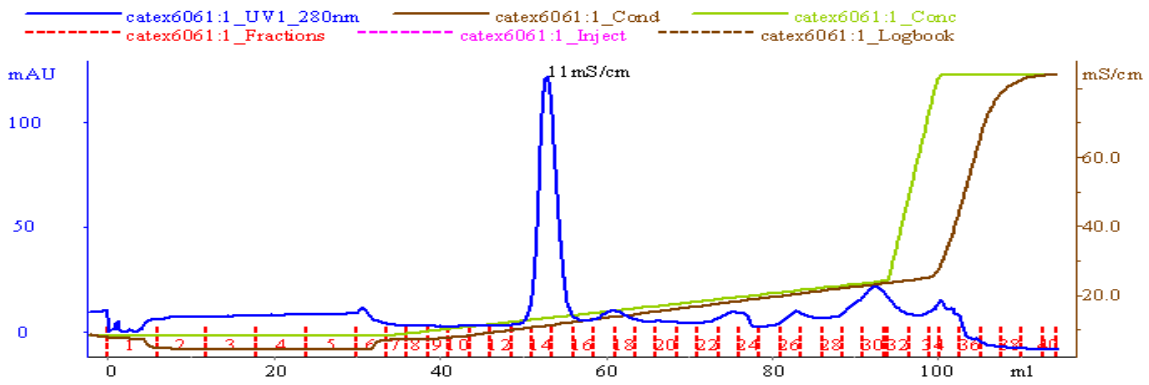


Fractions were analysed for protein by method of Bradford and by SDS-PAGE.
Fractions 20-23 containing hFEN were combined: V=10ml, C=1.4mg/ml, 14mg

Preparation for cation exchange chromatography. hFEN has pI 8.8 and is suitable for cation exchange chromatography. To allow hFEN to bind to SP matrix it has to be prepared at pH 6-6.5 and as low salt as possible. hFEN was eluted from Heparin column with about 0.35M NaCl 50mM tris pH 8.0. To avoid over night dialysis, concentration and dilution technique was employed.
Sample was concentrated using VivaSpin device to V=1.4ml, C=8.3mg/ml, 11.6mg
and then it was diluted in 30ml of 50mM MES-NaOH buffer pH 6.3
Some of the proteins precipitated and was removed by centrifugation at 70000g for 10 minutes.
Supernatant fraction: V=30ml, C=0.28mg/ml, 8.4mg

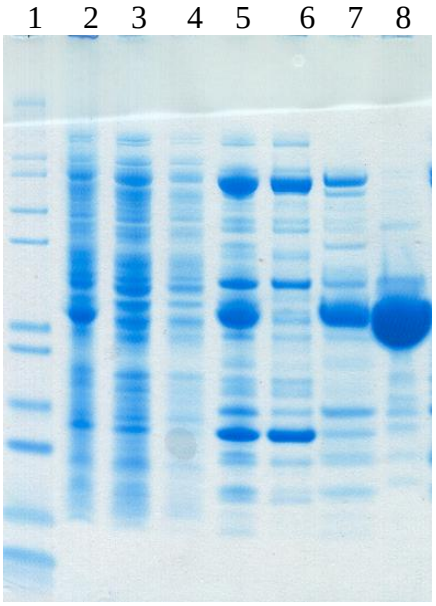
Cation Exchange Cromatography. Sample was applied on a 6ml Resource S column. Flow rate 4ml/min. Fractions of 2.5ml were collected. Elution was performed by 60ml gradient of NaCl concentration from 0.05 to 0.25M in 50mM MES buffer pH 6.3

```
No 1:   Sample Volume and Type
        hFEN 8.4mg
No 2:   Column
        resourceS 6ml
No 3:   Eluent A
        50mM MES pH 6.3
No 4:   Eluent B
        A+1MNaCl
No 5:   Remarks
        5-25%B 10CV   16.8.16
```

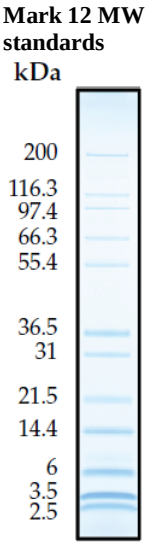


hFEN elutes with about 0.1M NaCl.
Fractions 14+15 were combined: V=5ml, C=0.53mg/ml, 2.6mg

Purification progress was analysed by SDS-PAGE (4-12% NuPage BT Novex gel)



1. Mark12
2. Cell debris
3. CFE
4. Heparin unbound fraction
5. Heparin fr20-23
6. Pellet occurred at pH 6.3
7. Sample applied on a Resource S column
8. Fr 14+15 from Resource S (final preparation)



Purity of the final preparation is about 85%. Yield is 2.6mg. Presuming 2 step purification has typical yield 50%, there was about 5mg of hFEN in CFE, which is 1.3% (5:380=0.013).

As you can see on gel there is some hFEN in cell debris, but it is difficult to find right band in CFE sample. Still, it was possible to purify hFEN to reasonable purity just in 2 step purification thanks to its affinity to Heparin and also its basic pI. It also was lucky to get significant amount of contaminations precipitated at pH 6.3.

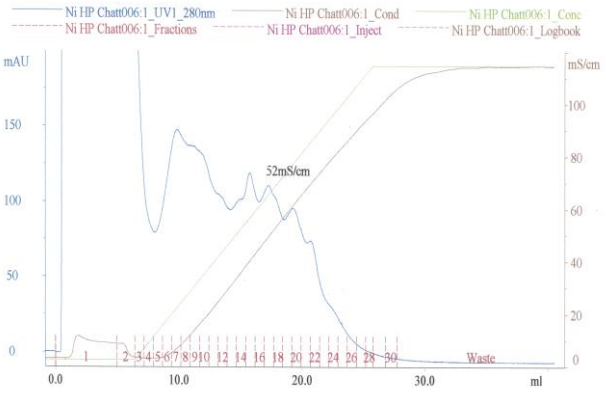
In need of nuclease treatment: SfsA

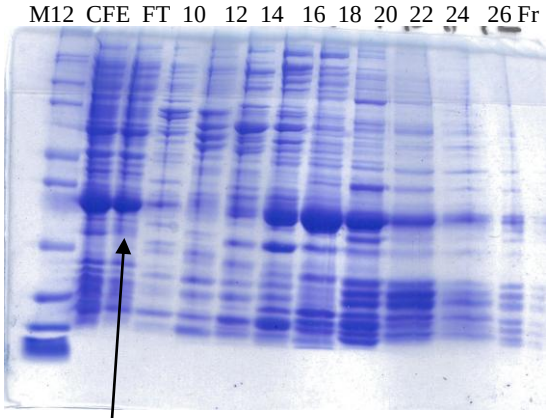
SfsA is sugar fermentation stimulation protein which function is positive regulation of transcription, DNA-templated. We had studied structure of SfsA from two organisms, *Pyrococcus furiosus* and *Escherichia coli*. This protein when over expressed in *E.coli* was found to interact with DNA very strongly. Nuclease treatment was needed to make the whole pool of TP to bind to Heparin, as described below.
However later we needed nuclease free sample for DNA-Protein complex studies. Ammonium sulphate precipitation was employed with good degree of success (see below).

SfsA *P. furiosus*:
MW: 26114
pI: 9.3
$A_{0.1\%}$: 0.4

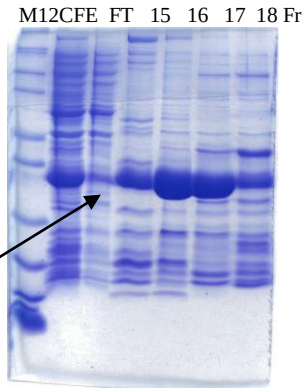
SfsA *E.coli*:
MW: 26229
pI: 6.5
$A_{0.1\%}$: 1.14

We started our study with Pyrococcal SfsA.
First CFE was received from collaborators in 50mM NaPhosphate pH 8.0.
About 30mg of the CFE was applied on 1ml Heparin-HP cartridge and eluted with 20ml of 0-1.5M NaCl gradient in Buffer A (50mM trs-HCl pH 8.0). 0.8ml fractions were collected and analysed by Bradford and SDS-PAGE:

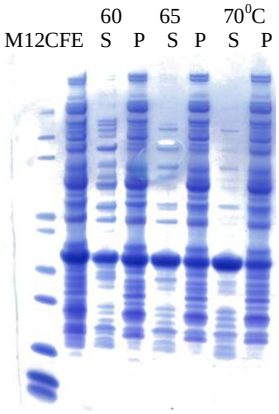




As you can see a lot of TP flows through column unbound, some of it was eluted in fractions 16 to 20 by about 0.5M NaCl. Fr.17-19 were combined, containing 2.2mg of total protein.
Rest of CFE was dialysed in 50mM tris pH 8.0, 2mM $MgCl_2$ and 25u of Benzonase was added per ml of GFE. After 30 min incubation at room temperature 30mg of CFE was applied on the same column again. The result was similar to the untreated sample. Rest of CFE was left for treatment over night at 4°C. Chromatography was repeated with 30mg of treated CFE.
This time very little of TP was found in unbound material and yield of TP in fr16+17 was 7mg.

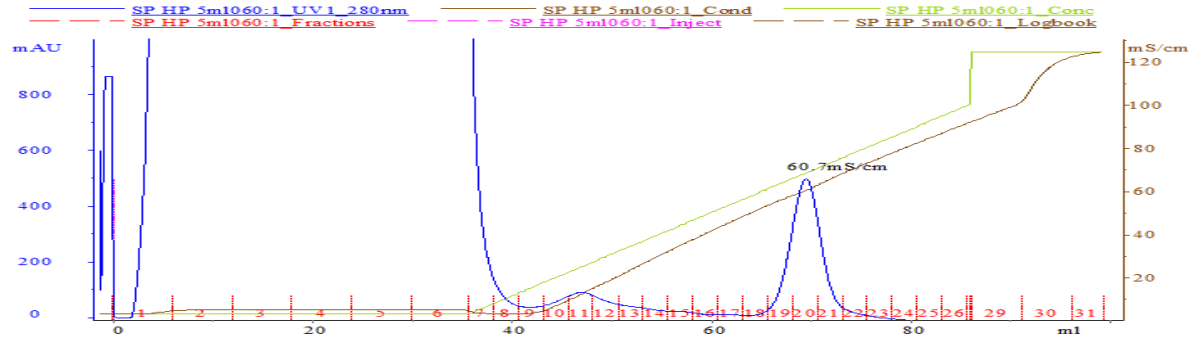


For Pyrococcal protein it is logical to try heat treatment to improve purity. Analytical experiment was performed on CFE. It was heated for 20 minutes at 60°C, 65°C and 70°C and precipitated material was spun down. Samples were run on SDS-PAGE. S-supernatant, P-pellet.
Best result was observed at 70°C.
As a second chromatography cation exchange was chosen as pI of TP is 9.3. It was performed on Resource S column.
Refined protocol presented below.

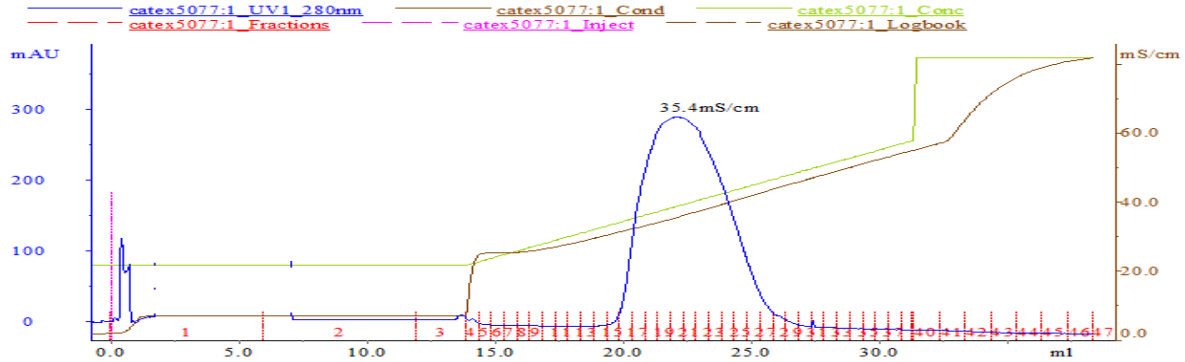


SfsA from *P. furiosus* purification 12.9.11

CFE from 2litre culture cells was prepared in buffer 20mM NaCl, 2mM $MgCl_2$, 50mM tris-HCl pH 8.0, 250 u of Benzonase was added and sample was dialysed against above buffer at 4^{o}C over night.
CFE after nuclease treatment (V=31ml, C=4mg/ml, 124mg) was heated at 70^{o}C for 20 minutes. Precipitate was removed by centrifugation (70000g, 10 minutes).
Sample after heat treatment (V=31m,l, C=1mg/ml, 31mg) was applied on a 5ml Heparin HP cartridge.
Heparin chromatography: Chromatography was performed on AKTA purifier system at flow rate 5ml/min. Elution was done by 50ml (10CV) of gradient of NaCl concentration from 0 to 1.2M in BufferA. 2.5ml fractions were collected.

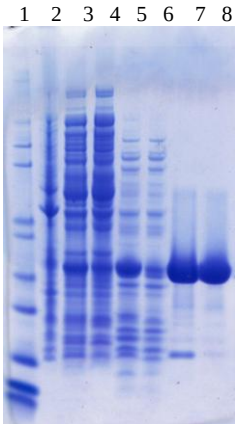


Fractions 20+21 were combined: V=5ml, C=3mg/ml, 15mg and concentrated using Viva Spin MWCO 10000 to 1ml.
Cation exchange chromatography: Sample was diluted in 15ml of 50mM MES-NaOH pH 6.5 buffer and applied on a 1ml Resource S column. Flow rate 2ml/min. Gradient: 18ml, 0.25-0.7M NaCl in above buffer. 0.5ml fractions were collected.



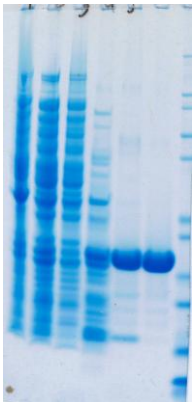
Fractions 18-26 were combined: V=4.5ml, C=2.6mg/ml, 11.7mg (final preparation)

Purification was analysed by SDS-PAGE (4-12% Nu Page Novex BT gel):



1. Mark12
2. Cell debris
3. CFE
4. Heat pellet
5. Heparin load
6. Heparin flow through
7. After Heparin
8. Final prep

Protocol was very effective even with lower expression level
Purity about 90%

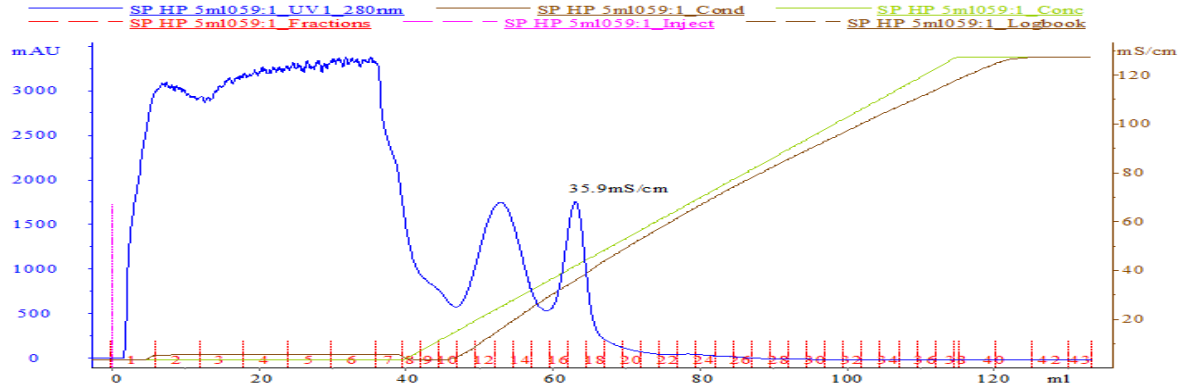


Purity of final preparation is better than 90%
Yield is 9% of total protein, 5.5mg/ litre of culture. Very good!

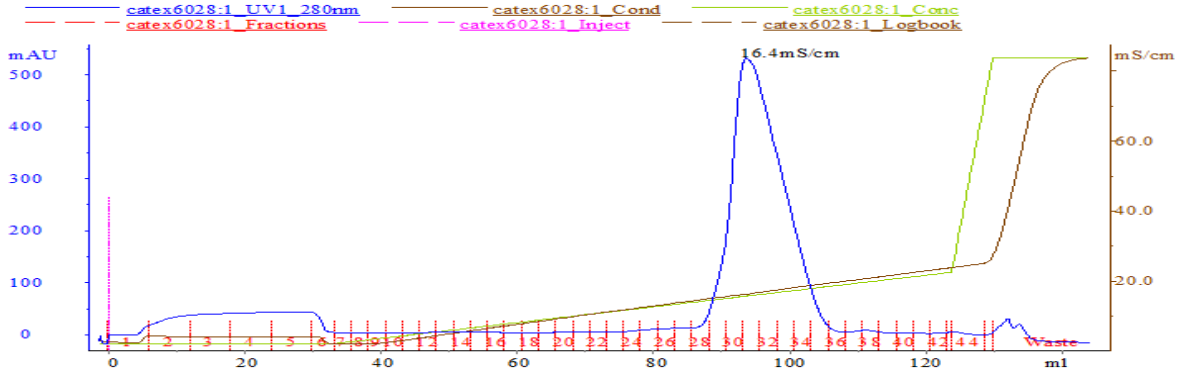
Similar protocol was applied for purification of *E.coli* SfsA except of heat treatment: CFE→Benzonase treatment→Heparin→Resource S. Cation exchange chromatography worked on *E.coli* SfsA protein despite its theoretical pI 6.5.

E.coli SfsA purification 8.9.11

CFE was prepared in 20mM NaCl, 5mM $MgCl_2$, 50mM tris-HCl pH 8.0 buffer and treated with Benzonase over night as described above.
CFE: V=35ml, C=2.45mg/ml, 86mg
Heparin chromatography. Flow rate 5ml/min. Gradient: 75ml (15CV) 0-1.5m NaCl in 50mM tris-HCl pH 8.0. Fractions 2.5ml.

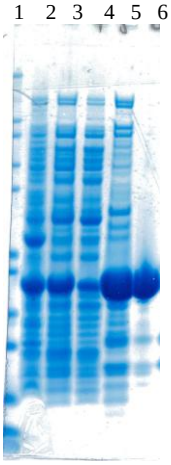


Fractions 16-19: V=10ml, C=2mg/ml, 20 mg
Sample was concentrated on VivaSpin MWCO10000 to 1.4ml and diluted with 30ml 50mM MES-NaOH pH 6.5 buffer.
Cation exchange chromatography. Sample was applied on a 6ml Resource S column. Flow rate 5ml/min. Gradient: 90ml (15CV) 0-0.25m NaCl in 50mM MES-NaOH buffer pH 6.5. Fractions 2.5ml.



Fractions 31-33: V=7.5ml, C=1.4mg/ml, 10mg (final preparation)

SDS-PAGE analysis (4-12% Nu Page Novex BT gel):

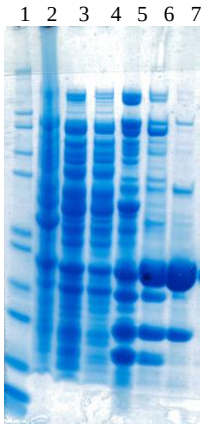


1.Mark12
2.Cell debris
3.CFE
4. Heparin flow through
5. After Heparin
6. Final prep

Purity of final preparation in this case is lower than for Pyrococcal SfsA, about 80-85%, but it is good enough for structural studies.
Yield is also good, 11% of total CFE protein

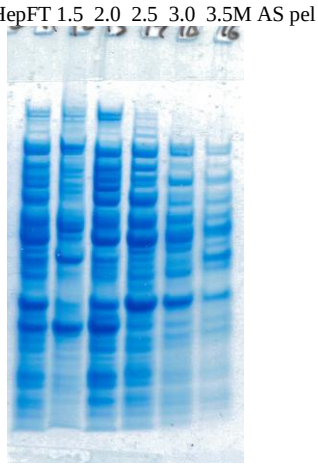
Nuclease free purification of *E.coli* SfsA

CFE was prepared from 5g of cells in 1M NaCl, 50mM tris-HCl pH 8.0 buffer.
CFE: V= 30ml, C=10mg/ml, 300mg
Heparin chromatography 1: CFE was diluted 5 fold with 50mM tris pH 8.0 buffer and applied on a 5ml Heparin-HP column. Chromatography was performed as described above yielded in 9.6mg.1ml Resource S column was used for the second chromatographic step producing 1.7mg of the TP. Gel analysis revealed that SfsA was not pure and so gel filtration was applied for further purification. SDS-PAGE analysis is presented below.

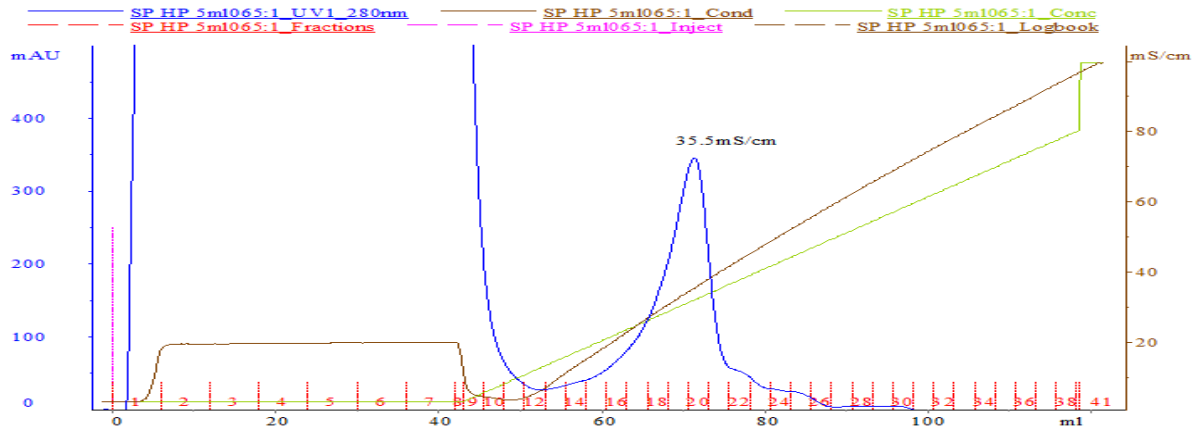


1.Mark 12
2.Cell Debris
3.CFE
4.Heparin flow through
5. After Heparin
6.After Resource S
7.After GF

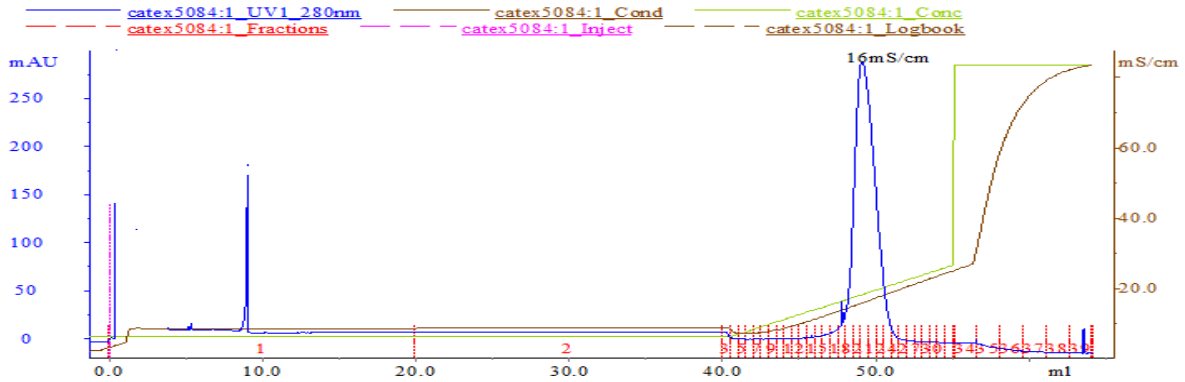
Only 0.3mg of TP was obtained after GF with purity not better than 70%.
It was not enough material for the experiments and so attempt was made to purify TP from the Heparin unbound material as it was a lot of SfsA revealed in this fraction.
Analytical ammonium sulphate cut (protocol 4) was performed on this material.
SfsA starts to precipitate with 2.0M AS and still is presented in 3.5M pellet.



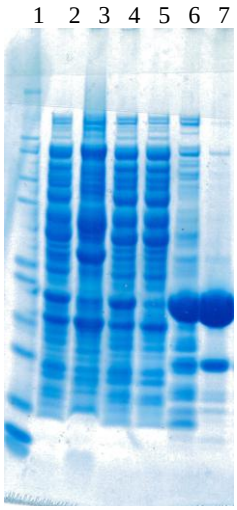
AS cut:43 ml of 4M AS was added to 65ml of Heparin 1 flow through fraction (2mg/ml, 130mg of total protein) (1.8M AS). Precipitated material was removed by centrifugation (5 minutes at 70000g). 21g of AS powder was added to a supernatant fraction gradually with stirring at 4^{o}C to bring AS concentration to about 3.2M. Solution was incubated for 10 minutes and precipitated material was collected by centrifugation as above. Pellet was dissolved in 40ml of 50mM tris-HCl pH 8.0 buffer (conductivity 18.8mS/cm, total protein 80mg) and applied on a 5ml Heparin-HP column.
Heparin chromatography 2: Chromatography was performed as described before.



Fractions 19-22 were combined, V=10ml, C= 0.72mg/ml, 7.2mg and concentrated to 1ml.
Cation exchange chromatography: Sample was diluted in 40ml of 50mM MES-NaOH pH 6.5 (8mS/cm) and applied on 1ml Resource S column. Flow rate 2ml/min. Gradient: 15ml(15CV) 0.05-0.3M NaCl in above buffer. Fractions 0.5ml. Fractions 22-24 were combined, yielded in 1.7mg of total protein



SDS-PAGE analysis (4-12% Nu Page Novex BT gel):



1. Mark12
2. Heparin 1 flow through
3. 1.8M AS pellet
4. 3.2M AS pellet
5. Heparin 2 unbound
6. After Heparin 2
7. Final prep

Application of AS cut was successful in attempt to purify SfsA without nuclease treatment. However purity was not as good as for purification with nuclease treatment. In the final preparation there is significant contamination and purity is not higher than 80%. Nevertheless crystallisation of the above prep with DNA was successful and structure was solved.

7. Tagged proteins

Proper use of a His-tag: Human protein PARP1 expressed in *E.coli*

Here is an example of purification of human protein PARP1. The level of expression of this protein in *E.coli* cells is extremely low. The rough estimation of expression level comes to about 0.15-0.2% of total protein. No any band can be seen on SDS gel in CFE corresponding to PARP1. Luckily the protein was His-tagged which allow me to purify it to acceptable purity in just too steps.

PARP1 (PolyADP-ribose polymerase):
MW =113083,
pI 9.0

The protein has two attractive properties : High MW and basic pI.
Both gel filtration and cation exchange chromatography potentially can produce a good result. It appeared that protein is dimeric and so gel filtration worked very well.

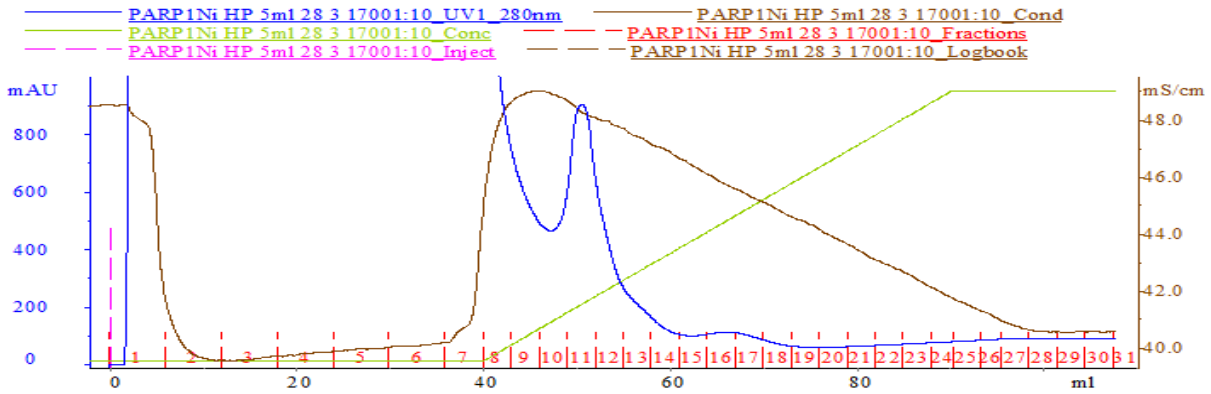
CFE from 1.5l culture was prepared as a standard procedure (sonication on ice 3x20sec at 16micron → spin down cell debris at 70000g for 10 min) in Buffer A+ 0.5M NaCl
Protein concentration was estimated by Bio-Rad assay.
CFE: V=35ml, C=7.5mg/ml, 260mg

Ni-NTA chromatography:Chromatography was performed on AKTA purifier system using a 5ml His-Trap column. CFE was applied and chromatography was run at flow rate 5ml/min. Proteins were eluted by 50ml gradient of imidazole concentration from 0 to 0.5M in starting buffer.
3ml fractions were collected.

```
No 1:   Sample Volume and Type
        PARP1 260mg

No 2:   Column
        Ni-HP 5ml

No 3:   Eluent A
        0.5M NaCl tris pH 8.0

No 4:   Eluent B
        A+0.5M Im

No 5:   Remarks
        0-100%B 10CV  28.3.17
```

BR For 20ul:

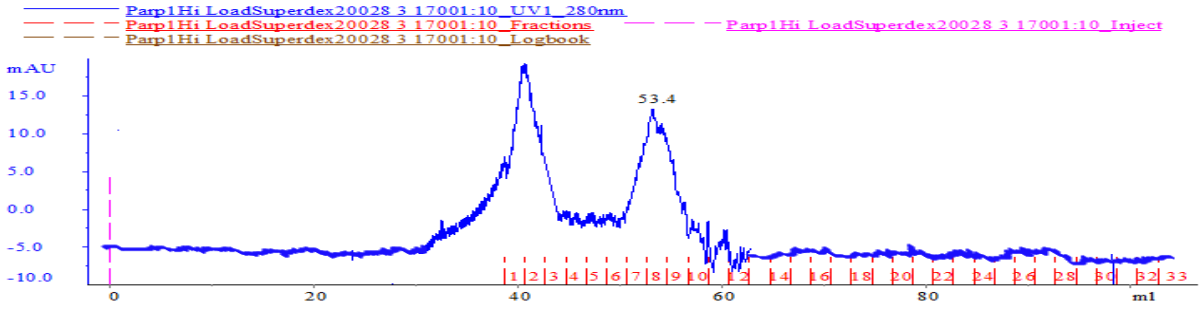
Fr	A595
12	1.1
13	1.0
14	0.74
15	0.48
16	0.44
17	0.46
18	0.24
19	0.06



The elution profile revealed a bigger peak in fractions 10 to 12 (normally containing *E.coli* proteins with low affinity to Ni) and small peak in fractions 15 to 18, which presumably contained His-tagged protein. Protein concentration in fractions was estimated by Bio-Rad assay.
Fractions 16 -18 were combined: V=9ml, C=0.225mg/ml, 2.2mg

To prepare sample for gel filtration the volume was reduced using VivaSpin device with MWCO 50000:
V=1ml, C=1.85mg/ml, 1.85mg
Gel filtration: Sample was applied on a 16x600 HiLoadSuperdex200 column equilibrated in Buffer A. Flow rate 1.5ml/min. Fractions (V=2ml) were collected after void volume.

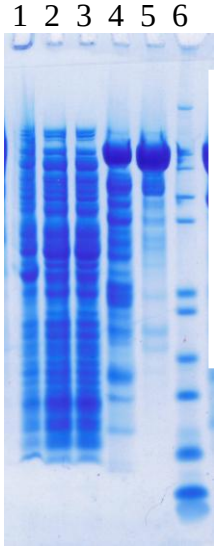
```
No 1:   Sample Volume and Type
        PARP1 1.8mg

No 2:   Column
        Superdex200 14

No 3:   Eluent A
        0.5M NaCl tris pH 8.0

No 4:   Eluent B

No 5:   Remarks
        28.3.17
```



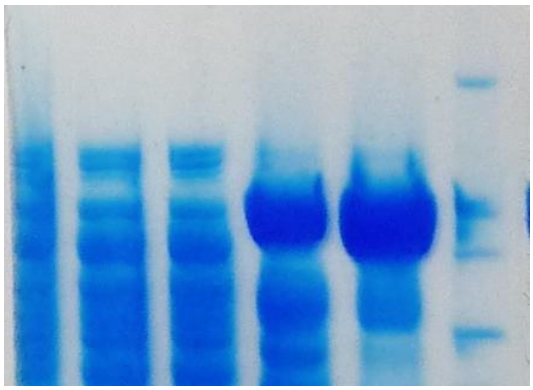
Fractions 7-9 were combined: V=6ml, C=0.09mg/ml, 0.54mg.

Sample was concentrated using VivaSpin device (MWCO50000):
V=0.55ml, C=0.76mg/ml, 0.42mg

The purification progress was monitored by SDS-PAGE:
NuPAGE 4-12% BT Novex gel run with MES buffer



1. CD
2. CFE
3. Flow through Ni column
4. GF loaded sample
5. Final preparation
6. Mark12



No band corresponding to purified protein can be seen neither in cell debris nor in CFE

Peak of PARP1 on GF column appeared at 53.4ml which corresponds to apparent MW of 280 kDa.
We can assume that PARP1 is an asymmetrical dimer in solution.
Purity of the final preparation is not ideal, about 70%, but it is good enough for the functional studies. Yield is about 0.5mg. Presuming about 50% yield and 70% purity very rough estimation of expression level is about 0.25%.
It seems, there is a degradation problem, so Protease Inhibitors have to be used during preparation of CFE.

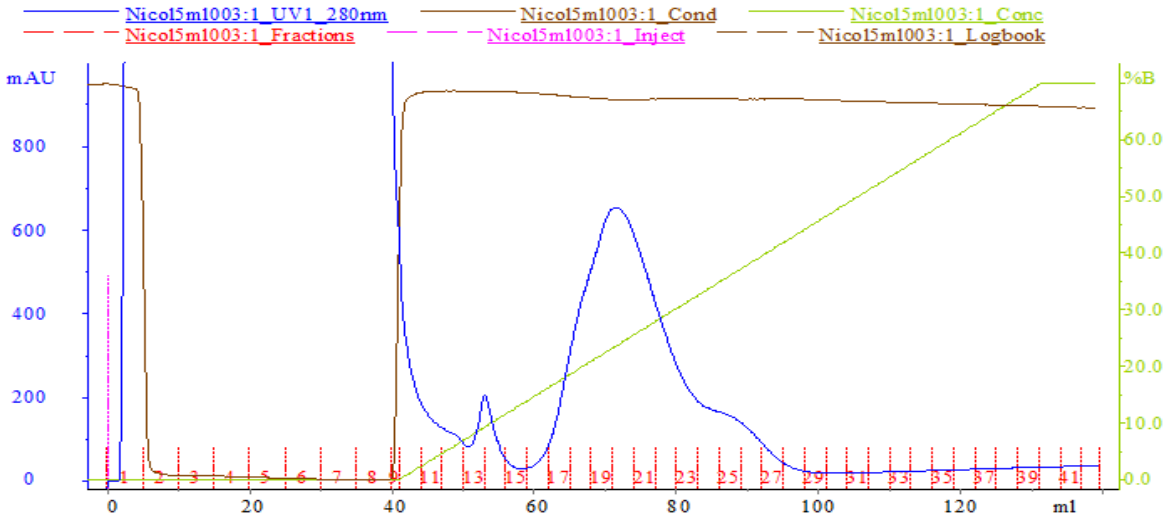
Removable tag: SAG19

SAG19 belong to group of *Eimeria tenella* surface antigens (SAGs). These proteins contain a number of cysteins which form disulphate bonds. In order to express rightly folded molecules in *E.coli* SAGs were fused with Therodoxin and expressed in Rossetta gami 2 strain. The construct also contained two His6 tags , S-tag and cleavage site for enterokinase (EK): Trx-His6-S tag-DDDDK-SAG19-His6. Obviously C-terminal His tag was not right thing to have and complicated purification of cleaved SAG (instead of second Ni-column after cleavage we had to use different method).
I chose to include this case to demonstrate protocols for handling of proteins with cleavable tags and also to show that unspecific cleavage for highly specific protease is not an unthinkable possibility.

	SAG19	Trx-His6-S-tag-DDDDK
MW	26061Da	17075Da
pI	5.14	5.5

SAG19 purification

CFE was prepared by a standard protocol (Protocols, 2) in Buffer A (50mM tris-HCl pH 8.0)
Ni-NTA chromatography: CFE was applied on a 5ml HisTrap-HP column. Flow rate 5ml/min. Fractions 3ml. Gradient: 90ml of Imidazole concentration from 0 to 0.35M in BufferA+0.5M NaCl.

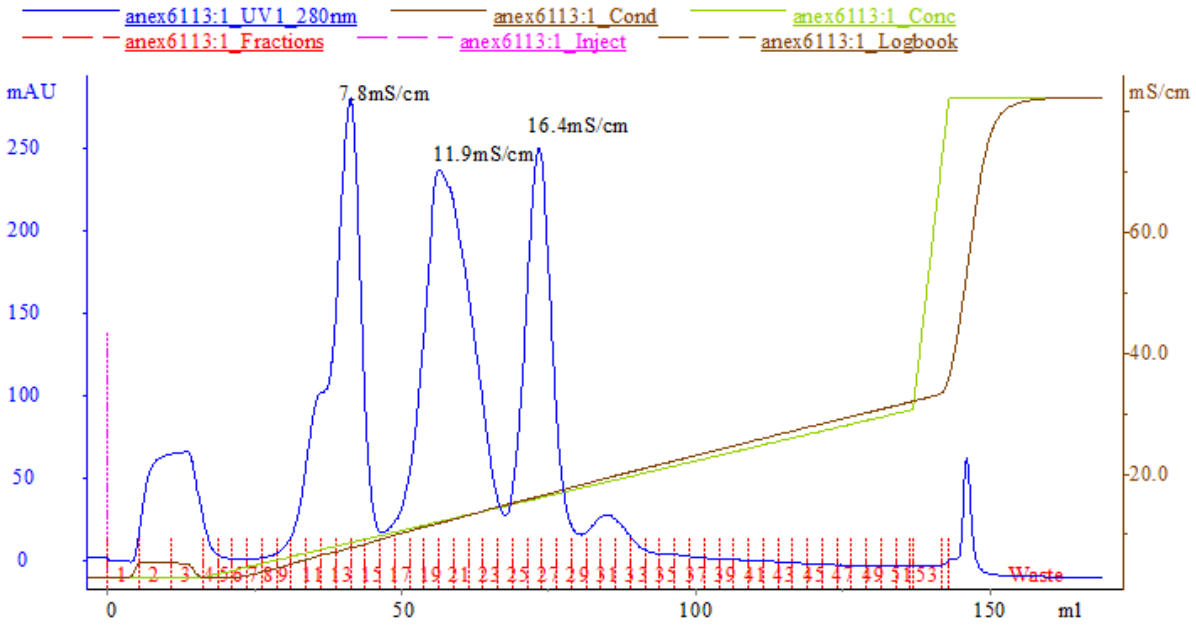


Fractions 18-24 were pooled: V=21ml, C=2.75mg/ml, 57.8mg

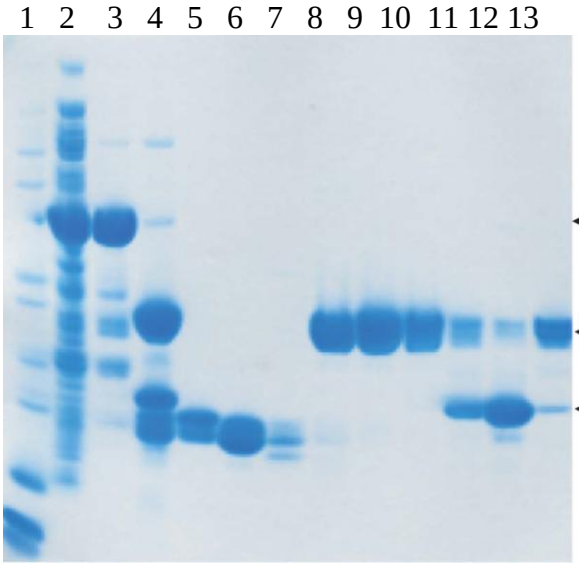
Preparation for cleavage: Sample was concentrated on VivaSpin device with MWCO 30000 and diafiltration cap was used for buffer exchange to cleavage buffer (20mM tris-HCl pH 7.4, 50mM NaCl, 2mM $CaCl_2$). Protein was concentrated to V=1.35ml, C=32.5mg/ml, 43.7mg

Cleavage: Preliminary cleavage experiments revealed that cleavage was not 100% specific, we always observed additional bands on gel, but best results was when we use protein at high concentration. Also, opposite to recommended EK : Protein ratio 1U : 20-100µg we used 1U per 1.5mg of SAG19. Cleavage was performed at room temperature for about 16 hours (overnight). Reaction was stopped by addition of 10mM of *p*-APMSF.

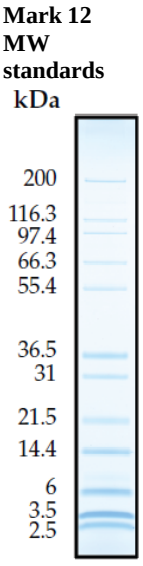
Purification of SAG19 (IEC): Protein concentration was checked in the sample after cleavage: C=26mg/ml, V=1.35ml, 35mg
Sample was diluted in 10ml of Buffer A and applied on a 6ml Resource Q column.
Flow rate 5ml/min. Fractions 2.5ml. Gradient:120ml, 0-0.35M NaCl in Buffer A



Fractions were analysed on SDS-PAGE (4-12% Nu-PAGE BT Novex gel)



1. Mark 12
2. CFE
3. After HisTrap column
4. After cleavage (sample applied on Resource Q column)
5. Fr 11
6. Fr 13
7. Fr 15
8. Fr 18
9. Fr 20
10. Fr 22
11. Fr 24
12. Fr 26
13. Fr30



So, despite close pIs SAG19 and Trx tag were perfectly separated on Resource Q column
First peak elutes with about 0.07M NaCl and most likely contains fragments of Trx tag
Second peak elutes with about 0.1M NaCl and is composed of SAG19 (the protein with apparent MW about 29kDa)
Third peak contains Trx tag and last small peak seems have some fragments which may be misfolded SAG19.
Fractions 18-22 were pooled: V=12ml, C=0.72mg/ml, 8.6mg
Protein was concentrated to about 11mg/ml and used for crystallisation.

The Mass Spectrometry analysis had shown that the purified protein was shorter than expected, 24565Da instead of 26061. N-terminal sequencing revealed that main component of the prep starts 15 aa residues later than predicted.

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAA
TKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGT*DDDDK**AMAAAPDFSSALSL
RLSSTATSQQNSLSTNIEASGDVSPQTPTPPQADEKTEDCLAIINKLRSENLKDLLGTLAKAEDTEVTESLKAIKIEEPASPTAPKIA
VTLAGSNVDTCESGEGANAKKYPGLVIPFPHDTEFNCNALIQATYTAGLDHLKQSNFEPSTGTYDVENAPFNNVNASNVAFLLS
EKSKKVSCAATKDCKAGHDVLFCYFIDPLRKEDKPFTAELYNALWGLEALEHHHHHH

DTNB reaction under denaturing conditions did not revealed any reduced cysteins indicating that protein was correctly folded. Structure was subserviently determined of the stable core of the protein which was found to be even shorter (started peptide is shown in green) as further degradation was observed during crystallisation.

So, this case revealed that specific protease may be not so specific. In case of SAGs EK is not just was very active to cleave target protein at a number of sites different in structure to the specific site, but Therodoxin tag, presumably well folded part of the construct seems was cleaved as well. SAG19 was the best example among the SAGs, a number of other SAGs from the same organism were destroyed by EK to a bunch of fragments.
In this particular case we had to use this kind of construct to produce specific eukaryotic proteins containing disulphate bridges, but in vast majority of cases if tag has to be removed it is better to try to express protein without tag rather than potentially have a trouble with unspecific cleavage.

Cleaver use of tag: TssA from *Burkholderia cepacia*

TssA is one of the proteins which form Type VI secretion system. It is protein of 41kDa which forms a big ring-like 32mer. The protein consists of about 30kDa N-terminal domain (NTD), a long flexible linker and 8.5kDa C-terminal domain (CTD) which involved in oligomerisation. Attempts to crystallise the whole protein did not succeed most likely due to flexibility of the linker. NTD was expressed separately and structure was solved. Expression of CTD was 100% insoluble. The clever move of Dr Mark Thomas was to fuse CTD to MBP (Maltose Binding Protein) which is close in size to NTD. Linker between MBP and TssA CTD contained a cleavage site for Factor Xa Protease. This approach works perfectly, huge soluble expression was achieved and MBP-TssACTD forms oligomers of the same size as a native protein. After MBP was cleaved off, TssA CTD rings were purified, crystallised and structure was solved.

TssA CTD (rings) purification

CFE was prepared by a standard protocol (Protocols, 2) in 0.2M NaCl, 50mM tris-HCl pH 8.0(starting buffer): V=20ml, C=4.5mg/ml, 90mg

Amylose chromatography: This chromatography was performed manually. 8ml column of Amylose resin (NEB) was made in PD-10 column (see picture) and equilibrated in the same buffer.

I have adapted a 20 ml syringe for sample application with a spare cap for PD-10 column and a piece of silicone tubing for a good seal. Works fine, sample can be applied in one go, no need to add sample ml by ml.

CFE was applied, unbound material was collected and column was washed with 20ml of the starting buffer. Unbound material was V=21ml, C=2.2mg/ml, 46mg

Elution was achieved with 10mM maltose in the same buffer. 2-2.5ml fractions were collected and protein concentration was estimated in them by method of Bradford:

For 2µl:

Fr	A_{595}
1	0
2	0.1
3	0.51
4	0.5
5	0.22
6	0.08

Fractions 2-5 were pooled:
V= 9ml, C=2.7mg/ml, 24mg

Amylose column was equilibrated with starting buffer again and unbound material was reapplied on the column. The reason for doing this was my previous experience that not all MBP fused protein would be bound to the column. It seems, binding conditions is not optimised. Also, column is losing some amylose with each use and capacity decreases.

I would recommend doing second application, especially in case if expression level of MBP fused protein is high.

Unbound material after second application: V=21ml, C=1mg/ml, 21mg.
Second set of eluted fractions was analysed for protein:
For 5µl:

Fr	A_{595}
1.	0
2.	0.65
3.	0.81
4.	0.5
5.	0.22
6.	0.1

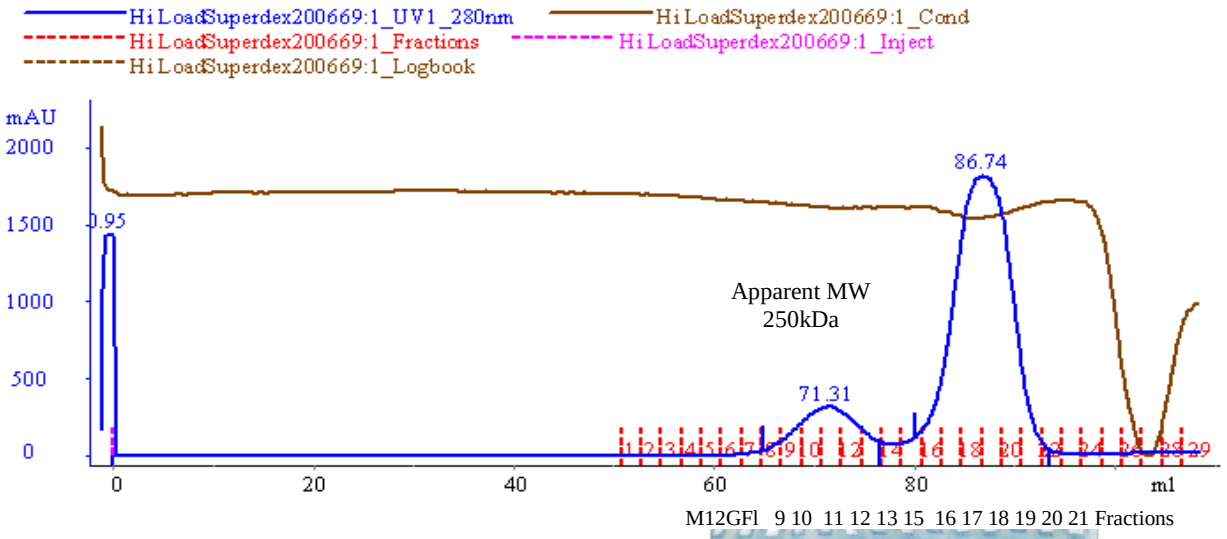
Fr 2-5: V=9.5ml, C=1.5mg/ml, 14.5mg

Eluates from both runs were combined and concentrated using VivaSpin device with MWCO 50000: V=1.6ml, C=14mg/ml, 30mg

Cleavage: The sample was supplemented with $CaCl_2$ (1.6µl of 2M solution) to make it 2mM. 150µl of 1mg/ml (150µg) of Factor Xa Protease (NEB) was added (5µg/1mg of MBP-CTD). Incubation: overnight at room temperature.

Gel filtration: Sample was applied on a 1.6x60cm gel filtration column which was Superdex200 in the past, but then it seems dextran moiety was destroyed and pores became bigger, more like for Superose 6. I use this column when I have to purify big proteins.
Gel filtration was performed in buffer 0.5M NaCl, tris-HCl pH 8.0. Flow rate 1.5ml/min. Fractions 2ml. Fractions were analysed for protein and by SDS-PAGE.

```
No 1:    Sample Volume and Type
         TssA rings  SM about 30mg
No 2:    Column
         Superdex200 09
No 3:    Eluent A
         0.5M NaCl tris pH 8.0
No 4:    Eluent B

No 5:    Remarks
         28.7.15
```

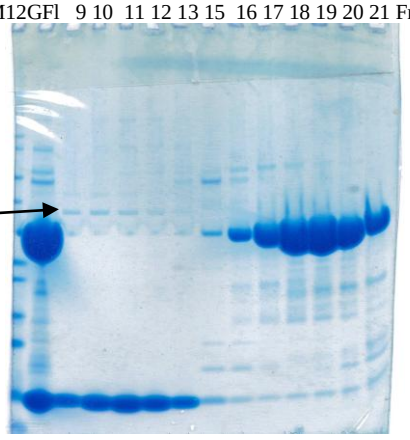


Bradford:
For 10µl:

Fr	A_{595}
7	0.06
8	0.17
9	0.36
10	0.61
11	0.7
12	0.61
13	0.4
14	0.2

Fractions 10-14: V=10ml
C=0.72mg/ml
7.2mg

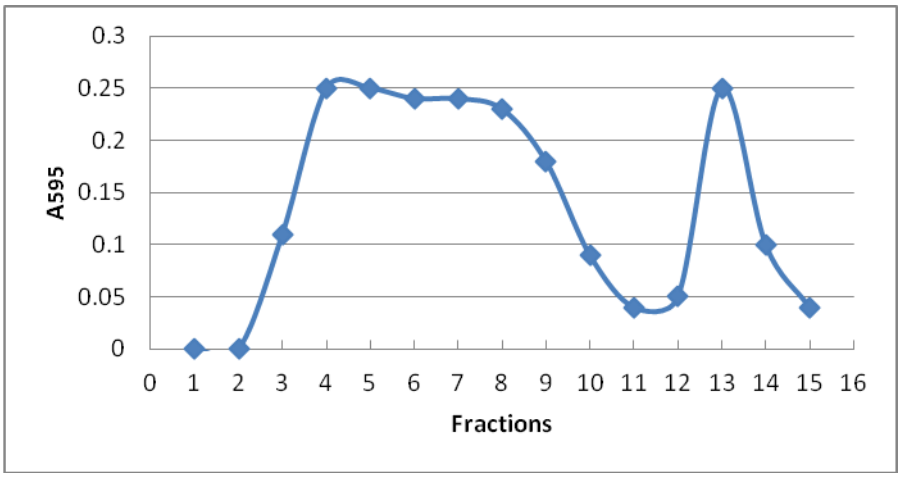
Uncleaved protein



As you can see on gel there is small amount of uncleaved protein which can not be separated from the cleaved TssA CTD because uncleaved molecules forms oligomers alongside of the cleaved ones. In our early attempts to crystallise TssA rings crystals did not difract well, most likely due to heterogenity as a result of having one or two uncleaved molecules in the ring.
To fight this problem we introduced Amylose chromotography as a last purification step.
Amylose Chromatography: Sample obtained after gel filtration was diluted twice to reduce salt concentration and was applied on a Amylose column. 2-2.5ml fractions were collected. Column was washed with 10ml of starting buffer and then eluted with 10mM maltose in the same buffer. All fractions were analysed by Bradford.

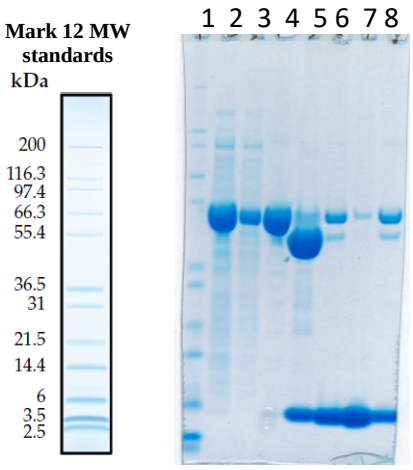
For 20ul:

Fr	A_{595}
1.	0
2.	0
3.	0.11
4.	0.25
5.	0.25
6.	0.24
7.	0.24
8.	0.23
9.	0.18
10.	0.09
11.	0.04
12.	0.05
13.	0.25
14.	0.1
15.	0.04



Unbound material was collected: fractions 3-9: V=19ml, C=0.3mg/ml, 5.7mg
Sample was concentrated, buffer exchanged and used for crystallisation. This time crystals were better and stucture was solved.

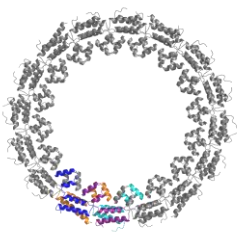
The purification progress was analysed by SDS-PAGE: 4-12% NuPage BT Novex gel



1. Mark12
2. CFE
3. Unbound from second Amylose chromatography
4. MBP-TssACTD
5. Cleaved sample
6. TssA CTD after gel filtration
7. Final prep
8. Fr 13 from last Amylose chromatography

Summary

As we can see on gel expression level of MBP-TssACTD is extremely high, may be up to 40-50%. After second Amylose column there still is significant amount of fused protein in unbound material...Are binding buffer conditions optimal?
Cleavage on this occasion was not ideal and so sample contained noticeable amount of uncleaved molecules. Small contamination of MBP also was left in the prep of CTD rings after gel filtration.
Last amylose chromatography improved purity significantly. Some of the rings probably containing one or two uncleaved molecules passed through Amylose column unbound but it seems amount of such molecules was not significant and crystals were ordered and diffracted well.



8. Special cases

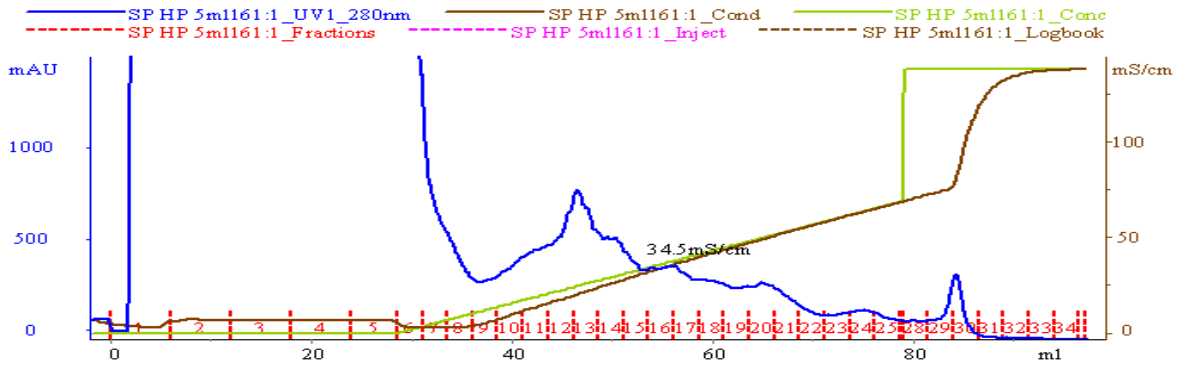
Phosphoglycerate Kinase from *Escherichia coli*

I include this case to show that it is possible to purify protein from the natural source even if it is not one of the main majors in CFE, but by chance has affinity to Heparin and also separates nicely from other proteins on Resource Q column.
The story begins when I was asked to purify DNA binding protein called Rv3616c, which has MW 39888 and pI 5.2. Protein was untagged and expression level was very low so no certain band could be identified as a target protein. So strategy in this case is clear: Run Heparin chromatography and find 40kDa protein as one of the significant or at least visible proteins in eluted fractions. Second step is IEC and then depending on result, HIC+GF or just one of them. If purification is successful in case like that we must to confirm identity of the protein (usually by Mass Spectrometry).

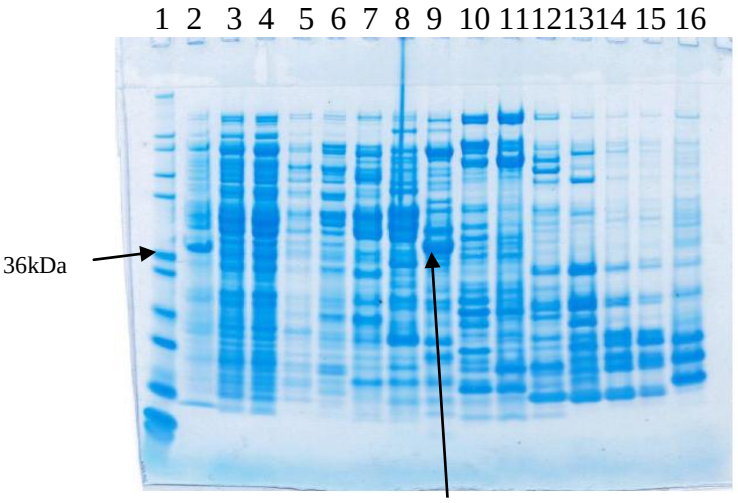
Purification

CFE was prepared from about 6g of cells in Buffer A (50mM tris-HCl pH 8.0).
CFE: V=24ml, C=13mg/ml, 310mg
Heparin Chromatography. CFE was applied on a 5ml Heparin column.
Flow rate 4ml/min. Fractions 2.5ml. Gradient: 50ml 0-1M NaCl in bufferA.

```
No 1:    Sample Volume and Type
         Rv1636c  310mg
No 2:    Column
         Heparin HP 5ml
No 3:    Eluent A
         50mM tris
No 4:    Eluent B
         A+2MNaCl
No 5:    Remarks
         0-50%B 10CV  21.4.16
```

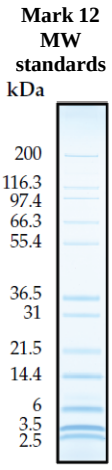


Fractions were analysed by SDS-PAGE (4-12% NuPageBT Novex gel)



This band of about 40kDa looks convincing

1. Mark12
2. Debris
3. CFE
4. Fr4
5. Fr8
6. Fr10
7. Fr12
8. Fr14
9. Fr16
10. Fr18
11. Fr20
12. Fr22
13. Fr24
14. Fr26
15. Fr28
16. Fr30



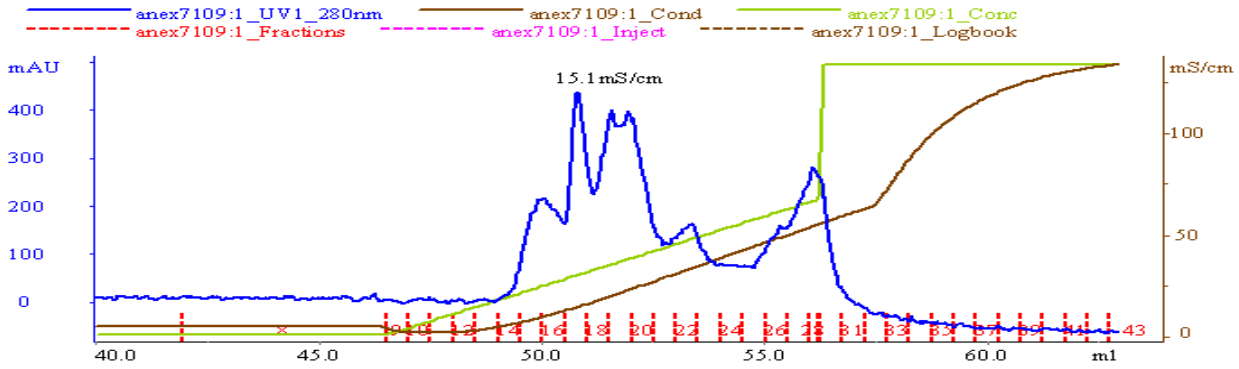
Fractions 16+17 were combined: V=5ml, C=1.8mg/ml, 9mg

Anion Exchange Chromatography. Sample was diluted to 5mS/cm and applied on a 1ml Resource Q column.

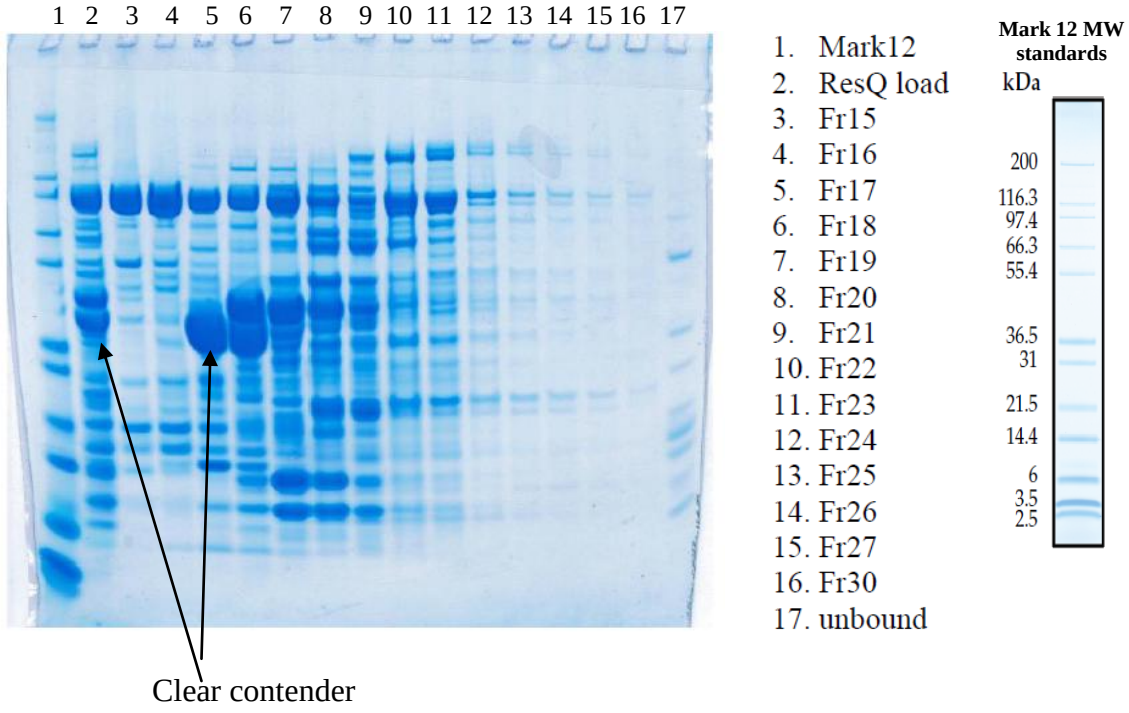
Flow rate 2ml/min, fractions 0.5ml.

Gradient: 10ml 0-1M NaCl in bufferA

```
No 1:    Sample Volume and Type
         Rv1636c 9mg
No 2:    Column
         ResQ 1ml
No 3:    Eluent A
         tris pH 8.0
No 4:    Eluent B
         A+2MNaCl
No 5:    Remarks
         0-50% 10CV  21.4.16
```



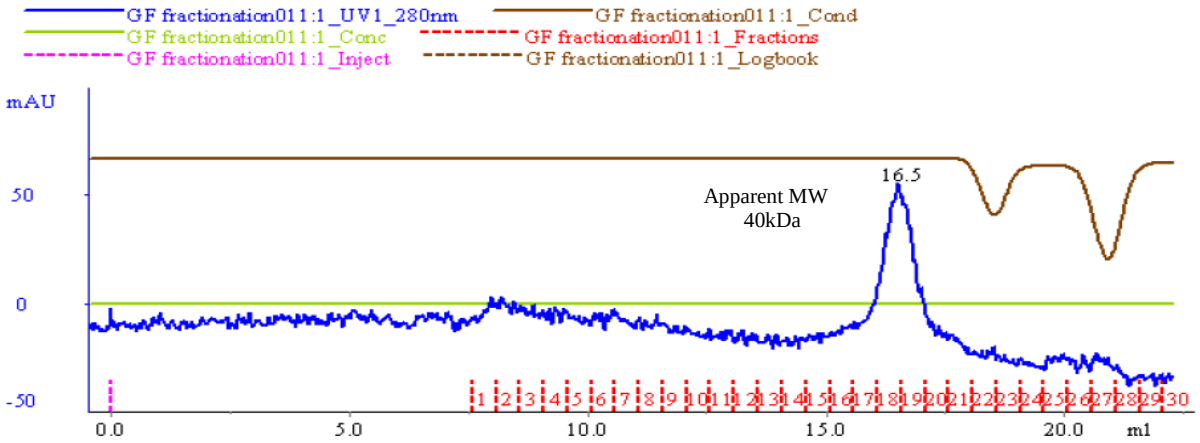
Fractions were analysed by SDS-PAGE:



Fraction 17: V=0.45ml, C=2.7mg/ml, 1. 2mg → GF

Gel filtration. Sample was applied on a Superdex200GL(24ml) column.
Buffer: 0.5M NaCl in bufferA
Flow rate 0.5ml/min
Fractions 0.5ml

```
No 1:    Sample Volume and Type
         Rv1636c    1.2mg
No 2:    Column
         superdex200 GL 11
No 3:    Eluent A
         0.5M Nacl tris
No 4:    Eluent B

No 5:    Remarks
         21.4.16
```



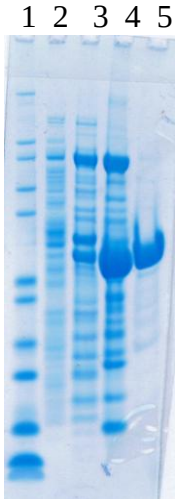
Peak fractions 18+19 were combined: V=1ml, C=0.5mg/ml, 0.5mg

Apparent MW of 40kDa looks suspicious as DNA-binding proteins usually are dimers.

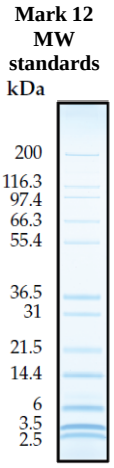
Sample was sent to Mass Spec and result was 40987Da, not 39757 as we would expect for the target protein.

Further identification was performed by Mass Spec of tryptic fragments and it revealed that purified protein was Phoshoglycerate Kinase (PGK) from *E.coli*.

So, below is summary on purification of PGM from *E.coli*



1. Mark12
2. CFE
3. After Heparin
4. After IEC
5. After GF (final prep)



Purity is about 80-85%. Yield is 0.5mg from 310mg of total protein. Presuming 30% yield from three steps purification protocol, PGK expression is about 0.5% of total cell protein.

What to say? PGK is a good protein to purify!

Low affinity chromatography: BPSL1958

BPSL1958 is one of proteins which found to be moderately important for the *Burkholderia pseudomallei* virulence. When structure was determined it appeared to be sugar binding protein. Further experiments revealed significant affinity of BPSL1958 to a number of sugars, including glucose.

MW 36126

pI 4.38

$A_{280\ 1mg/ml}$ 2.13

For protein with pI 4.38 we expect that it has high affinity to anion exchange matrix. In fact under our standard conditions of 50mM tris pH 8.0 this protein did not bind to DEAE column properly and start to pass through the column in the end of sample application. We increase pH to 9.0, but seems it did not help. In fact it was better interaction at pH 7.0!

So we decided to employ low affinity chromatography protocol.

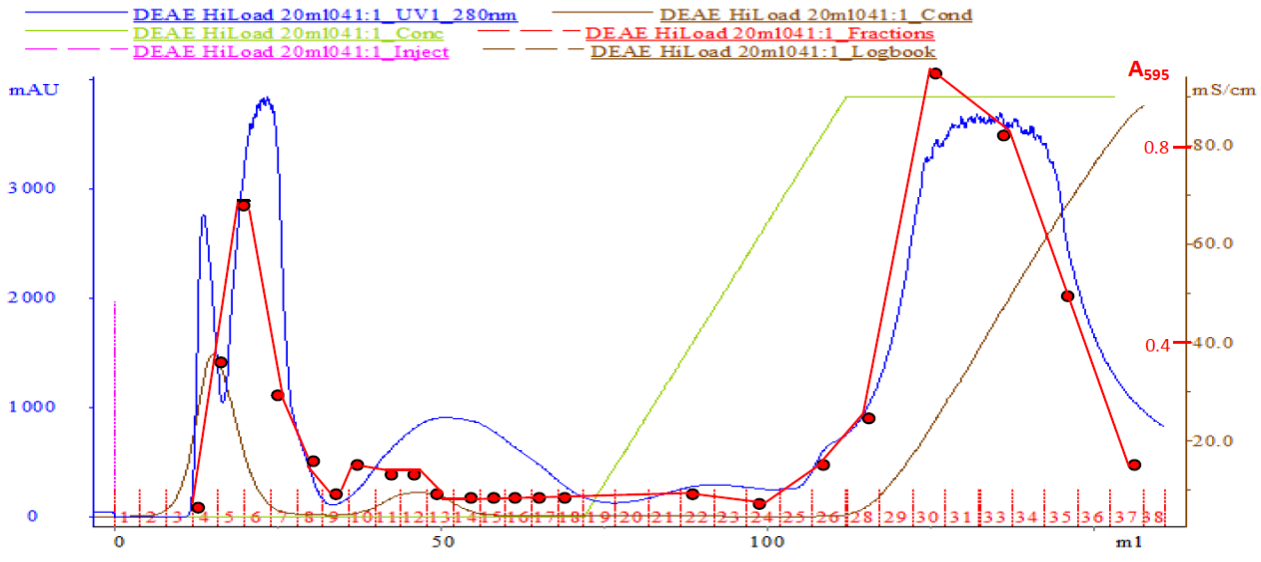
BPSL1958 purification

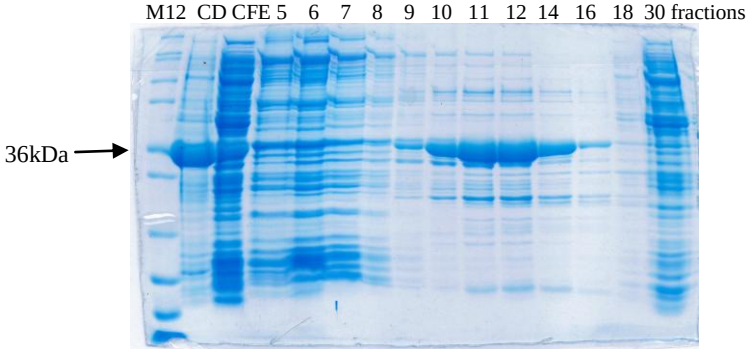
CFE was prepared in 30mM tris-HCl buffer pH 7.0 using standard protocol, but with modifications. The volume of CFE had to be reduced and so cells:buffer ratio was reduced, 3g of cells were suspended in about 12 ml of buffer. Sonication was extended to 4 cycles of 20 sec at 16micron amplitude and centrifugation was performed for 20 minutes at 70000g to compensate for higher viscosity of the sample.

CFE: V=12ml, C=10mg/ml, 120 mg

Anion Exchange chromatography. Sample was applied on a 50ml column with DEAE-Sepharose Fast Flow (1.6x25cm) equilibrated in the 30mM tris-HCl pH 7.0 at flow rate 5ml/min. 4ml fractions were collected. Column was washed with 75ml of the starting buffer and then a 50ml gradient of NaCl concentration from 0 to 1M was applied to elute rest of the proteins.

Fractions were analysed by Bradford (5ul)(presented on chart) and by SDS-PAGE (Laemmly, 12%)

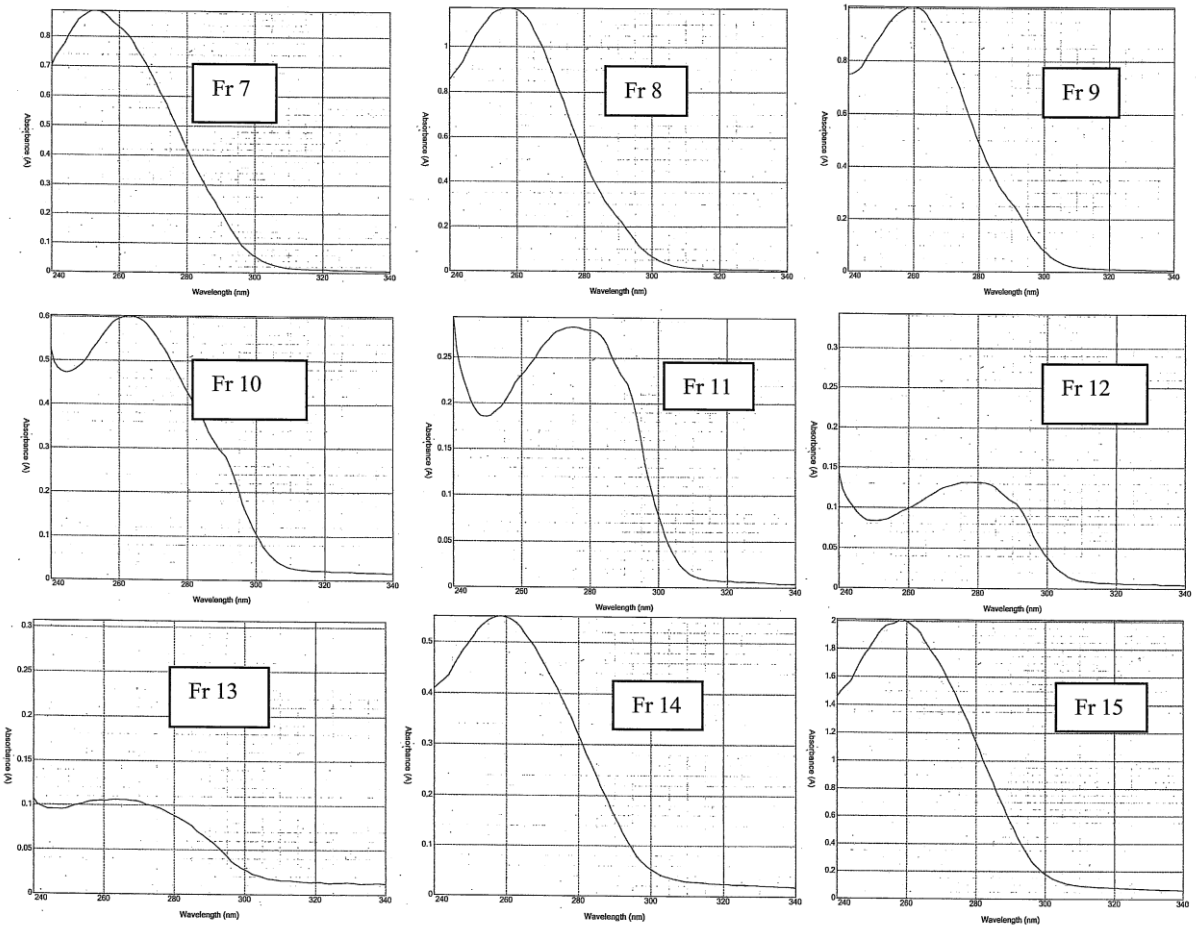




BPSL1958 is one of those proteins which show low level of Bradford reaction. So figures of A_{595} are low and do not really correspond to BPSL1958 distribution. So we would probably need to run SDS-PAGE every time to find exact fractions with the target protein.

Luckily in this case we can use another quick method as we found that UV spectra are very informative in this case.

UV spectra(240-340nm) were taken from the fractions 7-15:

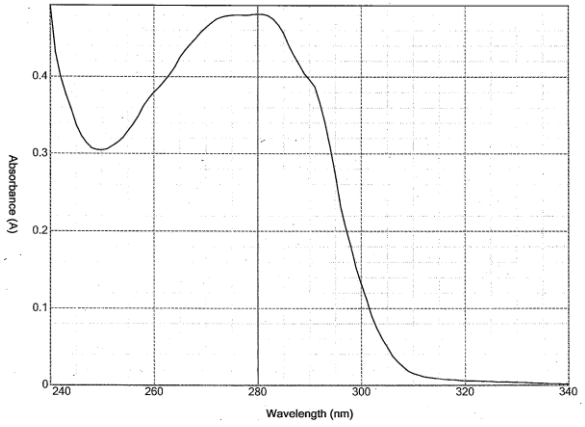


As you can see fractions 11 and 12 display protein spectra, fractions 10 shows mix of protein and nucleic acid while in other fractions nicleic acids are dominated. As we can see on gel image, fractions 11 and 12 contain most pure BPSL1958.

In subsequent purifications we were taken spectra from relevant fractions to find 2-3 fractions which show protein spectra and pooled them.

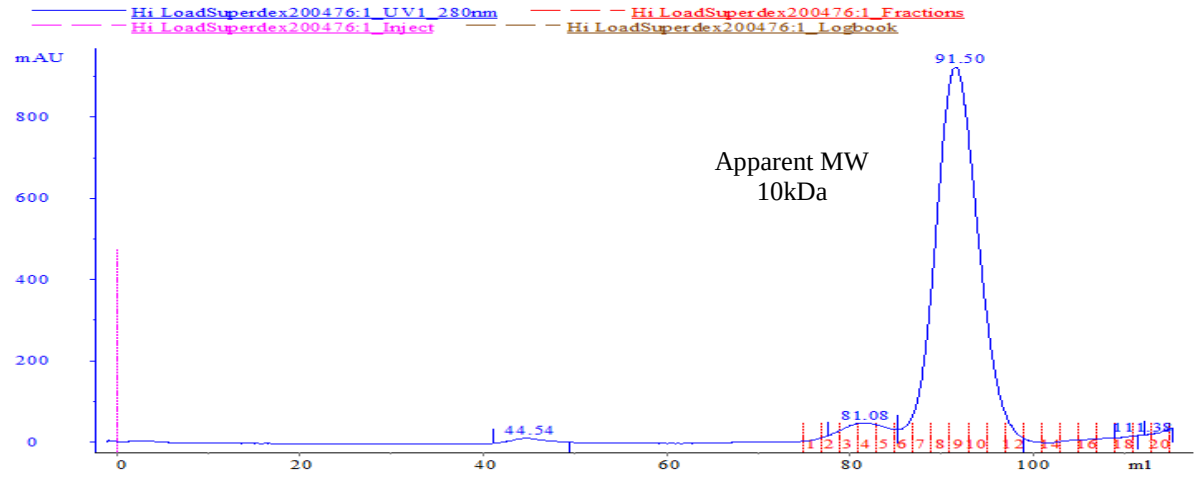
Fr 11+12: V=8ml, C=0.48mg/ml, 3.8mg (Bradford reaction)

Sample was concentrated using VivaSpin: V=1.4ml, C=3mg/ml (BR). Concentration also was measured by UV (dilution 1:50):

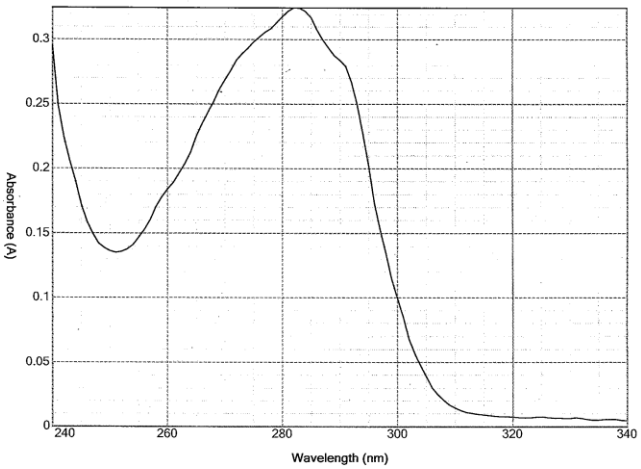


For BPSL1958 $A_{1mg/ml}$ = 2.13
(0.48x50)/2.13= 11.2mg/ml
So Bradford reaction underestimate
BPSL1958 concentration almost 4-fold.
Total protein: 1.4ml x11.2mg/ml= 15.7mg

Gel filtration. Sample obtained after concentration was applied on a 1.6x60 HiLoad Superdex200 column.
Buffer: 0.5M NaCl, 50mM tris-HCl pH 8.0
Flow rate 1.5ml/min
Fractions 2ml



Well, once again BPSL1958 displays unusual behaviour. Apparent MW from gel filtration is 10kDa instead of 36kDa. It seems, interaction with glucose residues in dextran moiety in the Superdex beads delays its elution from the Superdex column!
Fractions 8-10 were pooled and UVspectrum was taken with 10 fold dilution:

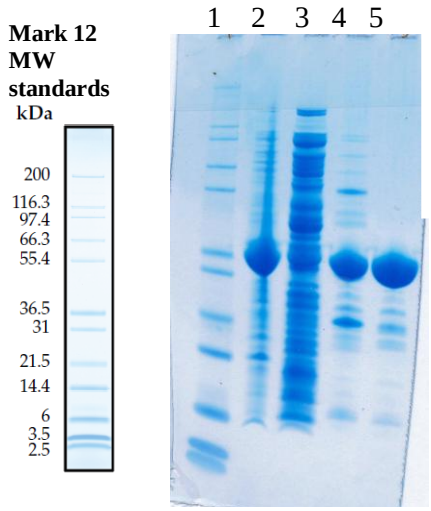


A280=0.31

C= (0.31x10)/ 2.13= 1.45mg/ml

Fr 8-10: V=6ml, C=1.45mg/ml (UV), 8.7mg

Progress of purification was analysed by SDS-PAGE (4-12% Nu Page BT Novex gel):



1. Mark12
2. CD
3. CFE
4. GF load
5. Final

Summary

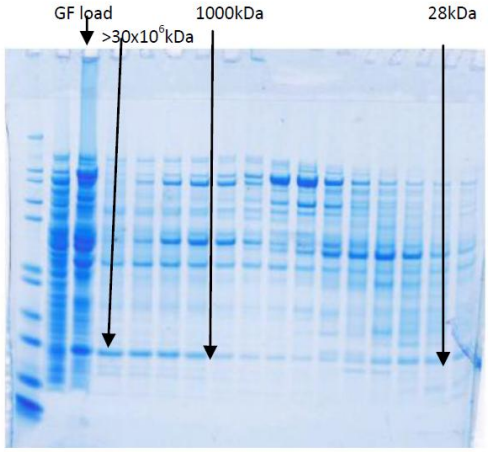
BPSL1958 is one of those odd proteins which display unusual behaviour in all possible ways:
Having pI 4.4 it does not bind to anion exchanger
Being 36kDa it runs as 10kDa on gel filtration column
It shows low signal in Bradford reaction

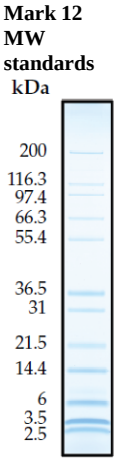
Expression of BPSL1958 was huge, but mainly insoluble.
Thanks to low affinity chromatography main purification was achieved on first step.
85-90% pure protein was obtained after 2 step purification despite of low level of soluble expression.

BacM from *Myxococcus Xanthus*

BacM is one of the weirdest proteins I ever got to purify. This protein forms filaments of a different size so to purify protein we had to use urea to destroy them. Previously His-tagged version of this protein was expressed in *E.coli* and it was purified by using Ni-column under denaturing conditions and then urea was dialysed out resulting in BacM forming big aggregates which came out of solution. Easy enough. But it was not easy for untagged BacM.

BacM : MW 13192, pI 8.2
BacM has no aromatic amino acid residues in its composition, so it displays no A_{280} absorbance. Luckily it reacts with Bradford reagent!
Level of expression of BacM in *E.coli* was relatively low, about 3% or so.
Attempts to use cation exchange chromatography under denaturing conditions as a first step of purification did not succeed. At pH 5 in 8M Urea BacM has very low affinity to SP-Sepharose. It mainly passed through the small column in first instance. When I tried to apply a low affinity chromatography it was bound to the bigger column and eluted in very first fractions of the NaCl gradient together with a lot of contaminations.
So I attempted to do some separations under native conditions.
When cells were destroyed in 50mM tris pH 8.0 and CFE was prepared using our standard centrifugation BacM was found in the pellet and in the supernatant. Decreasing speed of centrifugation to 12000g and time to 5minutes we managed to keep all BacM in supernatant fraction. We also get lucky with ammonium sulphate cut. All BacM precipitates by 1.5M ammonium sulphate displaying some purification.
When AS pellet was dissolved in native buffer and run on gel filtration column BacM was mainly found among particles bigger than 1000kDa, some smaller oligomers and also on monomers-dimers position:





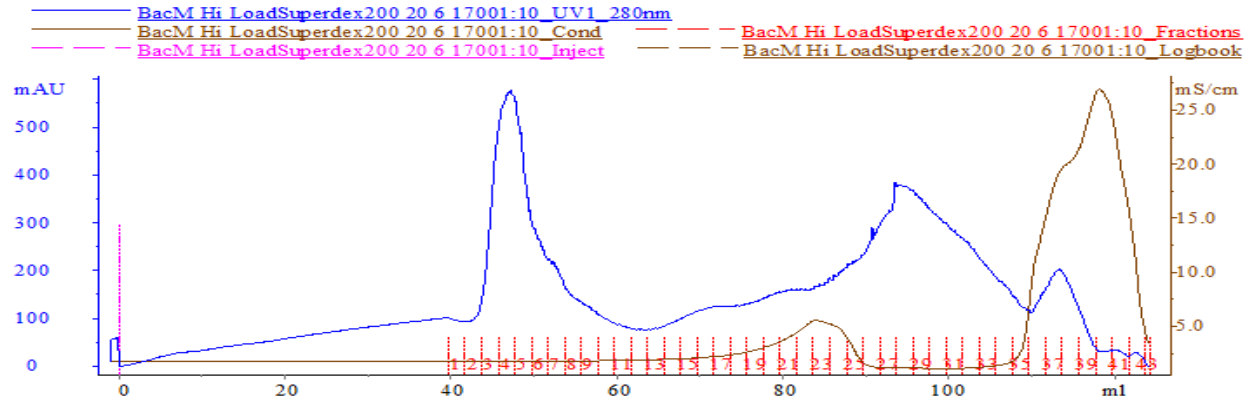
It was clear that upon this point we have to go under denaturing conditions.
So AS pellet was dissolved in 8M Urea and gel filtered in the presence of 7M Urea. Best purification was achieved on this stage. However protein still was not pure. Best purity of about 80% or so was only reached after additional negative IEC and second gel filtration as described below.

BacM purification 20.6.17

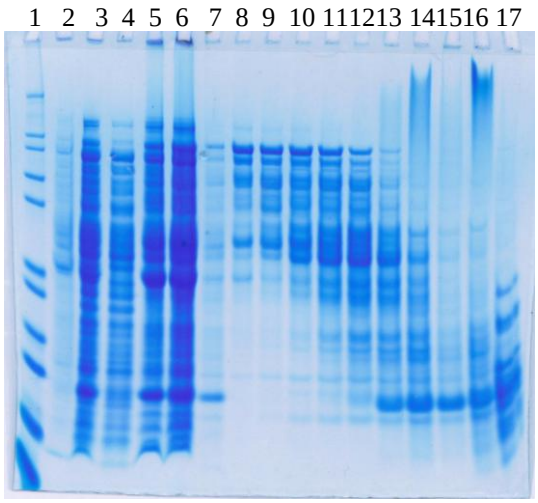
About 5-6g of cells were suspended in about 45ml of 50mM tris-HCl pH 8.0
Sonication: 3x20sec at 16 micron on Soniprep
Centrifugation: 12000g for 5min
CFE looked like a lot of DNA still is not destroyed. 3x10sec sonication was applied
Looks much better, less viscous.

CFE: V=48ml, C=11mg/ml, 520mg
37ml of 4M AS was added (to 1.7M) , incubated for 5 min on ice and spin down at 43000g for 10min. Supernatant fraction: V=80ml, C=3mg/ml, 240mg
2 pellets were obtained. One of them was placed on -20^{o}C and saved for the next time.
To the second AS pellet 3ml of Buffer U (8M Urea, 50mM HEPES pH 7)was added
Extraction of BacM was performed by stirring the mixture for 1 h at room temperature
Insoluble material was removed by centrifugation at 70000g for 20min
Supernatant fraction: V=3.4ml, C=8.7mg/ml, 32mg
3ml was applied on GF column in buffer U. Flow rate 1.5ml/min, fractions 2ml

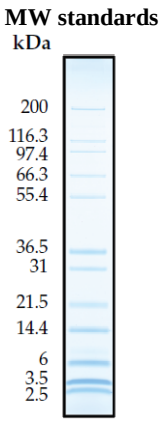
```
No 1:    Sample Volume and Type
         BacM 26mg

No 2:    Column
         Superdex200 09

No 3:    Eluent A
         8M urea HEPES pH 7

No 4:    Eluent B

No 5:    Remarks
         20.6.17
```



SDS-PAGE analysis (NuPage 4-12% BT Novex gel)

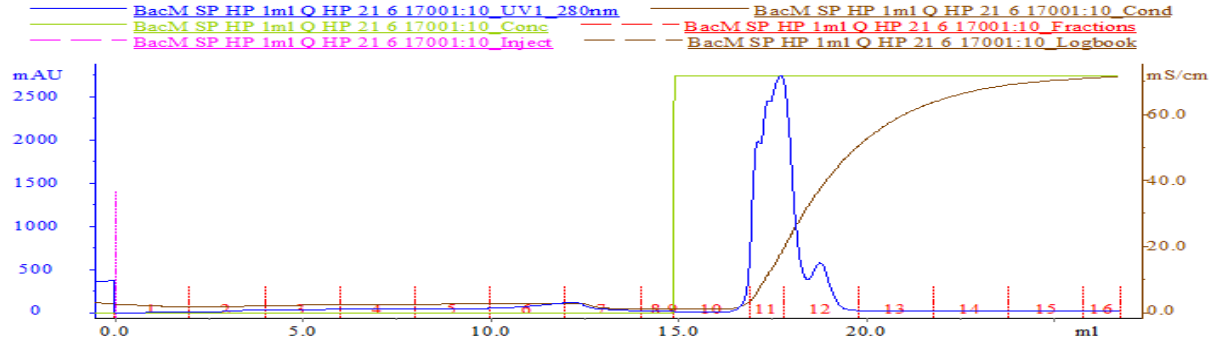


1. Mark12
2. CD
3. CFE
4. AS super
5. AS pellet
6. Urea pellet
7. Urea extract
8. Fr 16
9. Fr18
10. Fr20
11. Fr22
12. Fr24
13. Fr26
14. Fr28
15. Fr30
16. Fr32
17. Fr34



Fractions 26-32: V=11.5ml, C=0.95mg/ml, 10mg
Sample was subjected for double negative IEC: 1ml Q-HP and 1ml SP-HP cartridges were screwed together and equilibrated in buffer U
Flow rate 1ml/min, 2ml fractions were collected. Columns were washed with Buffer U, then with Buffer U+1M NaCl

```
No 1:    Sample Volume and Type
         BacM 10mg

No 2:    Column
         SP-HP+Q-HP 1+1ml

No 3:    Eluent A
         8M urea HEPES pH 7

No 4:    Eluent B
         A+1MNaCl

No 5:    Remarks
         collect unbound + 1M elution  21.6.17
```



Fractions were analysed for protein and by SDS-PAGE:

BR 10ul:

Fr	A595
2	0.22
3	0.32
4	0.3
5	0.35
6	0.34
7	0.3
8	0.04
10	0.0
11	0.04
12	1.2
13	0.24
14	0.04

M12 L 3 6 7 12 Fractions

Fr 2-7: V=11ml, C=0.45mg/ml

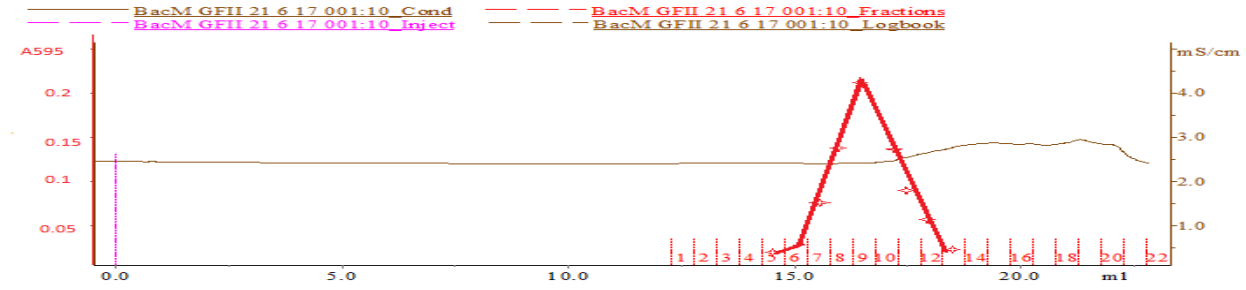
10ml, 4.5mg was concentrated on VivaSpin to V=0.5ml, C=5mg/ml, 2.5mg (out of 4.5!)

Sample was applied on a 24ml GF column equilibrated in Buffer U.

Flow rate 0.5ml/min, fractions 0.5ml

UV records failed on this occasion, so protein concentration was analysed in fractions and it is shown on chromatogram below.

```
No 1:    Sample Volume and Type
         BacM 2.5mg

No 2:    Column
         Superdex200 GL 11

No 3:    Eluent A
         8M Urea HEPES pH 7.0

No 4:    Eluent B

No 5:    Remarks
         21.6.17
```



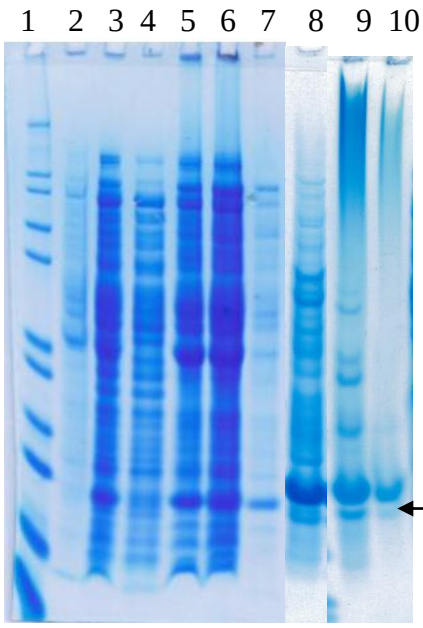
Fr 8-10 were combined:
V=1.5ml, C=0.33mg/ml, 0.5mg **Final preparation**

SDS-PAGE:

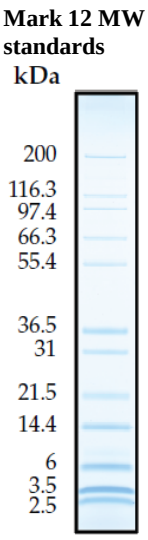
1. Mark12
2. Before last GF
3. Final prep
4. Second Urea extraction

Summary

Unfortunately for this purification I have not run gel to show purification progress, so below is a combination of different gels:



1. Mark12
2. CD
3. CFE
4. AS supernatant
5. AS pellet
6. Urea pellet
7. Urea extract
8. After first GF
9. After negative IEC
10. After second GF (final preparation)



Looking on gel for Urea extract we would estimate BacM to be about 10% or so, so having 32mg on this stage, we would expect to have about 1mg of pure protein after 3 step purification. However, BacM kept disappearing even when left in an eppendorf in the presence of Urea. Almost half of the protein was lost on VivaSpin device, presumably stick to a membrane. Only 0.5mg of the protein was obtained, very low yield with purity not better than 80%.
Also it seems significant amount of BacM was left in the pellet after Urea extraction. Second extraction was attempted but extract was much less enriched in BacM than the first extract and so it was dismissed.
Another unpleasant thing was that BacM co-purifies with another a bit smaller protein. It was identified as GroES!!!?
So, I cannot say that purification of BacM was a great success.
However, I have managed to optimise purification by using the same buffer (Buffer U) for all purification steps and achieved more or less acceptable purity. In this case, with very limited options for chromatography, purity completely depends on level of TP expression.

Perfect purity:
Imidazoleglycerol-phosphate dehydratase 1 from *Arabidopsis thaliana*

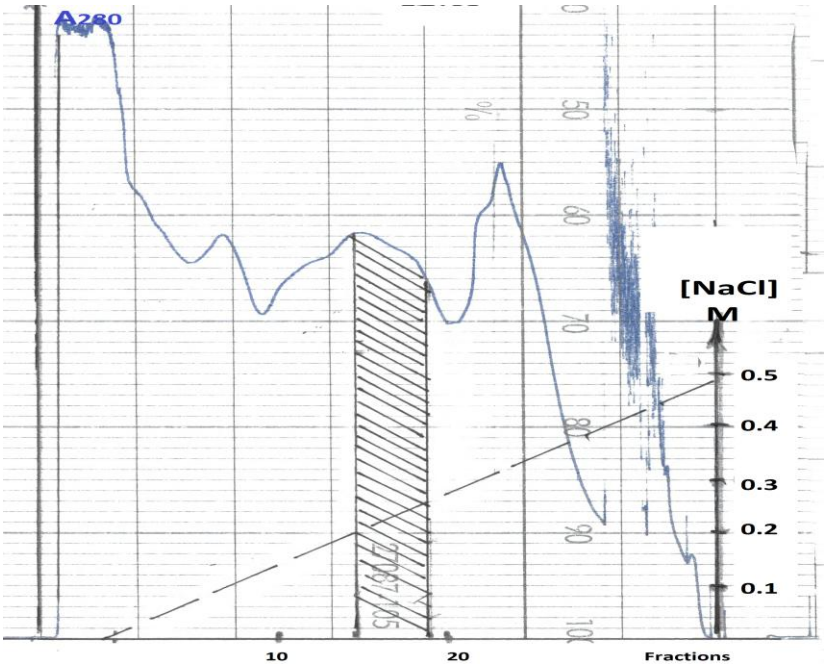
IGPD takes part in histidine biosynthesis in plants and bacteria. It is a homooligomer of 24 subunits of about 22-29kDa . Enzyme contains Mn important for its activity. There are two types of IGPD and *Arabidopsis thaliana* has both type 1 and type 2. Type 1 24mer dissociates to stable trimers when Mn is removed in the presence of EDTA and associates to 24mer when Mn is bound back.
Type 2 IGPD keeps its oligomeric 24mer form in the presence of EDTA and absence of Mn.
Thanks to such an unique property Type 1 IGPD could be purify to perfect 100% purity by employing two gel filtrations: first gel filtration is performed in the presence of EDTA, trimers are collected and then sample is supplied with Mn and subjected to gel filtration in the presence of Mn to obtain 100% pure 24mer.

Type 1 IGPD from *A.thaliana* purification

CFE was prepared from 4g of cells in buffer A (40mM tris-HCl pH 8.0, 2mM EDTA) using a standard protocol.
CFE: V=28ml, C=9mg/ml, total protein 252mg

Anion exchange chromatography

Sample was applied on a 20ml DEAE Sepharose FF column at flow rate about 4.5ml/min. Chromatography was performed using peristaltic pump, UV monitor UV-1 Pharmacia and 2110 Bio-Rad fraction collector (see page 15). Elution was performed with 300ml of gradient NaCl concentration from 0 to 0.5M in buffer A. 7.5ml fractions were collected.

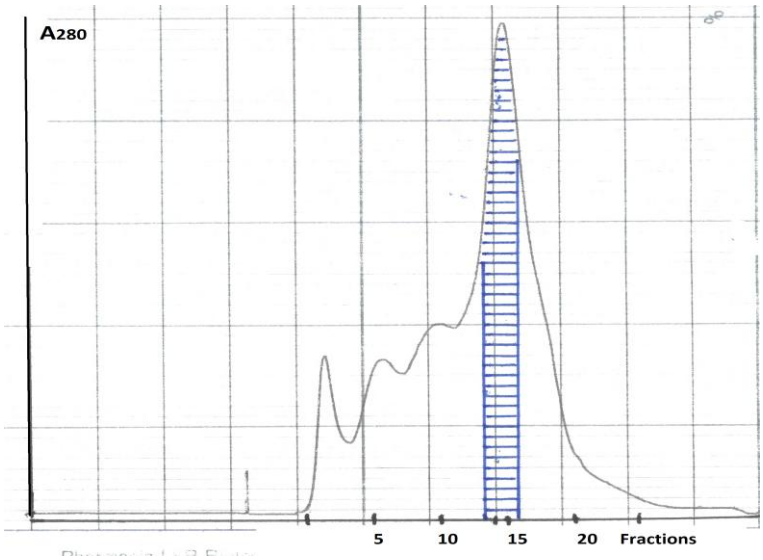


Fractions were analysed for protein by method of Bradford and four fractions with highest protein concentration were combined:
Fr 16-19: V=30ml,
C=2.2mg/ml,
Total protein 66mg

22ml of 4M ammonium sulphate solution was added to the sample obtained after IEC and so IGPD was precipitated by 1.5M AS. Pellets were collected by centrifugation (5minutes at 45000g) and dissolved in 1 ml of buffer A: V=1.35ml, C=23mg/ml, 32mg.

First gel filtration

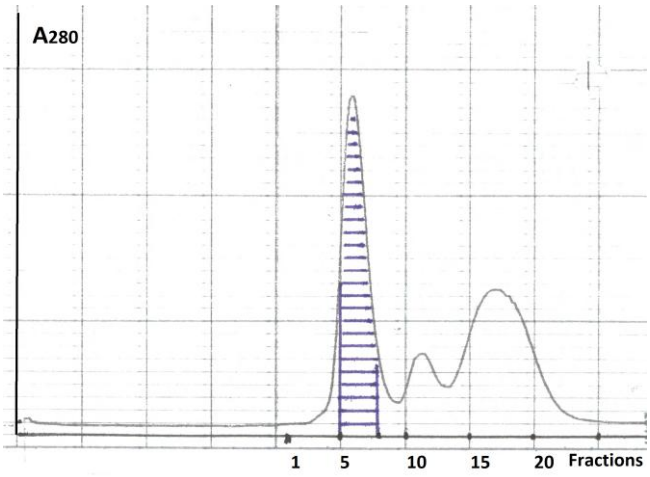
Gel filtration was performed on HiLoad Superdex200 column in buffer A+0.1M NaCl at flow rate 1ml/min. 2m fractions were collected after void volume.



Fractions were analysed for protein and four fractions with highest protein concentration were collected:
Fr 13-16: V=8ml, C=2.5mg/ml,
Total 20mg
Volume of the sample was reduced using VivaSpin 30000MWCO device:
V=0.6ml, C=29mg/ml, 17.5mg

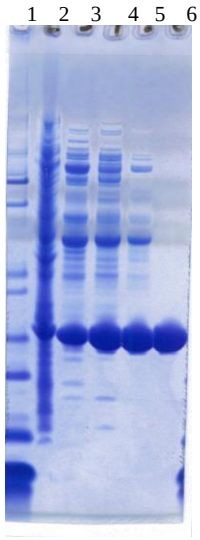
Second gel filtration

The same column was equilibrated in buffer 20mM tris-HCl pH 7.5, 2mM $MnCl_2$, 0.1M NaCl.
The sample obtained after first gel filtration was applied and gel filtration was performed under the same conditions as the first one.

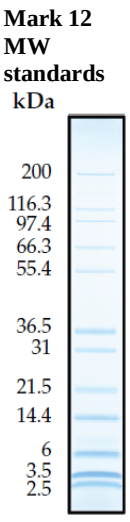


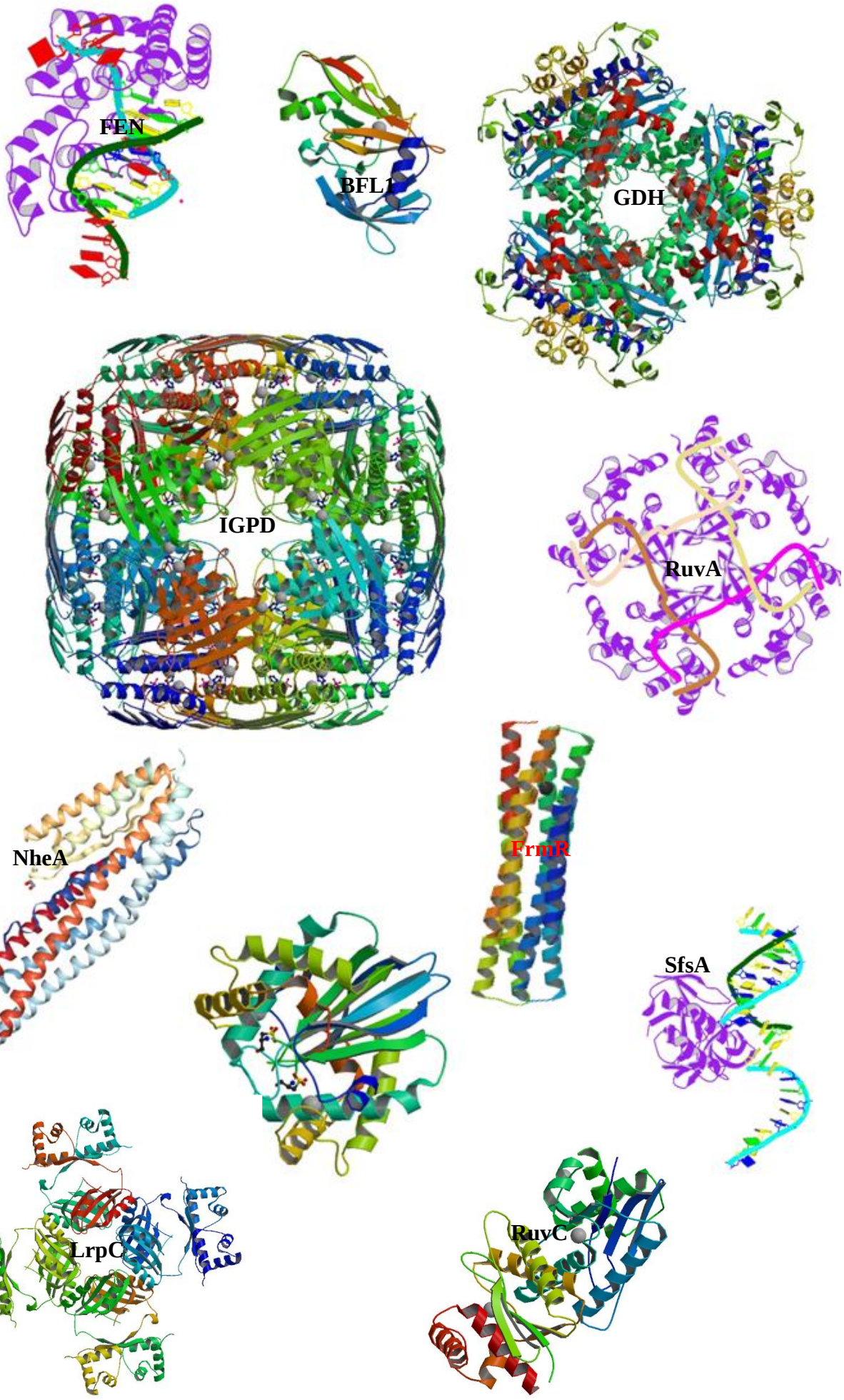
First peak was analysed by Bradford and peak fractions were combined:
Fr 5-7: V=6ml, C=2mg/ml, 12mg
Sample was concentrated using VivaSpin to prepare for crystallisation:
V=0.33ml, C=30mg/ml, 10mg

Purification progress was analysed by SDS-PAGE (4-12% NuPage BT Novex gel)



1. Mark12
2. CFE
3. After AEC
4. Sample for the first GF
5. Sample for the second GF
6. Final preparation




FEN
BFL1
GDH
IGPD
RuvA
NheA
FrmR
SfsA
LrpC
RuvC