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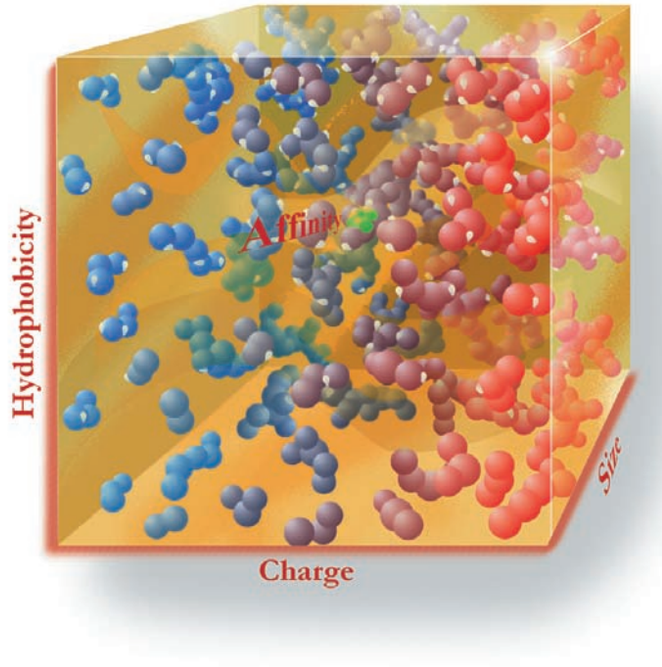
Protein Purification – Handbook

GE Healthcare



Protein Purification

Handbook

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Protein Purification

Handbook

Contents

Introduction

The development of techniques and methods for protein purification has been an essential pre-requisite for many of the advancements made in biotechnology. This handbook provides advice and examples for a smooth path to protein purification. Protein purification varies from simple one-step precipitation procedures to large scale validated production processes. Often more than one purification step is necessary to reach the desired purity. The key to successful and efficient protein purification is to select the most appropriate techniques, optimise their performance to suit the requirements and combine them in a logical way to maximise yield and minimise the number of steps required.
Most purification schemes involve some form of chromatography. As a result chromatography has become an essential tool in every laboratory where protein purification is needed. Different chromatography techniques with different selectivities can form powerful combinations for the purification of any biomolecule.
The development of recombinant DNA techniques has revolutionised the production of proteins in large quantities. Recombinant proteins are often produced in forms which facilitate their subsequent chromatographic purification. However, this has not removed all challenges. Host contaminants are still present and problems related to solubility, structural integrity and biological activity can still exist. Although there may appear to be a great number of parameters to consider, with a few simple guidelines and application of the Three Phase Purification Strategy the process can be planned and performed simply and easily, with only a basic knowledge of the details of chromatography techniques.

Advice codes:


general advice for any purification


advice for large scale purification


advice for micro scale purification


shortcuts


advice on media selection

Chapter 1

Purification Strategies – a simple approach

Apply a systematic approach to development of a purification strategy.

The first step is to describe the basic scenario for the purification. General considerations answer questions such as: What is the intended use of the product? What kind of starting material is available and how should it be handled? What are the purity issues in relation to the source material and intended use of the final product? What has to be removed? What must be removed completely? What will be the final scale of purification? If there is a need for scale-up, what consequences will this have on the chosen purification techniques? What are the economical constraints and what resources and equipment are available?

Most purification protocols require more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. For example, if a yield of 80% in each step is assumed, this will be reduced to only 20% overall yield after 8 processing steps as shown in Figure 1. Consequently, to reach the targets for yield and purity with the *minimum* number of steps and the *simplest* possible design, it is not efficient to add one step to another until purity requirements have been fulfilled. Occasionally when a sample is readily available purity can be achieved by simply adding or repeating steps. However, experience shows that, even for the most challenging applications, high purity and yield can be achieved efficiently in fewer than four well-chosen and optimised purification steps. Techniques should be organised in a logical sequence to avoid the need for conditioning steps and the chromatographic techniques selected appropriately to use as few purification steps as possible.

Limit the number of steps in a purification procedure.

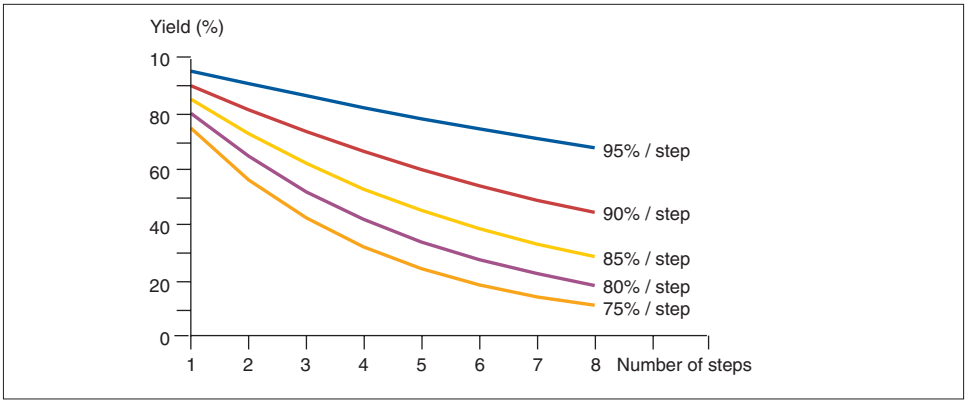


Fig 1. Yields from multistep purifications.

Preparation

The need to obtain a protein, efficiently, economically and in sufficient purity and quantity, applies to every purification. It is important to set *objectives* for purity, quantity and maintenance of biological activity and to define the economical and time framework for the work. All information concerning *properties of the target protein and contaminants* will help during purification development. Some simple experiments to characterise the sample and target molecule are an excellent investment. Development of fast and reliable *analytical assays* is essential to follow the progress of the purification and assess its effectiveness. *Sample preparation* and *extraction procedures* should be developed prior to the first chromatographic purification step.

With background information, assays and sample preparation procedures in place the Three Phase Purification Strategy can be considered.

Three Phase Purification Strategy

Imagine the purification has three phases Capture, Intermediate Purification and Polishing.

In the Three Phase Strategy specific objectives are assigned to each step within the process:

In the *capture* phase the objectives are to *isolate, concentrate and stabilise* the target product.

During the *intermediate purification* phase the objective is to *remove most of the bulk impurities* such as other proteins and nucleic acids, endotoxins and viruses.

In the *polishing* phase the objective is to *achieve high purity* by removing any remaining trace impurities or closely related substances.

The selection and optimum combination of purification techniques for Capture, Intermediate Purification and Polishing is crucial to ensure fast method development, a shorter time to pure product and good economy.

The final purification process should ideally consist of sample preparation, including extraction and clarification when required, followed by three major purification steps, as shown in Figure 2. The number of steps used will always depend upon the purity requirements and intended use for the protein.

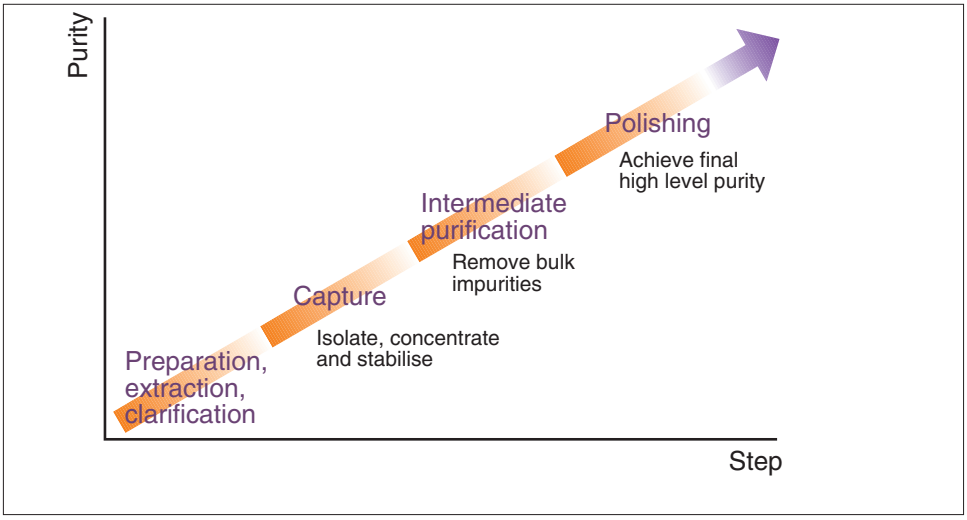


Fig 2. Preparation and the Three Phase Purification Strategy.

Guidelines for Protein Purification

The guidelines for protein purification shown here can be applied to any purification process and are a suggestion as to how a systematic approach can be applied to the development of an effective purification strategy. As a reminder these guidelines will be highlighted where appropriate throughout the following chapters.

Define objectives
for purity, activity and quantity required of final product to avoid over or under developing a method

Define properties of target protein and critical impurities
to simplify technique selection and optimisation

Develop analytical assays
for fast detection of protein activity/recovery and critical contaminants

Minimise sample handling at every stage
to avoid lengthy procedures which risk losing activity/reducing recovery

Minimise use of additives
additives may need to be removed in an extra purification step or may interfere with activity assays

Remove damaging contaminants early
for example, proteases

Use a different technique at each step
to take advantage of sample characteristics which can be used for separation (size, charge, hydrophobicity, ligand specificity)

Minimise number of steps
extra steps reduce yield and increase time, combine steps logically

KEEP IT SIMPLE!

Chapter 2

Preparation

Before You Start

The need to obtain a protein, efficiently, economically and in sufficient purity and quantity, applies to any purification, from preparation of an enriched protein extract for biochemical characterisation to large scale production of a therapeutic recombinant protein. It is important to set *objectives* for purity and quantity, maintenance of biological activity and economy in terms of money and time. Purity requirements must take into consideration the nature of the source material, the intended use of the final product and any special safety issues. For example, it is important to differentiate between contaminants which must be removed and those which can be tolerated. Other factors can also influence the prioritisation of objectives. High yields are usually a key objective, but may be less crucial in cases where a sample is readily available or product is required only in small quantities. Extensive method development may be impossible without resources such as an ÄKTAdesign™ chromatography system. Similarly, time pressure combined with a slow assay turnaround will steer towards less extensive scouting and optimisation. All information concerning *properties* of the target protein and contaminants will help during purification development, allowing faster and easier technique selection and optimisation, and avoiding conditions which may inactivate the target protein.

Development of fast and reliable analytical assays is essential to follow the progress of the purification and assess effectiveness (yield, biological activity, recovery).

Define objectives

Goal: To set minimum objectives for purity and quantity, maintenance of biological activity and economy in terms of money and time.

Define purity requirements according to the final use of the product.

Purity requirement examples are shown below.

Extremely high > 99%	Therapeutic use, in vivo studies	
High 95- 99 %		X-ray crystallography and most physico-chemical characterisation methods
Moderate < 95 %		Antigen for antibody production N-terminal sequencing

Identify 'key' contaminants

Identify the nature of possible remaining contaminants as soon as possible.

The statement that a protein is >95% pure (i.e. target protein constitutes 95% of total protein) is far from a guarantee that the purity is sufficient for an intended application. The same is true for the common statement "the protein was homogenous by Coomassie™ stained SDS-PAGE". Purity of 95% may be acceptable if the remaining 5% consists of harmless impurities. However, even minor impurities which may be biologically active could cause significant problems in both research and therapeutic applications. It is therefore important to differentiate between contaminants which must be removed completely and those which can be reduced to acceptable levels. Since different types of starting material will contain different contaminant profiles they will present different contamination problems.

It is better to over-purify than to under-purify.

Although the number of purification steps should be minimised, the quality of the end product should not be compromised. Subsequent results might be questioned if sample purity is low and contaminants are unknown.

Contaminants which degrade or inactivate the protein or interfere with analyses should be removed as early as possible.

The need to maintain biological activity must be considered at every stage during purification development. It is especially beneficial if proteases are removed and target protein transferred into a friendly environment during the first step.

A downstream production process must achieve the required purity and recovery with complete safety and reliability, and within a given economic framework.

Economy is a very complex issue. In commercial production the time to market can override issues such as optimisation for recovery, capacity or speed. Robustness and reliability are also of great concern since a batch failure can have major consequences.

Special safety issues may be involved in purification of biopharmaceuticals, such as detection or removal of infectious agents, pyrogens, immunogenic contaminants and tumorigenic hazards.

It may be necessary to use analytical techniques targetted towards specific contaminants in order to demonstrate that they have been removed to acceptable levels.

Define properties of target protein and critical impurities

Goal: To determine a 'stability window' for the target protein for easier selection and optimisation of techniques and to avoid protein inactivation during purification.

Check target protein stability window for at least pH and ionic strength.

All information concerning the target protein and contaminant properties will help to guide the choice of separation techniques and experimental conditions for
purification. Database information for the target, or related proteins, may give size, isoelectric point (pI) and hydrophobicity or solubility data. Native one and two dimensional PAGE can indicate sample complexity and the properties of the target protein and major contaminants. Particularly important is a knowledge of the *stability window* of the protein so that irreversible inactivation is avoided. It is advisable to check the target protein stability window for at least pH and ionic strength. Table 1 shows how different target protein properties can affect a purification strategy.

Table 1. Protein properties and their effect on development of purification strategies.

Sample and target protein properties	Influence on purification strategy
Temperature stability	Need to work rapidly at lowered temperature
pH stability	Selection of buffers for extraction and purification Selection of conditions for ion exchange, affinity or reversed phase chromatography
Organic solvents stability	Selection of conditions for reversed phase chromatography
Detergent requirement	Consider effects on chromatographic steps and the need for detergent removal. Consider choice of detergent.
Salt (ionic strength)	Selection of conditions for precipitation techniques, ion exchange and hydrophobic interaction chromatography
Co-factors for stability or activity	Selection of additives, pH, salts, buffers
Protease sensitivity	Need for fast removal of proteases or addition of inhibitors
Sensitivity to metal ions	Need to add EDTA or EGTA to buffers
Redox sensitivity	Need to add reducing agents
Molecular weight	Selection of gel filtration media
Charge properties	Selection of ion exchange conditions
Biospecific affinity	Selection of ligand for affinity medium
Post translational modifications	Selection of group-specific affinity medium
Hydrophobicity	Selection of medium for hydrophobic interaction chromatography

Develop analytical assays

Goal: To follow the progress of a purification, to assess effectiveness (yield, biological activity, recovery) and to help during optimisation.


Select assays which are fast and reliable.

To progress efficiently during method development the effectiveness of each step should be assessed. The laboratory should have access to the following assays:

- A rapid, reliable assay for the target protein
- Purity determination
- Total protein determination
- Assays for impurities which must be removed

The importance of a reliable assay for the target protein cannot be over-emphasised. When testing chromatographic fractions ensure that the buffers used for separation do not interfere with the assay. Purity of the target protein is most often estimated by SDS-PAGE, capillary electrophoresis, reversed phase chromatography or mass spectrometry. Lowry or Bradford assays are used most frequently to determine the total protein.
The Bradford assay is particularly suited to samples where there is a high lipid content which may interfere with the Lowry assay.

For large scale protein purification the need to assay for target proteins and critical impurities is often essential. In practice, when a protein is purified for research purposes, it is too time consuming to identify and set up specific assays for harmful contaminants. A practical approach is to purify the protein to a certain level, and then perform SDS-PAGE after a storage period to check for protease cleavage. Suitable control experiments, included within assays for bio-activity, will help to indicate if impurities are interfering with results.

Sample Extraction and Clarification

Minimise sample handling
Minimise use of additives
Remove damaging contaminants early

Definition: Primary isolation of target protein from source material.
Goal: Preparation of a clarified sample for further purification. Removal of particulate matter or other contaminants which are not compatible with chromatography.

The need for *sample preparation* prior to the first chromatographic step is dependent upon sample type. In some situations samples may be taken directly to the first capture step. For example cell culture supernatant can be applied directly to a suitable chromatographic matrix such as Sepharose™ Fast Flow and may require only a minor adjustment of the pH or ionic strength. However, it is most often essential to perform some form of *sample extraction and clarification* procedure.

If sample extraction is required the chosen technique must be robust and suitable for all scales of purification likely to be used. It should be noted that a technique such as ammonium sulfate precipitation, commonly used in small scale, may be unsuitable for very large scale preparation. Choice of buffers and additives must be carefully considered if a purification is to be scaled up. In these cases inexpensive buffers, such as acetate or citrate, are preferable to the more complex compositions used in the laboratory. It should also be noted that dialysis and other common methods used for adjustment of sample conditions are unsuitable for very large or very small samples.

For repeated purification, use an extraction and clarification technique that is robust and able to handle sample variability. This ensures a reproducible product for the next purification step despite variability in starting material.

Use additives only if essential for stabilisation of product or improved extraction. Select those which are easily removed. Additives may need to be removed in an extra purification step.

Use prepacked columns of Sephadex™ G-25 gel filtration media, for rapid sample clean-up at laboratory scale, as shown in Table 2.

Table 2. Prepacked columns for sample clean-up.

Prepacked column	Sample volume loading per run	Sample volume recovery per run	Code No.
HiPrep™ Desalting 26/10	2.5–15 ml	7.5–20 ml	17-5087-01
HiTrap™ Desalting	0.25–1.5 ml	1.0–2.0 ml	17-1408-01
Fast Desalting PC 3.2/10	0.05–0.2 ml	0.2–0.3 ml	17-0774-01
PD-10 Desalting	1.5–2.5 ml	2.5–3.5 ml	17-0851-01

Sephadex G-25 gel filtration media are used at laboratory and production scale for sample preparation and clarification of proteins >5000. Sample volumes of up to 30%, or in some cases, 40% of the total column volume are loaded. In a single step, the sample is desalted, exchanged into a new buffer, and low molecular weight materials are removed. The high volume capacity, relative insensitivity to sample concentration, and speed of this step enable very large sample volumes to be processed rapidly and efficiently. Using a high sample volume load results in a separation with minimal sample dilution (approximately 1:1.4). Chapter 8 contains further details on sample storage, extraction and clarification procedures.

Sephadex G-25 is also used for sample conditioning, e.g. rapid adjustment of pH, buffer exchange and desalting between purification steps.

 Media for consideration:

Sephadex G 25 gel filtration

For fast group separations between high and low molecular weight substances

Typical flow velocity 60 cm/h (Sephadex G-25 Superfine, Sephadex G-25 Fine), 150 cm/h (Sephadex G-25 Medium).

 **Combine Sample Clean-up and Capture in a single step**

If large sample volumes will be handled or the method scaled-up in the future, consider using STREAMLINE™ expanded bed adsorption. This technique is particularly suited for large scale recombinant protein and monoclonal antibody purification. The crude sample containing particles can be applied to the expanded bed without filtration or centrifugation. STREAMLINE adsorbents are specially designed for use in STREAMLINE columns. Together they enable the high flow rates needed for high productivity in industrial applications of fluidised beds. The technique requires no sample clean up and so combines sample preparation and capture in a single step. Crude sample is applied to an expanded bed of STREAMLINE media. Target proteins are captured whilst cell debris, cells, particulate matter, whole cells, and contaminants pass through. Flow is reversed and the target proteins are desorbed in the elution buffer.

 Media for consideration:

STREAMLINE (IEX, AC, HIC)

For sample clean-up and capture direct from crude sample.

STREAMLINE adsorbents are designed to handle feed directly from both fermentation homogenate and crude feedstock from cell culture/fermentation at flow velocities of 200 - 500 cm/h, according to type and application.

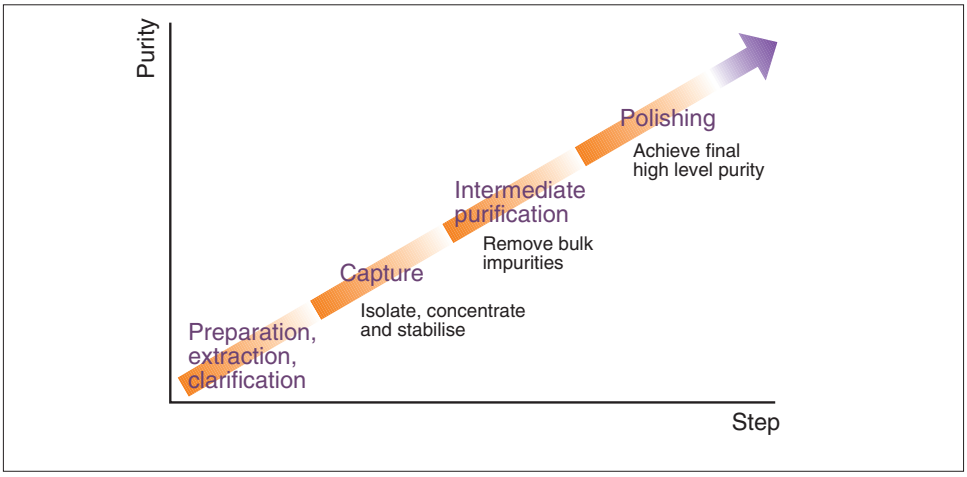
Note: *cm/h: flow velocity (linear flow rate) = volumetric flow rate/cross sectional area of column.*

Chapter 3

Three Phase Purification Strategy

Principles

With background information, assays, and sample preparation and extraction procedures in place the Three Phase Purification Strategy can be applied (Figure 3). This strategy is used as an aid to the development of purification processes for therapeutic proteins in the pharmaceutical industry and is equally efficient as an aid when developing purification schemes in the research



laboratory.

Fig 3. Preparation and the Three Phase Purification Strategy.

Assign a specific objective to each step within the purification process.

In the Three Phase Strategy a specific objective is assigned to each step. The purification problem associated with a particular step will depend greatly upon the properties of the starting material. Thus, the objective of a purification step will vary according to its position in the process, i.e. at the beginning for *isolation* of product from crude sample, in the middle for *further purification* of partially purified sample, or at the end for *final clean up* of an almost pure product.

The Three Phase Strategy ensures faster method development, a shorter time to pure product and good economy.

In the *capture phase* the objectives are to *isolate, concentrate and stabilise* the target product. The product should be concentrated and transferred to an environment which will conserve potency/activity. At best, significant removal of

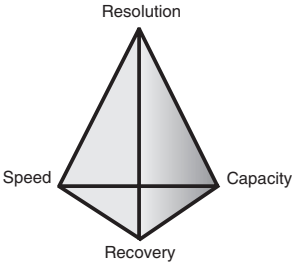
other critical contaminants can also be achieved.
During the *intermediate purification phase* the objectives are to *remove most of the bulk impurities,* such as other proteins and nucleic acids, endotoxins and viruses.

In the *polishing phase* most impurities have already been removed except for trace amounts or closely related substances. The objective is to *achieve final purity.*

It should be noted that this Three Phase Strategy does not mean that all strategies must have three purification steps. For example, capture and intermediate purification may be achievable in a single step, as may intermediate purification and polishing. Similarly, purity demands may be so low that a rapid capture step is sufficient to achieve the desired result, or the purity of the starting material may be so high that only a polishing step is needed. For purification of therapeutic proteins a fourth or fifth purification step may be required to fulfil the highest purity and safety demands.

The optimum selection and combination of purification techniques for *Capture, Intermediate Purification and Polishing* is crucial for an efficient purification process.

Selection and Combination of Purification Techniques



Every technique offers a balance between resolution, capacity, speed and recovery.

Minimise sample handling
Minimise number of steps
Use different techniques at each step

Goal: Fastest route to a product of required purity.

For any chromatographic separation each different technique will offer different performance with respect to recovery, resolution, speed and capacity. A technique can be optimised to focus on one of these parameters, for example resolution, or to achieve the best balance between two parameters, such as speed and capacity. A separation optimised for one of these parameters will produce results quite different in appearance from those produced using the same technique, but focussed on an alternative parameter. See, for example, the results shown on page 49 where ion exchange is used for a capture and for a polishing step.

Select a technique to meet the objectives for the purification step.

Capacity, in the simple model shown, refers to the amount of target protein loaded during purification. In some cases the amount of sample which can be loaded may be limited by volume (as in gel filtration) or by large amounts of contaminants rather than the amount of the target protein.

Speed is of the highest importance at the beginning of a purification where contaminants such as proteases must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery is influenced by destructive processes in the sample and unfavourable conditions on the column.

Resolution is achieved by the selectivity of the technique and the efficiency of the chromatographic matrix to produce narrow peaks. In general, resolution is most difficult to achieve in the final stages of purification when impurities and target protein are likely to have very similar properties.

Every technique offers a balance between resolution, speed, capacity and recovery and should be selected to meet the objectives for each purification step. In general, optimisation of any one of these four parameters can only be achieved at the expense of the others and a purification step will be a compromise. The importance of each parameter will vary depending on whether a purification step is used for capture, intermediate purification or polishing. This will steer the optimisation of the critical parameters, as well as the selection of the most suitable media for the step.

Proteins are purified using chromatographic purification techniques which separate according to differences in specific properties, as shown in Table 3.

Table 3. Protein properties used during purification.

Protein property	Technique
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)
Charge, ligand specificity or hydrophobicity	Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC

Choose logical combinations of purification techniques based on the main benefits of the technique and the condition of the sample at the beginning or end of each step.

Minimise sample handling between purification steps by combining techniques to avoid the need for sample conditioning.

A guide to the suitability of each purification technique for the stages in the Three Phase Purification Strategy is shown in Table 4.

Technique	Main features	Capture	Intermediate	Polish	Sample Start condition	Sample End condition
IEX	high resolution high capacity high speed	★★★	★★★	★★★	low ionic strength sample volume not limiting	high ionic strength or pH change concentrated
HIC	good resolution good capacity high speed	★★	★★★	★	high ionic strength sample volume not limiting	low ionic strength concentrated
AC	high resolution high capacity high speed	★★★	★★★	★★	specific binding conditions sample volume not limiting	specific elution conditions concentrated
GF	high resolution using Superdex™		★	★★★	limited sample volume (<5% total column volume) and flow rate range	buffer exchanged (if required) diluted
RPC	high resolution		★	★★★	requires organic solvents	in organic solvent, risk loss of biological activity concentrated

Table 4. Suitability of purification techniques for the Three Phase Purification Strategy

Avoid additional sample conditioning steps.

The product should be eluted from the first column in conditions suitable for the start conditions of the next column.

The start conditions and end conditions for the techniques are shown in Table 4. For example, if the sample has a low ionic strength it can be applied to an IEX column. After elution from IEX the sample will usually be in a high ionic strength buffer and can be applied to a HIC column (if necessary the pH can be adjusted and further salt can be added). In contrast, if sample is eluted from a HIC column, it is likely to be in high salt and will require dilution or a buffer exchange step in order to further decrease the ionic strength to a level suitable for IEX. Thus it is more straightforward to go from IEX to HIC than vice-versa.

Ammonium sulfate precipitation is a common sample clarification and concentration step at laboratory scale and in this situation HIC (which requires high salt to enhance binding to the media) is ideal as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a Sephadex G-25 desalting column will prepare it for the next IEX or AC step.

GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC) since the target protein will be eluted in a reduced volume and the components from the elution buffer will not affect the gel filtration separation (gel filtration is a non-binding technique with limited volume capacity and unaffected by buffer conditions).

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 4.

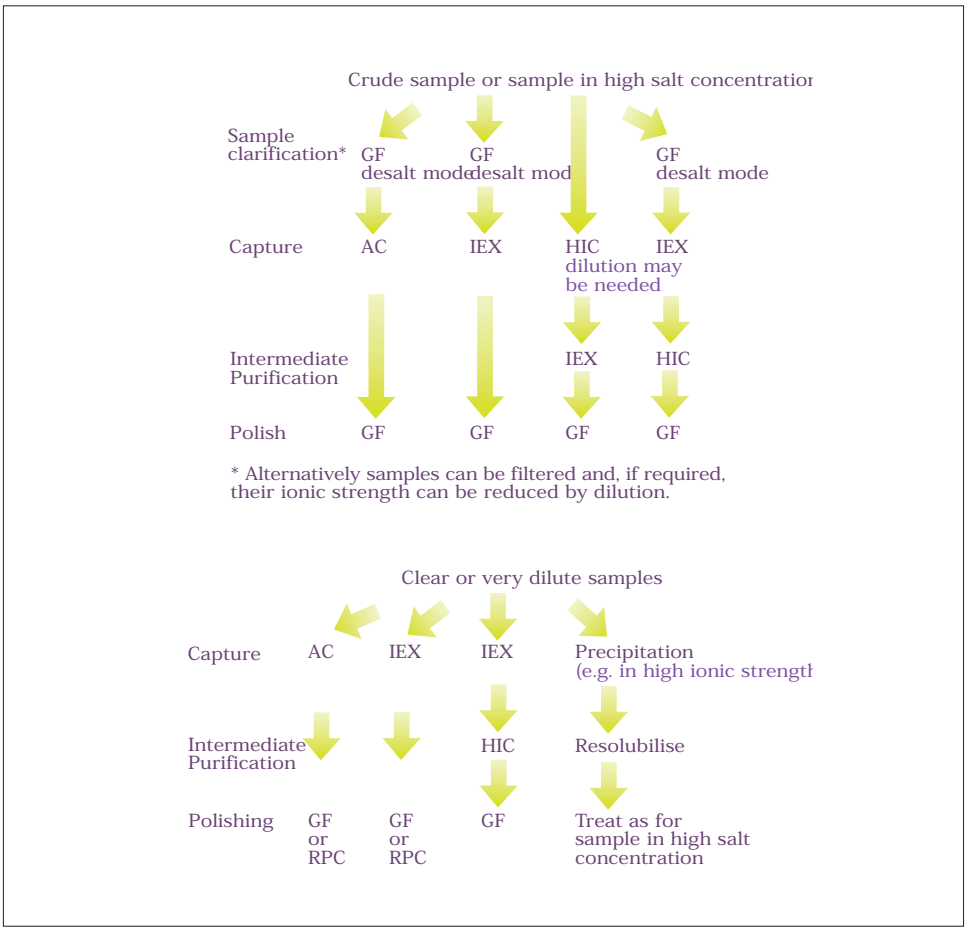


Fig 4. Logical combinations of chromatographic steps.

For any capture step, select the technique showing the strongest binding to the target protein while binding as few of the contaminants as possible, i.e. the technique with the highest selectivity and/or capacity for the protein of interest.

A sample is purified using a combination of techniques and alternative selectivities. For example, in an IEX-HIC-GF Three Phase Strategy the capture step selects according to differences in charge (IEX), the intermediate purification step according to differences in hydrophobicity (HIC) and the final polishing step according to differences in size (GF). Figure 5 shows a standard Three Phase strategy purification.

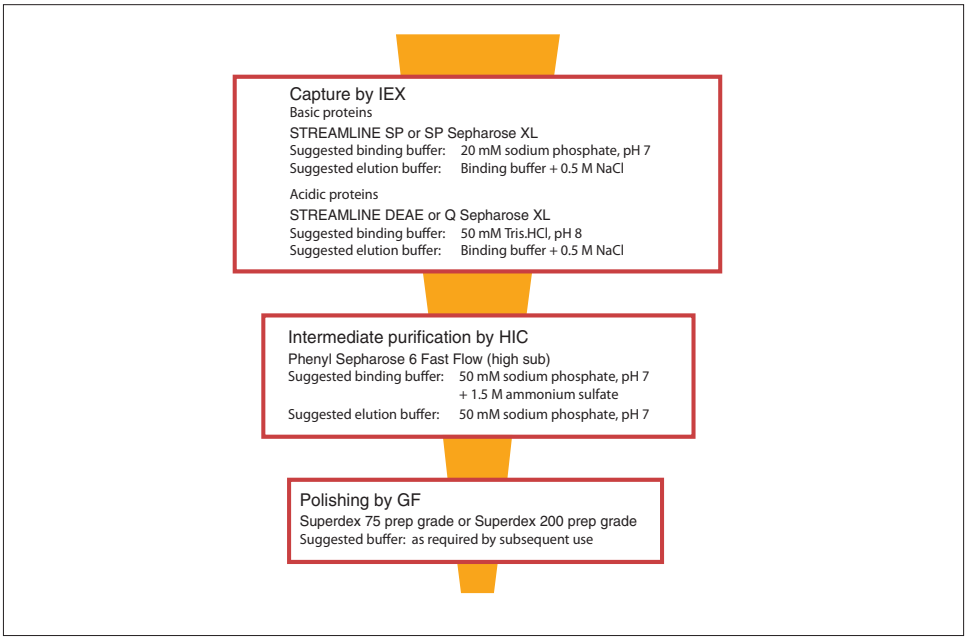


Fig 5. A standard purification protocol.

If nothing is known about the target protein use IEX-HIC-GF. This combination of techniques can be regarded as a standard protocol.

Consider the use of both anion and cation exchange chromatography to give different selectivities within the same purification strategy.

IEX is a technique which offers different selectivities using either anion or cation exchangers. The pH of the separation can be modified to alter the charge characteristics of the sample components. It is therefore possible to use IEX more than once in a purification strategy, for capture, intermediate purification or polishing. IEX can be used effectively both for rapid separation in low resolution mode during capture, and in high resolution mode during polishing in the same purification scheme. Figure 6 shows an example for the purification of cellulase in which advantage is taken of the different selectivities of anion and cation exchange to create a simple two step process.

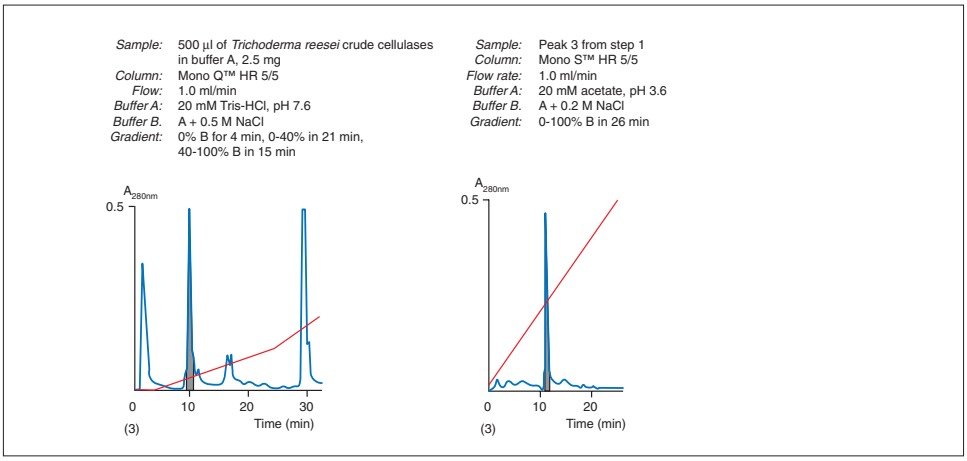


Fig 6. Two step purification of a cellulase.

Consider RPC for a polishing step provided that the target protein can withstand the run conditions.

Reversed phase chromatography (RPC) separates proteins and peptides on the basis of hydrophobicity. RPC is a high selectivity (high resolution) technique, requiring the use of organic solvents. The technique is widely used for purity check analyses when recovery of activity and tertiary structure are not essential. Since many proteins are denatured by organic solvents, the technique is not generally recommended for protein purification where recovery of activity and return to a correct tertiary structure may be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be excellent, particularly for small target proteins which are often not denatured by organic solvents.

If a purification is not intended for scale up (i.e. only milligram quantities of product are needed), use high performance, prepacked media such as Sepharose High Performance (IEX, HIC), SOURCE™ (IEX, HIC), MonoBeads™ (IEX), or Superdex (GF) for all steps.

Recommended media for a standard protocol

Purification step	Media	Quantity	Code No.
Capture	STREAMLINE SP	300 ml	17-0993-01
Capture	STREAMLINE DEAE	300 ml	17-0994-01
Capture	HiPrep 16/10 SP XL	1 column	17-5093-01
Capture	HiPrep 16/10 Q XL	1 column	17-5092-01
Intermediate purification	HiPrep 16/10 Phenyl FF (high sub)	1 column	17-5095-01
Polishing	HiLoad™ 16/60 Superdex 75 prep grade	1 column	17-1068-01
Polishing	HiLoad 16/60 Superdex 200 prep grade	1 column	17-1069-01
Sample clarification/conditioning	Prepacked PD-10 Column	30 columns	17-0851-01
Sample clarification/conditioning	HiTrap Desalting	5 columns	17-1408-01
Sample clarification/conditioning	HiPrep 26/10 Desalting	1 column	17-5087-01

Sample Conditioning

Although additional sample handling between purification steps should be avoided, it may be necessary to adjust the buffer conditions of an eluted product (pH, ionic strength and/or buffering ions) to ensure compatibility with the following purification technique.

Sephadex G-25 is an ideal media for *rapid desalting and pH adjustment* by buffer exchange between purification steps. Sample volumes of up to 30%, or in some cases 40%, of the total column volume are loaded. In a single step, the sample is desalted, exchanged into a new buffer, and low molecular weight materials are removed. Figure 7 shows a typical desalt/buffer exchange separation. The high volume capacity and speed of this step enable very large sample volumes to be processed rapidly and efficiently. The high sample volume load results in a separation with minimal sample dilution. Sephadex G-25 is also used for rapid sample clean-up at laboratory scale.

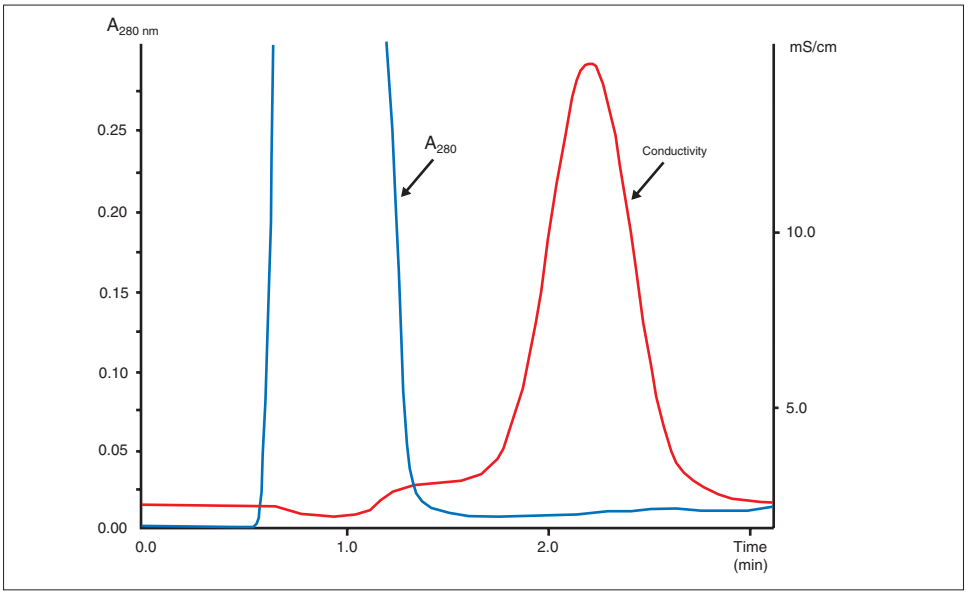


Fig 7. Buffer exchange of mouse plasma on HiPrep 26/10 Desalting.

Use prepacked columns of Sephadex G-25 for rapid sample conditioning at laboratory scale, as shown in Table 5.

Table 5. Prepacked columns for rapid desalting and buffer exchange.

Prepacked column	Sample volume loading per run	Sample volume recovery per run	Code No.
HiPrep Desalting 26/10	2.5 -15 ml	7.5 - 20 ml	17-5087-01
HiTrap Desalting	0.25 - 1.5 ml	1.0 - 2.0 ml	17-1408-01
Fast Desalting PC 3.2/10	0.05 - 0.2 ml	0.2 - 0.3 ml	17-0774-01
PD-10 Desalting	1.5 - 2.5 ml	2.5 - 3.5 ml	17-0851-01

Dilution can be used as an alternative to desalting before application to an ion exchange column.

Media for consideration:

Sephadex G-25 Gel filtration

For fast group separations between high and low molecular weight substances.

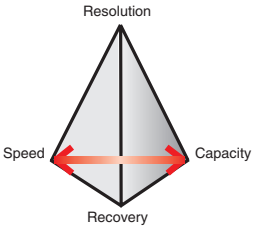
Typical flow velocities 60 cm/h (Sephadex G-25 Superfine, Sephadex G-25 Fine), 150 cm/h (Sephadex G-25 Medium).

In the following chapters Capture, Intermediate Purification and Polishing are discussed in more detail.

Note: *cm/h: flow velocity (linear flow rate) = volumetric flow rate/cross sectional area of column.*

Chapter 4

Capture



Remove damaging contaminants early

Definition: Initial purification of the target molecule from crude or clarified source material.
Goals: Rapid isolation, stabilisation and concentration.

Use a high capacity, concentrating technique to reduce sample volume, to enable faster purification and to allow the use of smaller columns.

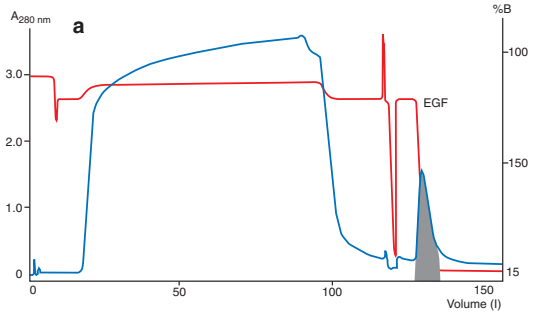
Focus on robustness and simplicity in the first purification step. Do not try to solve all problems in one step when handling crude material.

In the capture phase, the objective is to isolate, concentrate and stabilise the target product efficiently by optimising speed and capacity. The product is concentrated and transferred to an environment which will conserve activity. Capture is often a group separation using a step elution on ion exchange or affinity chromatography. Ideally, removal of critical contaminants is also achieved. It is sometimes possible to achieve a high level of purification if a highly selective affinity media is used.

Binding capacity for the protein in the presence of the impurities will be one of the most critical parameters to optimise and reduce the scale of work. For example, when ion exchange chromatography is used as a capture step, the goal is to adsorb the target protein quickly from the crude sample and isolate it from critical contaminants such as proteases and glycosidases. Conditions are selected to avoid binding of contaminants so that the capacity for the target protein is maximised. High speed may be required to reduce sample application time, particularly if proteolysis or other destructive effects threaten the integrity of the target protein.

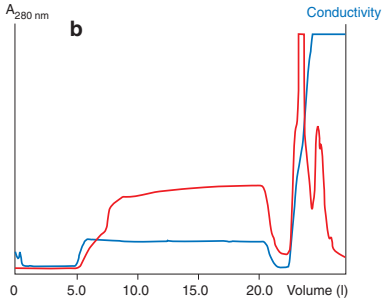
Transfer to a step elution during method development to increase speed and capacity of the capture step.

The most common technique for a capture step is ion exchange chromatography (IEX) which has high binding capacity. IEX media are resistant to harsh cleaning conditions which may be needed after purification of crude samples. Typically proteins are eluted from an IEX column using a salt gradient. However, during method development, a transfer to a step elution will give a simple, robust separation with a shorter run time and decreased buffer consumption. It is often possible to use high sample loadings since the focus is not on resolution (high sample loadings will decrease resolution). High speed and capacity and low buffer consumption are particularly advantageous for large scale purification, as shown in Figure 8.



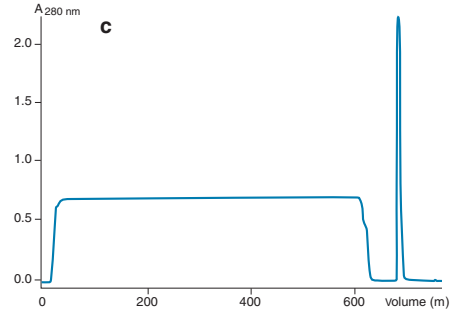
Column: BPG™ 300/500 packed with Phenyl Sepharose 6 Fast Flow (high sub)
Sample: EGF in yeast supernatant ammonium sulfate added to 0.5 M
Sample load: 80 L containing 2.56 g EGF
Starting buffer: 20 mM sodium phosphate, pH 7.0 + 0.5 M ammonium sulfate
Elution buffer: 20 mM sodium phosphate, pH 7.0
Flow loading: 210 L/h, 300 cm/h

a) Purification of recombinant epidemal growth factor (EGF) - capture step.



Column: INdEX™ 70 (70 mm i.d.)
Adsorbent: Q Sepharose XL, 385 mL bed volume
Sample: Recombinant α-amylase produced in *E. coli*, homogenized, 2.2 L diluted in distilled water to 15.4 L, 7.2 mS/cm, 10 mM $CaCl_2$, centrifuged
Buffer A: 20 mM Tris-HCl, pH 8, 10 mM $CaCl_2$
Buffer B: 20 mM Tris-HCl, pH 8, 1 M NaCl, 10 mM $CaCl_2$
Flow: 300 cm/h, 12 L/h
Gradient: 20 bed volumes 0-1 M NaCl
Eluate: 1.48 L, 3.8 bed volumes
Spec. act. α-amylase: 6420 U/L

b) Pilot scale purification of recombinant α-amylase from *E. coli* - capture step.



Column: rProtein A Sepharose Fast Flow, XK 16/20, bed height 4.8 cm (9.6 mL)
Sample: clarified cell culture containing IgG_{2a}
Sample volume: 600 mL containing 87.6 mg IgG_{2a}
Starting buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 20 mM sodium citrate, pH 4.0
Flow: 5 mL/min (150 cm/h)

c) Purification of IgG_{2a} from clarified cell culture - capture step.

Fig. 8. Examples of capture steps.

For large scale capture, throughput will often be the focus during method development. It is important to consider all aspects: sample extraction and clarification, sample loading capacity, flow rate during equilibration, binding, washing, elution and cleaning, and the need for cleaning-in-place procedures.

In principle, a capture step is designed to maximise capacity and/or speed at the expense of some resolution. However, there is usually significant resolution and purification from molecules which have significant physicochemical differences compared to the target protein. Recovery will be of concern in any preparative situation, especially for production of a high value product, and it is important to assay for recovery during optimisation of the capture step. Examples of capture steps are shown on page 30.

Media for capture steps should offer high speed and high capacity.

Sepharose XL (IEX)

For capture steps handling crude mixtures at laboratory and process scale. Fast removal and a combination of high capacity and good resolution at high flow rates are the main characteristics. Recommended flow velocity is 100-500 cm/h.

Particle size: 90 µm. Available in prepacked columns and as bulk media.

Sepharose Big Beads (IEX)

For capture steps handling viscous samples or very large sample volumes. Sepharose Big Beads are for the capture step in processes where high sample viscosity precludes the use of ion exchange media with smaller bead sizes. Recommended flow velocity is up to 300 cm/h. This medium should be chosen when fast adsorption is required and resolution is of less importance. The flow characteristics of Big Beads may also be useful when processing very large volumes under conditions requiring an extremely high volumetric throughput. Flow velocities in these situations can exceed 1000 cm/h.

Particle size: 200 µm. Available as bulk media.

STREAMLINE (IEX, AC, HIC)

For sample clean-up and capture direct from crude sample. STREAMLINE adsorbents are designed to handle feed directly from both fermentation homogenate and crude feedstock from cell culture/fermentation at flow velocities of 200 - 500 cm/h, according to type and application.

Particle size: 200 µm. Available as bulk media.

Other media for consideration:

Sepharose Fast Flow (IEX, HIC)

These media offer the widest range of selectivities and an excellent alternative for purification of crude samples at any scale. They offer a fast separation combined with good resolution. Recommended flow velocity is 100-300 cm/h.

Particle size: 90 µm. Available in prepacked columns and as bulk media.

Note: *cm/h: flow velocity (linear flow rate) = volumetric flow rate/cross sectional area of column.*

If a purification is not intended for scale up (i.e. milligram quantities of product are needed), use high performance media such as Sepharose High Performance (IEX, HIC) or MonoBeads (IEX), or SOURCE (IEX, HIC). All these media are available in prepacked columns.

For microscale purification use MonoBeads or MiniBeads™ (IEX), Phenyl Superose™ (HIC) or NHS-activated Superose (AC) columns.

For 'one time' purification or with a readily available sample, sacrifice yield for purity by taking a narrow cut from a chromatographic peak during the first purification step.

Use HiTrap Ion Exchange and HiTrap HIC Test Kits for media screening and simple method optimisation.

If the starting material is reasonably clean, a single step purification on highest resolution MonoBeads (IEX) may be sufficient to achieve required purity at laboratory scale.

If a biospecific ligand is available, consider using affinity chromatography as the capture step. If the media is to be used routinely, ensure that any contaminants from the crude sample can be removed by column regeneration procedures which do not damage the affinity ligand. AC will give a highly selective capture step to improve resolution from contaminants, but speed may need to be reduced to maintain a high binding capacity.

If the starting material is reasonably clean a single step purification on a prepacked HiTrap affinity column may be sufficient to achieve required purity at the milligram scale, as shown in Figure 9. HiTrap affinity columns are available in a wide range of selectivities (see Table 6, page 34).

If the starting material is concentrated, has a low volume and there is no intention to scale up, Superdex gel filtration media can offer a mild first step, requiring little or no optimisation. Conversely, gel filtration is not suitable in a typical capture step where the sample volume is large or will be scaled up.

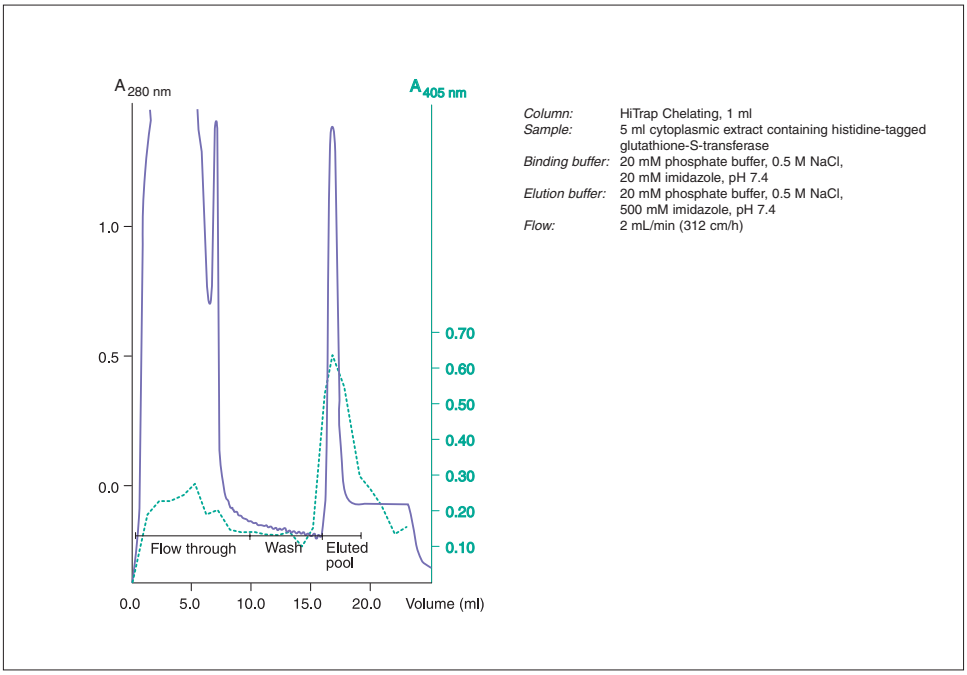


Fig 9. HiTrap Chelating column used to purify histidine-tagged glutathione-S-transferase from cytoplasmic extract.

Table 6. Recommended HiTrap affinity columns for laboratory scale separation.

Application	HiTrap column	Code No.	Quantity/ components	Approximate binding capacity per ml gel
Isolation of immunoglobulins IgG classes, fragments and subclasses	HiTrap rProtein A	17-5079-01 17-5080-01 17-5029-02	5 x 1 ml 1 x 5 ml 2 x 1 ml	human IgG 50 mg/ml
IgG classes, fragments and subclasses	HiTrap Protein A	17-0402-01 17-0402-03 17-0403-01	5 x 1 ml 2 x 1 ml 1 x 5 ml	human IgG 20 mg/ml
IgG classes, fragments and subclasses including human IgG_3 strong affinity for monoclonal mouse IgG_1 and rat IgG	HiTrap Protein G	17-0404-01 17-0404-03 17-0405-01	5 x 1 ml 2 x 1 ml 1 x 5 ml	human IgG 25 mg/ml
Monoclonal and polyclonal IgG from ascites fluid, serum and cell culture supernatant	MAbTrap™ GII	17-1128-01	HiTrap Protein G column (1 ml), accessories, pre-made buffers for 10 purifications	as above
Mouse recombinant Single chain antibody Fragment variable (ScFv) produced in *E. coli*	RPAS Purification Module	17-1362-01	HiTrap Anti-E column, accessories, pre-made buffers for 20 purifications	0.17 mg ScFv/5 ml
IgY antibodies from egg yolk	HiTrap IgY Purification	17-5111-01	1 x 5 ml	IgY 20 mg/ml
IgM	HiTrap IgM Purification	17-5110-01	5 x 1 ml	IgM 5 mg/ml

Application	HiTrap column	Code No.	Quantity/ components	Approximate binding capacity per ml gel
Group Specific Media	HiTrap Blue	17-0412-01	5 x 1 ml	
Various Nucleotide-requiring enzymes, coagulation factors, DNA binding proteins, α_2-macroglobulin		17-0413-01	1 x 5 ml	HSA 20 mg/ml
Proteins and peptides with exposed amino acids: His (Cys, Trp) e.g. α_2-macroglobulin and interferon	HiTrap Chelating	17-0408-01	5 x 1 ml	
		17-0409-01	1 x 5 ml	histidine-tagged protein (27.6 kD) 12 mg /ml
Histidine-tagged fusion proteins	HisTrap™	17-1880-01	HiTrap Chelating column (1 ml), accessories, pre-made buffers	as above
Biotin and biotinylated substances	HiTrap Streptavidin	17-5112-01	5 x 1 ml	biotinylated BSA 6 mg/ml
Coagulation factors, lipoprotein lipases, steroid receptors, hormones, DNA binding proteins, interferon, protein synthesis factors	HiTrap Heparin	17-0406-01	5 x 1 ml	
		17-0407-01	1 x 5 ml	ATIII (bovine) 3 mg/ml
Matrix for preparation of affinity media Coupling of primary amines	HiTrap NHS-activated	17-0716-01	5 x 1 ml	
		17-0717-01	1 x 5 ml	ligand specific

Recommended separation conditions
All HiTrap columns are supplied with a detailed protocol to ensure optimum results
Maximum flow rates: HiTrap 1 ml column: up to 4 ml/min
HiTrap 5 ml column: up to 20 ml/min

For crude, large volume samples containing particles, consider using STREAMLINE expanded bed adsorption

STREAMLINE expanded bed adsorption is particularly suited for large scale recombinant protein and monoclonal antibody purification. STREAMLINE adsorbents are specially designed for use in STREAMLINE columns. The technique requires no sample clean up and so combines sample preparation and capture in a single step. As shown in Figure 10, crude sample is applied to an expanded bed of STREAMLINE media, target proteins are captured whilst cell debris, particulate matter, whole cells, and contaminants pass through. Flow is reversed and the target protein is desorbed in the elution buffer.

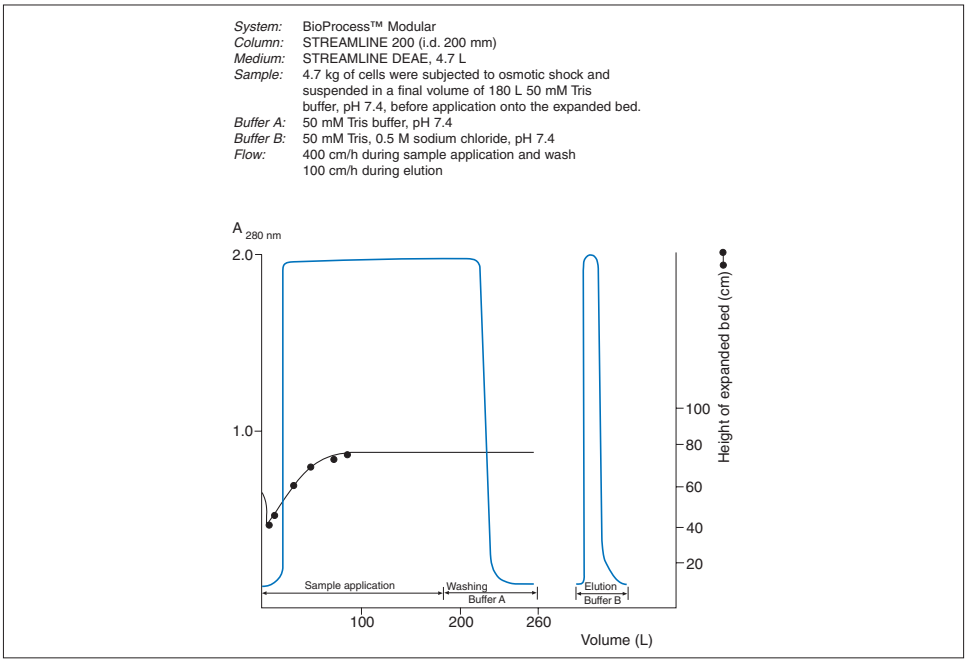
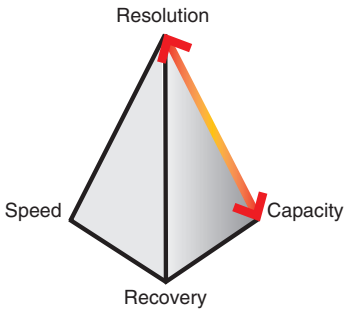


Fig. 10. Purification of a recombinant protein *Pseudomonas aeruginosa* exotoxin A - capture step.

See Chapter 3, page 23 for suggested logical combinations of techniques for Capture, Intermediate Purification and Polishing.

Chapter 5

Intermediate Purification



Use different techniques at each step
Minimise number of steps

Definition: Further removal of bulk contaminants.
Goal: Purification and concentration.

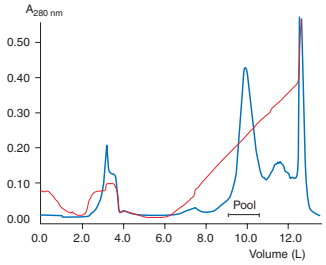
In the intermediate purification phase the focus is to separate the target protein from most of the bulk impurities such as other proteins, nucleic acids, endotoxins and viruses. The ability to resolve similar components is of increased importance. The requirements for resolution will depend upon the status of the sample produced from the capture step and the purity requirements for the final product. Capacity will still be important to maintain productivity.
Speed is less critical in intermediate purification since the impurities causing proteolysis or other destructive effects should have been removed, and sample volume should have been reduced, in the capture step.
The optimal balance between capacity and resolution must be defined for each specific application. This then decides how the separation conditions should be optimised during method development.

The technique must give a high resolution separation. Elution by a continuous gradient will usually be required.

As in a capture step, selectivity during sample adsorption will be important, not only to achieve high binding capacity, but also to contribute to the purification by achieving a further separation during sample application. However, in contrast to a capture step, selectivity during sample desorption from the column is also important and is usually achieved by applying a more selective desorption principle, such as a continuous gradient or a multistep elution procedure, as shown in Figure 11. Examples of Intermediate Purification steps are shown on page 38.

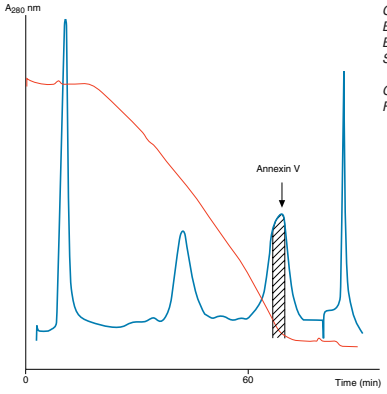
Use a technique with a complementary selectivity to that which was used for the capture step.

a) Purification of recombinant protein *Pseudomonas aeruginosa* exotoxin A -intermediate purification step.



Column: FineLINE™ 100 (i.d. 100 mm)
Medium: SOURCE 30Q, 375 mL (50 mm bed height)
Sample: from the previous pool, diluted 1 to 3 with distilled water 1.5 L/cycle were applied
Buffer A: 20 mM phosphate, pH 7.4
Buffer B: Buffer A + 1.0 M sodium chloride
Gradient: 0 to 50% B, 20 column volumes
Flow: 600 cm/h

b) Purification of recombinant Annexin V - intermediate purification step.



Column: XK 16/20 Butyl Sepharose 4 Fast Flow
Buffer A: 20 mM Sodium phosphate, pH 7.0, 1 M $(NH_4)_2SO_4$
Buffer B: 20 mM Sodium phosphate, pH 7.0,
Sample: Partially purified Annexin V expressed in *E. coli*, 5 ml
Gradient: 0 to 50% B, 20 column volumes
Flow: 100 cm/h

Fig 11. Intermediate purification steps.

Media for intermediate purification should offer high capacity and high resolution with a range of complementary selectivities.

SOURCE (IEX)

For fast, high resolution and high capacity intermediate purification.
SOURCE media are for high throughput, high capacity and high resolution purification. Frequently, if filtered samples are used, the intermediate purification step can be combined with the capture step. A flow velocity up to 2000 cm/h is possible. SOURCE 30 is also a good choice at large scale for intermediate purification.

Particle size: 15 µm. Available in prepacked columns and as bulk media.
Particle size: 30 µm. Available as bulk media.

Sepharose High Performance (IEX, HIC, AC)

For high resolution and high capacity intermediate purification.
These media are ideal for intermediate purification at large scale and should be used when resolution and capacity are a priority. Recommended flow velocity is up to 150 cm/h.

Particle size: 34 µm. Available in prepacked columns and as bulk media.

Sepharose Fast Flow (IEX, HIC, AC)

Proven in large scale production of pharmaceuticals during intermediate purification steps.
These media are the accepted standard for general applications in the laboratory and at large scale. They are available in the widest range of techniques and selectivities and are able to withstand harsh cleaning-in-place conditions. They offer a fast separation combined with good resolution. Recommended flow velocity is 100-300 cm/h.

Particle size: 90 µm. Available in prepacked columns and as bulk media.
Note: *cm/h: flow velocity (linear flow rate) = volumetric flow rate/cross sectional area of column.*

Use HiTrap IEX, HiTrap HIC and RESOURCE™ HIC Test Kits for media screening and simple method optimisation. See the table below.

Kit	Code No.
HiTrap IEX Test Kit	17-6001-01
HiTrap HIC Test Kit	17-1349-01
RESOURCE™ HIC Test Kit	17-1187-01

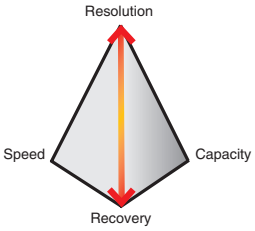
If a purification is not intended for scale up (i.e. only milligram quantities of product are needed), use high performance media such as Sepharose High Performance (IEX, HIC) MonoBeads (IEX) or SOURCE 15 (IEX, HIC).

For microscale purification use MonoBeads, MiniBeads (IEX) or Phenyl Superose PC (HIC) columns.

See Chapter 3, page 23 for suggested logical combinations of techniques for Capture, Intermediate Purification and Polishing.

Chapter 6

Polishing



Use different techniques at each stage

Definition: Final removal of trace contaminants. Adjustment of pH, salts or additives for storage.
Goal: End product of required high level purity.

In the polishing phase the focus is almost entirely on high resolution to achieve final purity. Most contaminants and impurities have already been removed except for trace impurities such as leachables, endotoxins, nucleic acids or viruses, closely related substances such as microheterogeneous structural variants of the product, and reagents or aggregates. To achieve resolution it may be necessary to sacrifice sample load or even recovery (by peak cutting).

Recovery of the final product is also a high priority and a technique must be selected which ensures the highest possible recovery. Product losses at this stage are more costly than in earlier stages. Ideally the product should be recovered in buffer conditions ready for the next procedure.

The technique chosen must discriminate between the target protein and any remaining contaminants

The high resolution required to achieve this discrimination is not always reached by using a high selectivity technique alone, but usually requires selection of a high efficiency medium with small, uniform bead sizes.

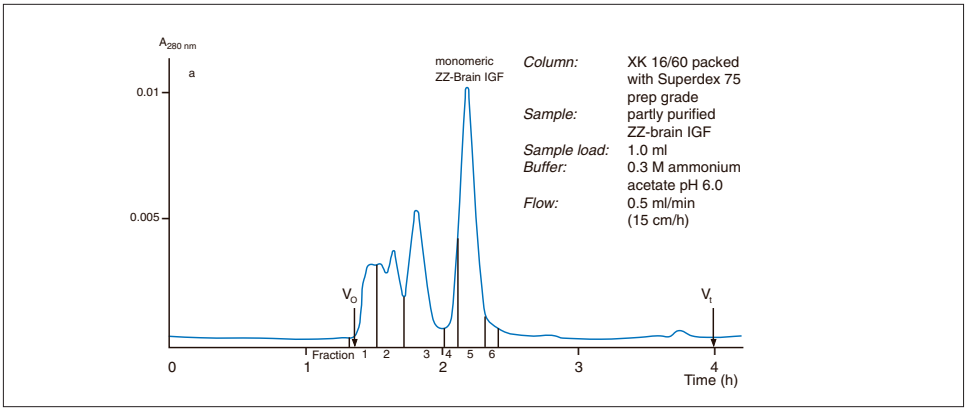


Fig 12. Separation of dimers and multimers -polishing step.

Typically, separations by charge, hydrophobicity or affinity will have already been used so that high resolution gel filtration is ideal for polishing. The product is purified and transferred into the required buffer in one step and dimers or aggregates can often be removed, as shown in Figure 12.

To remove contaminants of similar size, an alternative high resolution technique using elution with shallow gradients is usually required, as shown in Figure 13.

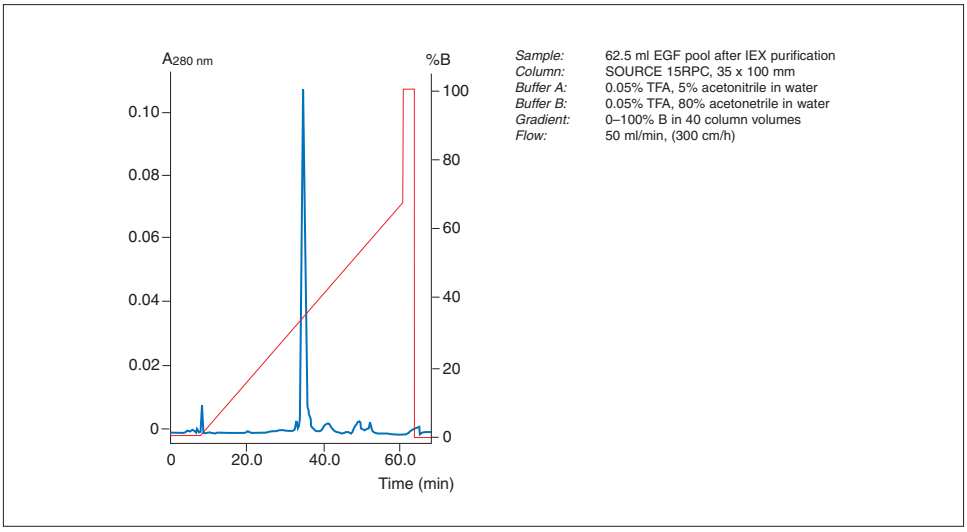


Fig 13. Final polishing step of recombinant epidermal growth factor, using reversed phase chromatography. Method developed on prepacked RESOURCE RPC and scaled up on SOURCE 15RPC.

Gel filtration is also the slowest of all chromatography techniques and the size of the column determines the volume of sample that can be applied. It is therefore most logical to use gel filtration after techniques which reduce sample volume so that smaller columns can be used.

When scaling up a purification it is important to verify that the high resolution achieved from the laboratory scale polishing step is maintained when applying preparative sample volumes to large scale columns.

Media for polishing steps should offer the highest possible resolution.

Superdex (GF)

High productivity gel filtration media for polishing.
Superdex media are high resolving gel filtration media for short run times and good recovery. Superdex is the first choice at laboratory scale and Superdex prep grade for large scale applications. Typical flow velocity is up to 75 cm/h.

Particle size Superdex: 13 µm. Available in prepacked columns.
Particle size Superdex prep grade: 34 µm. Available in prepacked columns and as bulk media.

MonoBeads (IEX)

Media for polishing at laboratory scale when highest resolution is essential.
These media offer high capacity and high resolution separations at laboratory scale. Typical flow velocity is 150-600 cm/h.

Particle size: 10 µm. Available in prepacked columns.

SOURCE 15 (IEX, HIC, RPC)

Media for rapid high resolution polishing.
SOURCE 15 are for rapid high capacity, high resolution separations for laboratory and large scale applications. The pore structure of these media enables resolution to be maintained at high loading and high flow rates. Recommended flow velocity is 150-1800 cm/h.

Particle size: 15µm. Available in prepacked columns and as bulk media.

Other media for consideration.

SOURCE 30 (IEX)

SOURCE 30 media are for high throughput, high capacity and high resolution purification. However, these media can be an alternative choice for polishing offering a flow velocity of up to 2000 cm/h at large scale.

Particle size: 30 µm. Available as bulk media.

Note: *cm/h: flow velocity (linear flow rate) = volumetric flow rate/cross sectional area of column.*

For microscale purification use Superdex PC (GF), MiniBeads (IEX) or Phenyl Superose PC (HIC) columns.

See Chapter 3, page 23 for suggested logical combinations of techniques for Capture, Intermediate Purification and Polishing.

Chapter 7

Examples of Protein Purification Strategies

The Three Phase Purification Strategy has been successfully applied to many purification schemes from simple laboratory scale purification to large, industrial scale production. Examples highlighted in this chapter demonstrate applications in which a standard protocol was applied, i.e. sample extraction and clarification, capture, intermediate purification and polishing. There are also examples where strategies were developed requiring even fewer steps, by following the general guidelines for protein purification given in this handbook and selecting the most appropriate technique and media to fulfil the purification objectives. In most of these examples methods were developed using ÄKTAdesign chromatography systems.

Example 1.
Three step purification of a recombinant enzyme

This example demonstrates one of the most common purification strategies: IEX for capture, HIC for intermediate purification and GF for the polishing step. The objective of this purification was to obtain highly purified protein for crystallisation and structural determination.

A more detailed description of this work can be found in Application Note 18-1128-91.

Target Molecule

Deacetoxycephalosporin C synthase (DAOCS), an oxygen-sensitive enzyme.

Source Material

Recombinant protein over-expressed in soluble form in the cytoplasm of *E. coli* bacteria.

Sample Extraction and Clarification

Cells were suspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 0.2 M benzamidine-HCl, 0.2 mM PMSF, pH 7.5) and lysed using ultrasonication.
Streptomycin sulfate (1%) and polyethyleneimine (0.1%) were added to precipitate DNA. The extract was clarified by centrifugation.

EDTA, DTT, Benzamidine-HCl and PMSF were used in the lysis buffer to inhibit proteases and to minimise damage to the oxygen-sensitive enzyme. Keeping the sample on ice also reduced protease activity.

Capture

The capture step focused on the rapid removal of the most harmful contaminants from the relatively unstable target protein. This, together with the calculated isoelectric point of DAOCS (pI = 4.8), led to the selection of an anion exchange purification. A selection of anion exchange columns, including those from HiTrap IEX Test Kit, were screened to select the optimum medium (results not shown) before using a larger column for the optimisation of the capture step. Q Sepharose XL, a high capacity medium, well suited for capture, was chosen.

As shown in Figure 14, optimisation of the capture step allowed the use of a step elution at high flow rate to speed up the purification. This was particularly advantageous when working with this potentially unstable sample.

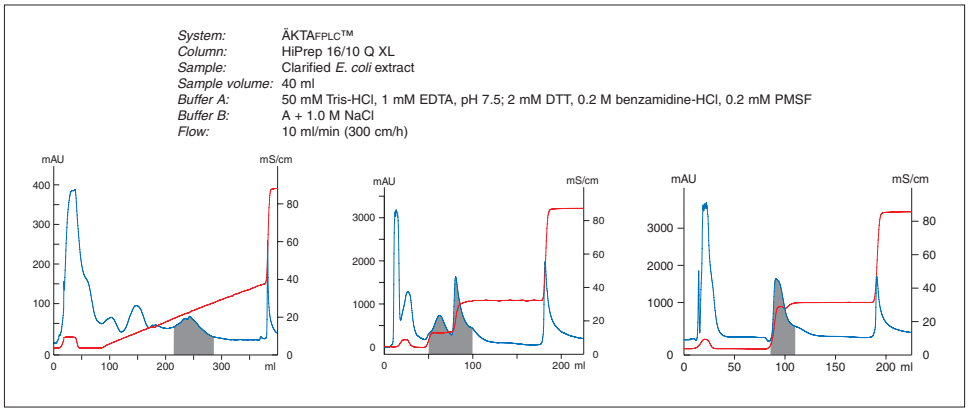


Fig 14. Capture using IEX and optimisation of purification conditions. The elution position of DAOCS is shaded.

Intermediate Purification

Hydrophobic interaction chromatography (HIC) was selected because the separation principle is complementary to ion exchange and because a minimum amount of sample conditioning was required. Hydrophobic properties are difficult to predict and it is always recommended to screen different media. The intermediate purification step was developed by screening prepacked hydrophobic interaction media (RESOURCE HIC Test Kit) to select the optimum medium for the separation (results not shown). SOURCE 15ISO was selected on the basis of the resolution achieved. In this intermediate step, shown in Figure 15, the maximum possible speed for separation was sacrificed in order to achieve higher resolution and to allow significant reduction of remaining impurities.

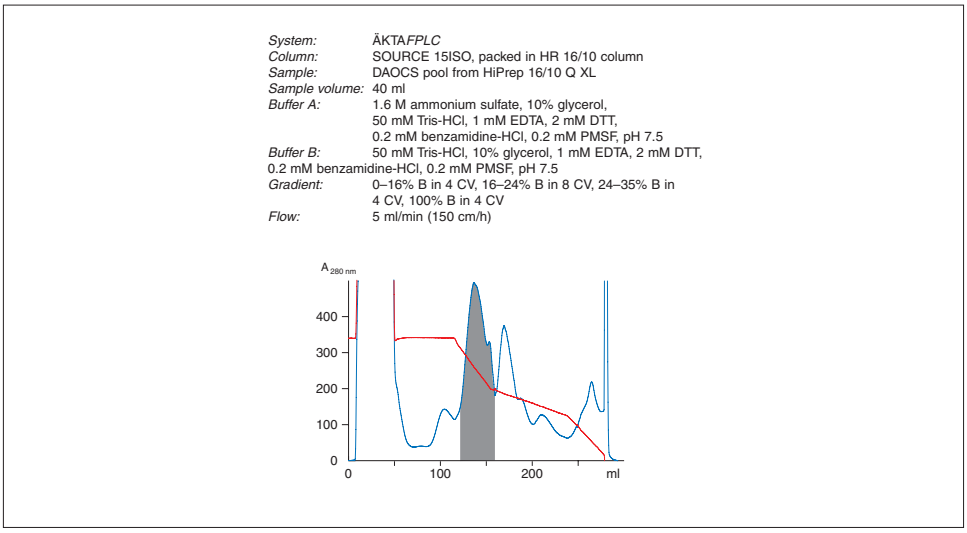


Fig 15. Intermediate purification using HIC.

Polishing

The main goal of the polishing step was to remove aggregates and minor contaminants and to transfer the purified sample into a buffer suitable for use in further structural studies. Superdex 75 prep grade, a gel filtration medium giving high resolution at relatively short separation times, was selected since the molecular weight of DAOCS (34 500) is within the optimal separation range for this medium. Figure 16 shows the final purification step.

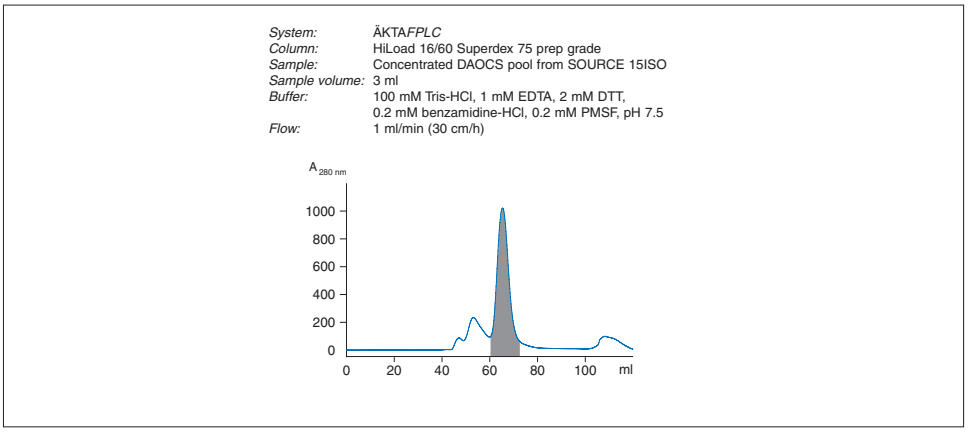
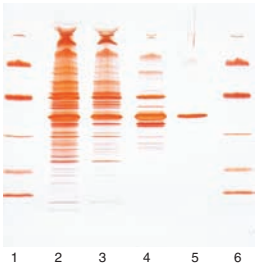


Fig 16. Polishing using gel filtration.

Analytical assays

Figure 17 shows the analysis of collected fractions by SDS-PAGE and silver staining using Multiphor™ II, following the separation and staining protocols supplied with the instruments.



Lane 1, 6: LMW Marker Kit

Lane 2: Cell homogenate

Lane 3: DAOCS pool from Q Sepharose XL

Lane 4: DAOCS pool from Source 15ISO

Lane 5: DAOCS pool from Superdex 75 prep grade

Fig 17. Analysis of purification steps using ExcelGel™ SDS Gradient 8-18.

The final product was used successfully in X-ray diffraction studies, Figure 18.

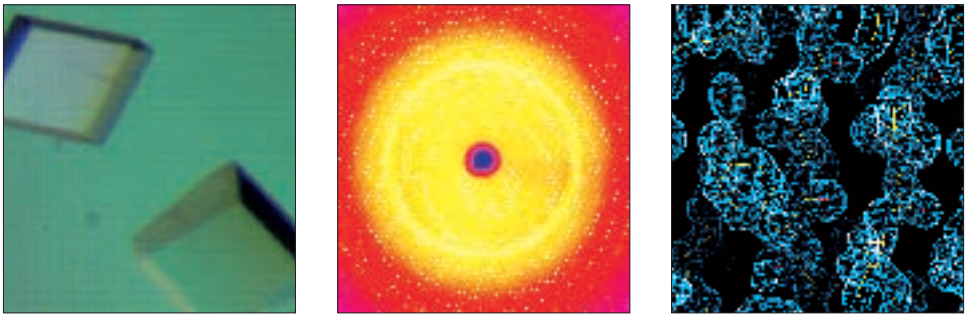


Fig 18. Crystals, diffraction pattern and high resolution electron density map of purified DAOCS. Figures supplied by Prof. I. Andersson and Dr A. Terwisscha van Scheltinga, Swedich University of Agricultural Sciences, and Dr K. Valegård, Uppsala University, Uppsala, Sweden.

Example 2.

Three step purification of a recombinant antigen binding fragment

This example demonstrates a three stage purification strategy in which the same purification principle is used in two different modes in the capture and polishing step : IEX for capture, HIC for intermediate purification and IEX for the polishing step. The objective of this purification was to scale up the purification for use as a routine procedure.

A more detailed description of this work can be found in Application Note 18-1111-23.

Target Molecule

Recombinant antigen binding fragment (Fab) directed against HIV gp-120.

Source Material

The anti-gp 120 Fab was expressed in the periplasm of the *E. coli* strain BM170 MCT61. *E. coli* pellets were stored frozen after being harvested and washed once.

Sample Extraction, Clarification and Capture

Thawed cells were lysed and the lysate was treated with DNase in the presence of 2 mM $MgCl_2$ at pH 7.5, before the capture step.

The Fab fragment was captured from non-clarified homogenate by using expanded bed adsorption with STREAMLINE SP (cation exchanger).

Expanded bed adsorption was chosen because the target protein could be captured directly from the crude homogenate in a single step, without centrifugation or other preparatory clean-up steps. The technique is well suited for large scale purification.

The result of the capture step is shown in Figure 19. The Fab fragment is concentrated and transferred rapidly into a stable environment, using step elution.

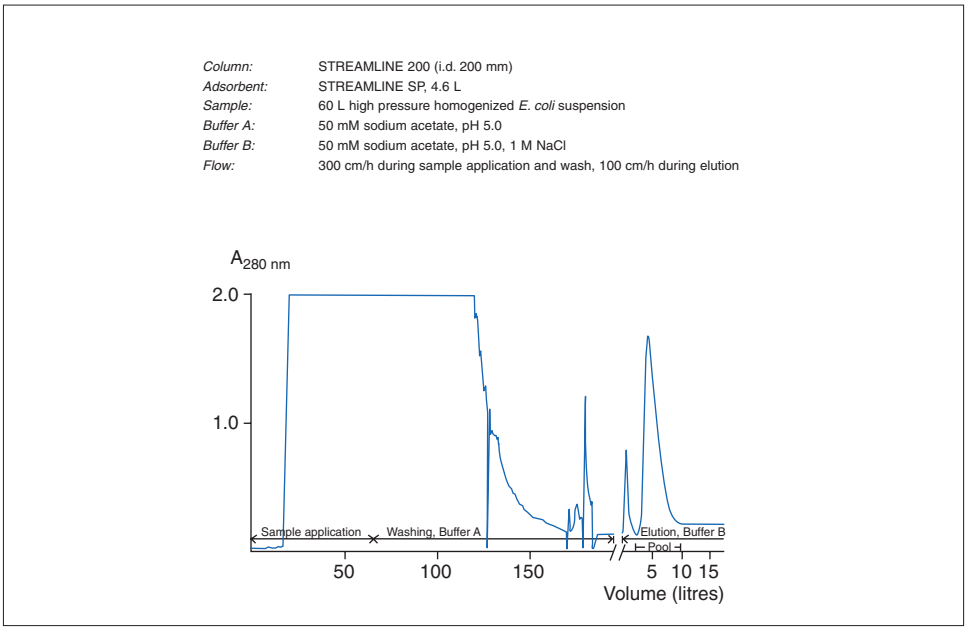


Fig 19. Capture step using expanded bed adsorption.

Intermediate Purification

Hydrophobic interaction chromatography (HIC) was selected because the separation principle is complementary to ion exchange and because a minimum amount of sample conditioning was required since the sample was already in a high salt buffer after elution from STREAMLINE SP.

Hydrophobic properties are difficult to predict and it is always recommended to screen different media. A HiTrap HIC Test Kit (containing five 1 ml columns prepacked with different media suitable for production scale) was used to screen for the most appropriate medium. Buffer pH was kept at pH 5.0 to further minimise the need for sample conditioning after capture. Results of the media screening are shown in Figure 20.

System: ÄKTAexplorer™
Sample: Fab fraction from STREAMLINE SP, 2 ml
Columns: HiTrap HIC Test Kit (1 ml columns), Phenyl Sepharose High Performance, Phenyl Sepharose 6 Fast Flow (low sub), Phenyl Sepharose 6 Fast Flow (high sub), Butyl Sepharose 4 Fast Flow, Octyl Sepharose 4 Fast Flow
Buffer A: 1 ml $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Buffer B: 50 mM NaAc, pH 5.0
Gradient: 20 column volumes
Flow: 2 ml/min (300 cm/hr)

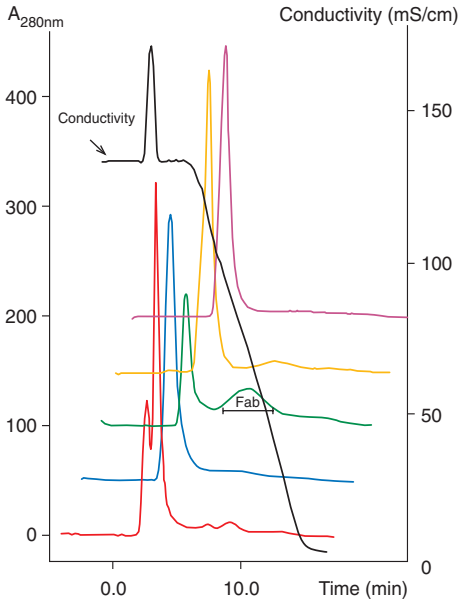


Fig 20. HIC media scouting using HiTrap HIC Test Kit.

Phenyl Sepharose 6 Fast Flow (high sub) was selected since the medium showed excellent selectivity for the target protein thereby removing the bulk contaminants. Optimisation of elution conditions resulted in a step elution being used to maximise the throughput and the concentrating effect of the HIC purification technique. Figure 21 shows the optimised elution and the subsequent scale up of the intermediate purification step.

System:	ÄKTAexplorer
Sample:	Fab fraction from STREAMLINE SP, 80 ml
Columns:	Phenyl Sepharose 6 Fast Flow (high sub) in XK 16/20 (10 cm bed height)
Buffer A:	1 M $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Buffer B:	50 mM NaAc, pH 5.0
Gradient:	Step gradient to 50% B
Flow:	5 ml/min

System:	ÄKTAexplorer
Sample:	Fab fraction from STREAMLINE SP, 800 ml
Columns:	Phenyl Sepharose 6 Fast Flow (high sub) in XK 50/20
Buffer A:	1 M $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Buffer B:	50 mM NaAc, pH 5.0
Gradient:	Step gradient to 50% B
Flow:	Equilibration: 100 ml/min Loading and elution: 50 ml/min

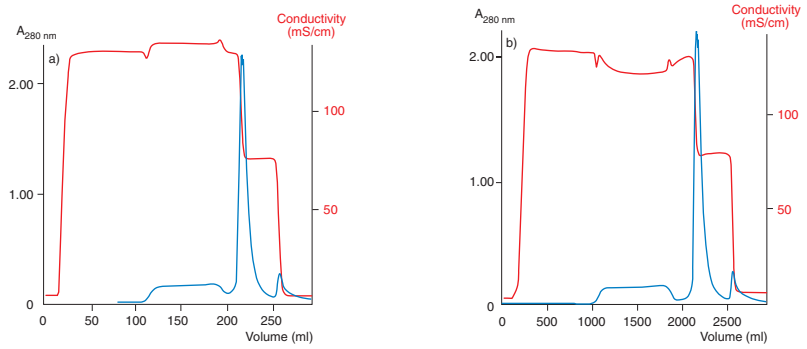


Fig 21. Intermediate purification using HIC: optimisation and scale-up.

Polishing

Gel filtration was investigated as the natural first choice for a final polishing step to remove trace contaminants and transfer the sample to a suitable storage conditions. However, in this example, gel filtration could not resolve a contaminant (M_r 52 000) from the Fab fragment (M_r 50 000) (results not shown).

As an alternative another cation exchanger SOURCE 15S was used. In contrast to the cation exchange step at the capture step, the polishing cation exchange step was performed using a shallow gradient elution on a medium with a small, uniform size (SOURCE 15S) to give a high resolution result, as shown in Figure 22.

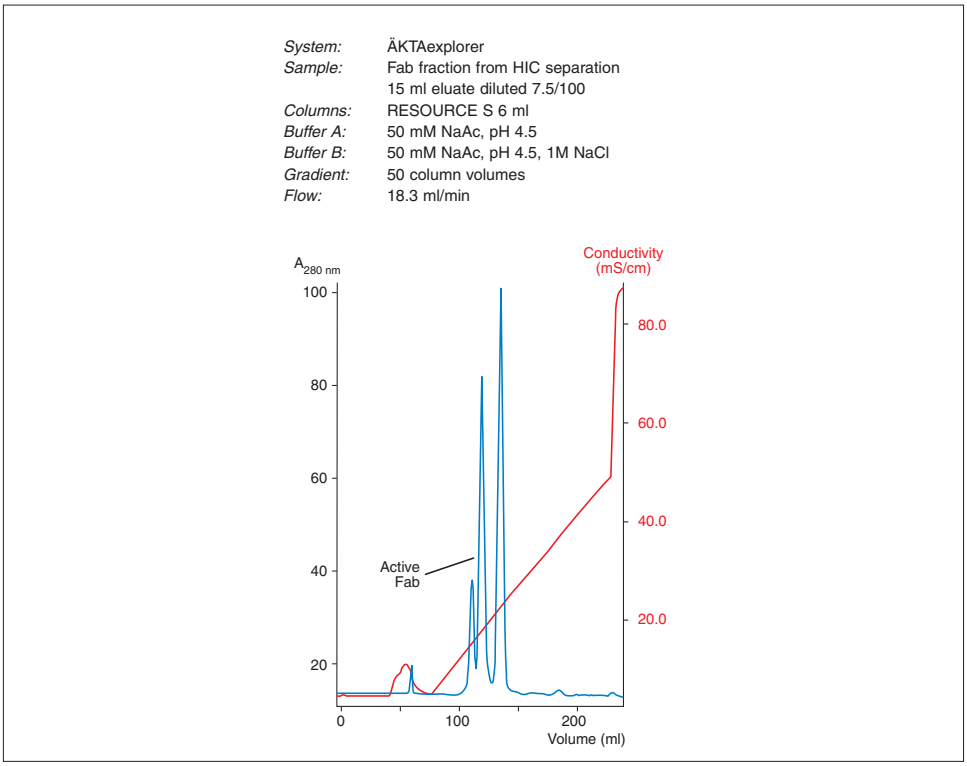


Fig 22. Optimised Fab polishing step.

Analytical assays

Collected fractions were separated by SDS-PAGE and stained by Coomassie™ using PhastSystem™, following the separation and staining protocols supplied with the instrument.

Fab was measured by a goat-anti-human IgG Fab ELISA, an anti-gp120 ELISA and an *in vitro* assay which measured the inhibition of HIV-1 infection of T-cells.

Nucleic acid was routinely monitored by measuring A_{260}/A_{280}. The correlation of a high A_{260}/A_{280} ratio (>1) with the presence of DNA was verified for selected samples by agarose gel electrophoresis and ethidium bromide staining. Endotoxin determination employed a kinetic chromogenic Limulus assay (Coamatic™, Chromogenix AB, Mölndal, Sweden).

Example 3.

Two step purification of a monoclonal antibody

This example demonstrates the effectiveness of using a high selectivity affinity chromatography technique as a capture step, since only a gel filtration polishing step was needed to achieve the required level of purity.

The objective of this work was to produce an efficient, routine procedure for monoclonal antibody purification.

A more detailed description of this work can be found in Application Note 18-1128-93.

Target Molecule

Mouse monoclonal IgG_1 antibodies.

Source Material

Cell culture supernatant.

Sample Extraction and Clarification

Salt concentration and pH were adjusted to those of the binding buffer in the capture step. Samples were filtered through a 0.45 µm filter before chromatography.

Capture

Affinity or ion exchange chromatography are particularly suitable for samples such as cell culture supernatants as they are binding techniques which concentrate the target protein and significantly reduce sample volume. For monoclonal antibody purification capture of the target protein can be achieved by using a highly selective affinity chromatography medium. In this example a HiTrap rProtein A column was used.

Although general standard protocols were supplied with this prepacked columns, it was decided to further optimise the binding and elution conditions for the specific target molecule.

Most mouse monoclonal antibodies of the IgG_1 sub-class require high salt concentrations to bind to immobilised Protein A, therefore a salt concentration was selected which gave the largest elution peak area and absence of antibodies in the flow-through. Results from the scouting for optimal binding conditions are shown in Figure 23. Scouting for the optimum elution pH also helped to improve antibody recovery.

Optimisation of binding and elution conditions gave a well resolved peak containing IgG_1, as shown in Figure 24.

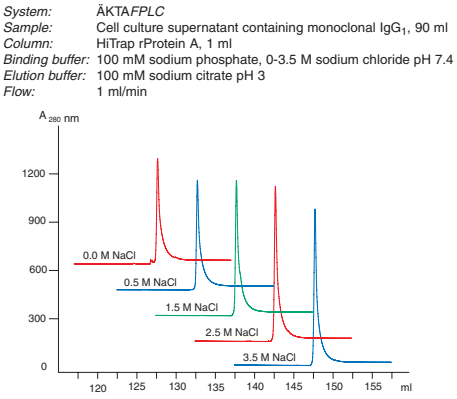
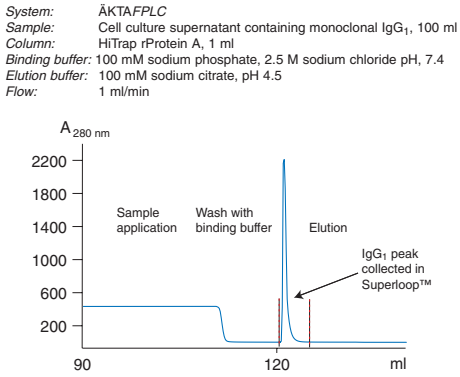


Fig 23. Automatic scouting for optimal binding conditions.

Fig 24. Optimised capture step on HiTrap rProtein A.



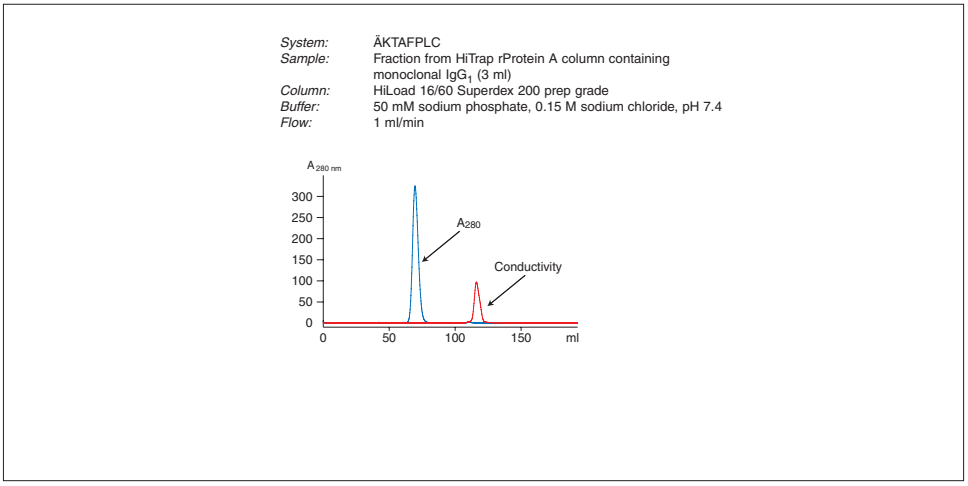
Intermediate Purification

No intermediate purification was required as the high selectivity of the capture step also removed contaminating proteins and low-molecular substances giving a highly efficient purification.

Polishing

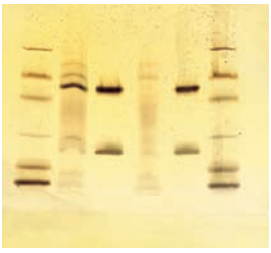
In most antibody preparations there is a possibility that IgG aggregates and/or dimers are present. It was therefore essential to include a gel filtration polishing step, despite the high degree of purity achieved during capture. The polishing step removes low or trace levels of contaminants. Superdex 200 prep grade gel filtration media was selected as it has the most suitable molecular weight separation range for IgG antibodies. Figure 25 shows the final purification step.

Fig 25. Gel filtration on HiLoad 16/60 Superdex 200 prep grade.



Analytical assay

Collected fractions were separated by SDS-PAGE and silver stained using PhastSystem, following the separation and staining protocols supplied with the instrument.



Lane 1. LMW-standard
Lane 2. Starting material (diluted 2 x)
Lane 3. Eluted IgG_1 peak from HiTrap rProtein A column (diluted 10 x)
Lane 4. Flow through, HiTrap rProtein A
Lane 5. Eluted IgG_1 peak from HiLoad Superdex 200 prep grade (diluted 6 x)
Lane 6. LMW-standard
Gel: 10–15 % SDS-PAGE PhastGel
System: PhastSystem™

Analysis of purification steps using SDS PAGE.

Example 4.

One step purification of an integral membrane protein

This example demonstrates that, with the use of a suitably tagged recombinant protein, selected detergents and an appropriate chromatographic medium, a successful purification can be achieved in a single chromatographic step. The objective was to purify a recombinant histidine-tagged integral membrane protein to allow characterisation under non-denaturing conditions. A more detailed description of this work, including results for size and charge homogeneity, can be found in Application Note 18-1128-92.

Target Molecule

Histidine-tagged cytochrome *bo* 3 ubiquinol oxidase from *E. coli*.

Source Material

The histidine-tagged cytochrome *bo* 3 ubiquinol oxidase accumulated in the membrane of *E. coli*.

Sample Extraction and Clarification

Membrane Preparation
Integral membrane proteins require the use of detergents for extraction. The concentration and type of detergent that is suitable for a particular extraction must be tested for each situation.

Cells were harvested by centrifugation and frozen at –80 °C.
Frozen cells were mixed with 200 mM Tris-HCl, pH 8.8, 20 mM Na_2-EDTA, 500 mM sucrose and brought to room temperature, stirring gently.
10 mg/ml lysozyme in buffer was added and the solution was stirred for 30 min.
Cells were sedimented by centrifugation and supernatant was removed.
Pellets were resuspended in 5 mM Na_2-EDTA, pH 8.0, with PMSF, and stirred for 10 min.
$MgCl_2$ (final concentration 10 mM) and a few crystals of DNase I were added and stirred for 5 min.
The solution was sonicated for 3 x 1 min.
Unbroken cells were removed by centrifugation
Membrane particles were isolated by high speed centrifugation, resuspended in 50 mM Tris-HCl (pH 8.0), 250 mM NaCl and sedimented again at high speed.
Membrane pellets were stored frozen.

Membrane Solubilisation
Membrane pellets were thawed, ice cold 1% dodecyl-β-D-maltoside (a non-ionic detergent) in 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM imidazole was added. The solution was stirred on ice for 30 min.
Insoluble material was removed by centrifugation.
The presence of non-ionic detergent avoided denaturing conditions and interference with purification steps whilst maintaining membrane protein solubility.

Capture

Due to the instability of membrane proteins and their tendency to associate it is often essential to use fast purification protocols at low temperatures. Attachment of a histidine tag allowed the use of a HiTrap Chelating column giving a highly selective affinity chromatography capture step, shown in Figure 26. This technique also removed contaminating proteins, DNA, lipids and low-molecular substances and allowed equilibration of detergent-protein complexes with the detergent solution. The technique was unaffected by the presence of the non-ionic detergent. Buffers and separation procedure followed the recommendations provided with the HiTrap Chelating column.

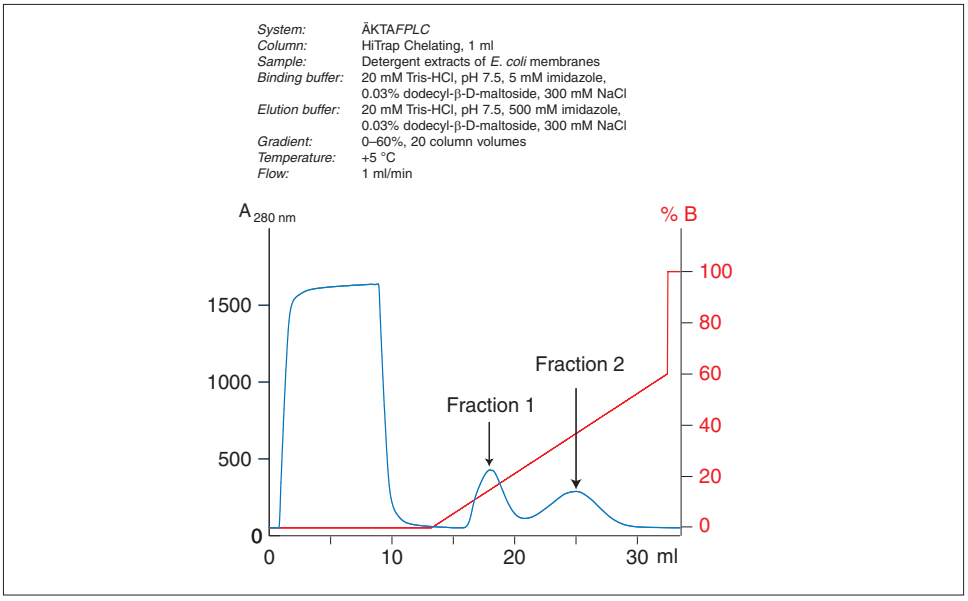


Fig 26. One Step Purification on a HiTrap Chelating column.

Intermediate Purification and Polishing

No intermediate or polishing steps were needed as the high selectivity of the capture step produced a membrane protein of sufficient purity to allow further characterisation, i.e. a single step purification was achieved (as shown by electrophoresis).

Analytical assay

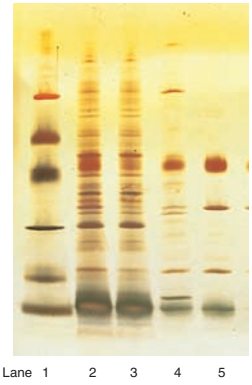


Fig 27. SDS electrophoresis on PhastSystem using PhastGel 8–25%, silver staining.

To confirm final purity, collected fractions were separated by SDS-PAGE and silver stained by PhastSystem, following the separation and staining protocols supplied with the instrument. Figure 27 shows that four subunits of cytochrome *bo* 3 were present in both fractions. Fraction 2 was essentially pure, whereas contaminants were seen in Fraction 1.

Chapter 8

Sample Storage Conditions

Recommendations for biological samples

Keep refrigerated in a closed vessel to minimise bacterial growth and protease activity. Avoid conditions close to stability limits (for example, extreme pH, pH values close to the isoelectric point of the target protein or salt concentrations, reducing or chelating agents).
For storage times longer than 24 hours a bacteriostatic agent may be added, but this should be selected with care to ensure compatability with subsequent procedures. For long term storage keep proteins frozen or freeze dried in small aliquots (to avoid repeated freeze/thawing or freeze drying/re-dissolving which may reduce biological activity). Samples which will be freeze dried should be dissolved in volatile buffers, examples shown in Table 7. It should also be noted that concentration gradients can develop during freezing and thawing which may create extreme conditions causing protein denaturation.
If essential add stabilising agents. These are more often required for storage of purified proteins.

Table 7. Volatile buffer systems.

	Volatile buffer systems used in ion exchange chromatography		
pH range	**Buffer system**	**Counter-ion**	**pK-values for buffering ions**
2.0	Formic acid	H^+	3.75
2.3-3.5	Pyridine/formic acid	$HCOO^-$	3.75; 5.25
3.0-5.0	Trimethylamine/formic acid	$HCOO^-$	3.75; 9.25
3.0-6.0	Pyridine/acetic acid	CH_3COO^-	4.76; 5.25
4.0-6.0	Trimethylamine/acetic acid	CH_3COO^-	4.76; 9.25
6.8-8.8	Trimethylamine/HCl	Cl^-	9.25
7.0-8.5	Ammonia/formic acid	$HCOO^-$	3.75; 9.25
8.5-10.0	Ammonia/acetic acid	CH_3COO^-	4.76; 9.25
7.0-12.0	Trimethylamine/carbonate	CO_3^{2-}	6.50; 9.25
7.9	Ammonium bicarbonate	HCO_3^-	6.50; 9.25
8.0-9.5	Ammonium carbonate/ammonia	CO_3^{2-}	6.50; 9.25
8.5-10.5	Ethanolamine/HCl	Cl^-	10.0
8.5	Ammonium carbonate	CO_3^{2-}	6.50; 9.25

Recommendations for purified proteins

Store in high concentration of ammonium sulfate (e.g. 4 M). Freeze in 50% glycerol, especially suitable for enzymes. Add stabilising agents, e.g. glycerol (5-20%), serum albumin (10 mg/ml), ligand (concentration is selected based on the concentration of the active protein). Sterile filter to avoid bacterial growth.

Sample Extraction and Clarification Procedures

Sample extraction

Extraction procedures should be selected according to the source of the protein, such as bacterial, plant or mammalian, intracellular or extracellular.
Use procedures which are as gentle as possible since disruption of cells or tissues leads to the release of proteolytic enzymes and general acidification. Selection of an extraction technique is dependent as much upon the equipment available and scale of operation as on the type of sample. Examples of common extraction processes are shown in Table 8. Extraction should be performed quickly, at sub-ambient temperatures, in the presence of a suitable buffer to maintain pH and ionic strength and to stabilise the sample. Samples should be clear and free from particles before beginning a chromatographic separation.

Table 8. Common sample extraction processes.

Extraction process	Typical conditions	Protein source	Comment
Gentle Cell lysis (osmotic shock)	2 volumes water to 1 volume packed pre-washed cells	erythrocytes, *E.coli* periplasm: intracellular proteins	lower product yield but reduced protease release
Enzymatic digestion	lysozyme 0.2 mg/ml, 37 °C, 15 mins.	bacteria: intracellular proteins	lab scale only, often combined with mechanical disruption
Hand homogenisation	follow equipment instructions	liver tissue	
Mincing (grinding)	"	muscle	
Moderate Blade homogeniser	follow equipment instructions	muscle tissue, most animal tissues, plant tissues	
Grinding with abrasive e.g. sand	"	bacteria, plant tissues	
Vigorous Ultrasonication or bead milling	follow equipment instructions	cell suspensions: intracellular proteins in cytoplasm, periplasm, inclusion bodies	small scale, release of nucleic acids may cause viscosity problems inclusion bodies must be resolubilised
Manton-Gaulin homogeniser	follow equipment instructions	cell suspensions	large scale only
French press	follow equipment instructions	bacteria, plant cells	
Fractional precipitation	see section on fractional precipitation	extracellular: secreted recombinant proteins, monoclonal antibodies, cell lysates	precipitates must be resolubilised

Details from Protein Purification, Principles and Practice, R.K. Scopes and other sources.

Buffers and additives

With knowledge of the target protein stability window and other properties, additives can be kept to a minimum. This can help to avoid problems of interference with assays or other procedures and will avoid the need for an extra purification step to remove additives at a later stage in purification. Examples of buffers and additives, together with their use, are shown in Table 9.

Table 9. Common substances used in sample preparation.

	Typical conditions for use	Purpose
Buffer components Tris	20 mM, pH 7.4	maintain pH, minimise acidification caused by lysosomal disruption
NaCl	100 mM	maintain ionic strength of medium
EDTA	10 mM	reduce oxidation damage, chelate metal ions
Sucrose or glucose	25 mM	stabilise lysosomal membranes, reduce protease release
Detergents Ionic or non-ionic detergents	See Table 10	extraction and purification of integral membrane proteins solubilisation of poorly soluble proteins
DNase and RNase	1 µg/ml	degradation of nucleic acids, reduce viscosity of sample solution
*Protease inhibitors** PMSF	0.5–1 mM	*Inhibits* serine proteases
APMSF	0.4–4 mM	serine proteases
Benzamidine-HCl	0.2 mM	serine proteases
Pepstatin	1 µM	aspartic proteases
Leupeptin	10–100 µM	cysteine and serine proteases
Chymostatin	10–100 µM	chymotrypsin, papain, cysteine proteases
Antipain-HCl	1–100 µM	papain, cysteine and serine proteases
EDTA	2–10 mM	metal dependent proteases, zinc and iron
EGTA	2–10 mM	metal dependent proteases e.g. calcium
Reducing agents 1,4 dithiothreitol, DTT	1–10 mM	keep cysteine residues reduced
1,4 dithioerythritol, DTE	1–10 mM	"
Mercaptoethanol	0.05%	"
Others Glycerol	5–10%	for stabilisation, up to 50% can be used if required

PMSF — Phenylmethylsulfonyl fluoride
APMSF — 4-Aminophenyl-methylsulfonyl fluoride
PMSF is a hazardous chemical. Half-life time in aqueous solution is 35 min. PMSF is usually stored as 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at –20° C.
* Protease inhibitors are available in pre-made mixes from several suppliers.
Details taken from Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer., Protein Purification, Principles, High Resolution Methods and Applications, J-C. Janson and L. Rydén, 1998, 2nd ed. Wiley VCH and other sources.

Detergents

Non-ionic detergents are used most commonly for extraction and purification of integral membrane proteins. Selection of the most suitable detergent is often a case of trial and error. The detergent should be used at concentrations near or above its critical micelle concentration, i.e. the concentration at which detergent monomers begin to associate with each other. This concentration is dependent upon the type of detergent and the experimental conditions. Examples of ionic and non-ionic detergents are shown in Table 10. Adjustment of the detergent concentration necessary for optimum results is often a balance between the activity and yield of the protein. During purification procedures it may be possible to reduce the concentration of detergent compared to that used for extraction. However, some level of detergent is usually essential throughout purification procedures to maintain solubility. Detergents can be exchanged by adsorption techniques (Ref: Phenyl Sepharose mediated detergent exchange chromatography: its application to exchange of detergents bound to membrane protein. Biochemistry 23, 1984, 6121–6126, *Robinson N.C., Wiginton D., Talbert L.)*

Table 10. Examples of ionic and non-ionic detergents.

Sodium dodecyl sulfate	0.1–0.5%	denatures proteins, used for SDS-PAGE use non-ionic detergents to avoid denaturation
Triton™ X-100	0.1 %	non-ionic detergent for membrane solubilisation. **Note:** *may absorb strongly at 280 nm!*
NP-40	0.05–2%	"
Dodecyl β D-maltoside	1%	"
Octyl β D-glucoside	1–1.5%	"

For further information on detergents: Protein Purification, Principles, High Resolution Methods and Applications, *J-C. Janson and L. Rydén,* 1998, 2nd ed. Wiley VCH.

Sample Clarification

Centrifugation

Use before first chromatographic step
Removes lipids and particulate matter

For small sample volumes and those which adsorb non-specifically to filters:
Centrifuge at 10000g for 15 minutes
For cell homogenates:
Centrifuge at 40 000–50 000g for 30 minutes

Ultrafiltration

Use before first chromatographic step
Removes salts, concentrates sample

Ultrafiltration membranes are available with different cut off limits for separation of molecules from M_r1000 up to 300000. The process is slower than gel filtration and membranes may clog.

Check the recovery of the target protein in a test run.
Some proteins may adsorb non-specifically to filter surfaces.

Filtration

Use before first chromatographic step
Removes particulate matter
Suitable for small sample volumes.

For sample preparation before chromatography select filter size according to the bead size of the chromatographic medium.

Filter size	Bead size of chromatographic medium
1 µm	90 µm and upwards
0.45 µm	3, 10, 15, 34 µm
0.22 µm	sterile filtration or extra clean samples

Check the recovery of the target protein in a test run. Some proteins may adsorb non-specifically to filter surfaces.

Gel filtration (for sample clarification or conditioning)

Use before or between chromatographic purification steps.
For rapid processing of small or large sample volumes.
Removes salts from samples M_r >5000.

Sephadex G-25 is used at laboratory and production scale for sample preparation and clarification. Typically sample volumes of up to 30% of the total column volume are loaded. In a single step, the sample is desalted, exchanged into a new buffer, and low molecular weight materials are removed. The high volume capacity and speed of this step enable very large sample volumes to be processed rapidly and efficiently. The high sample volume load results in a separation with minimal sample dilution. A typical elution is shown in Figure 28.

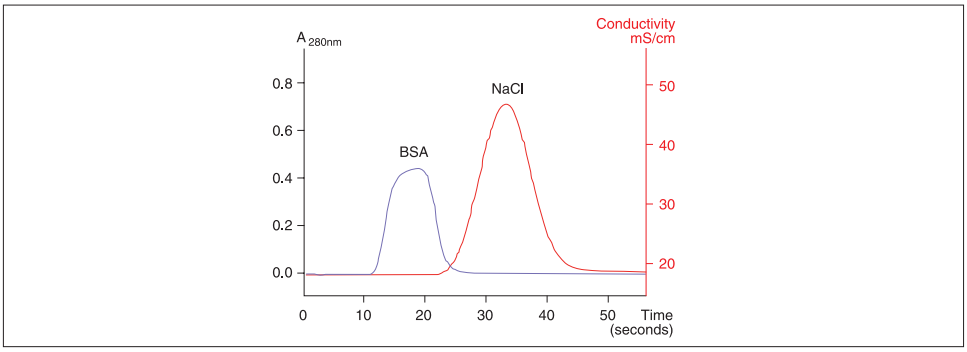


Fig 28. Typical elution profile for sample desalting and buffer exchange.

Methodology

Select a prepacked desalting column from the table below or pack a column.

Prepacked column	Sample volume loading per run	Sample volume recovery per run	Code No.
HiPrep Desalting 26/10	2.5–15 ml	7.5–20 ml	17-5087-01
HiTrap Desalting	0.25–1.5 ml	1.0–2.0 ml	17-1408-01
Fast Desalting PC 3.2/10	0.05–0.2 ml	0.2–0.3 ml	17-0774-01
PD-10 Desalting	1.5–2.5 ml	2.5–3.5 ml	17-0851-01

Column packing

The following guidelines apply at all scales of operation:
Column dimensions = typically 10 - 20 cm bed height.
Quantity of gel = five times volume of sample.

For column packing Sephadex G-25 is available in a range of bead sizes (Superfine, Fine, Medium and Coarse). Changes in bead size alter flow rates and sample volumes which can be applied (see Figure 29). For laboratory scale separations use Sephadex G-25 Fine with an average bed height of 15 cm.

Individual product packing instructions contain more detailed information on packing Sephadex G-25.

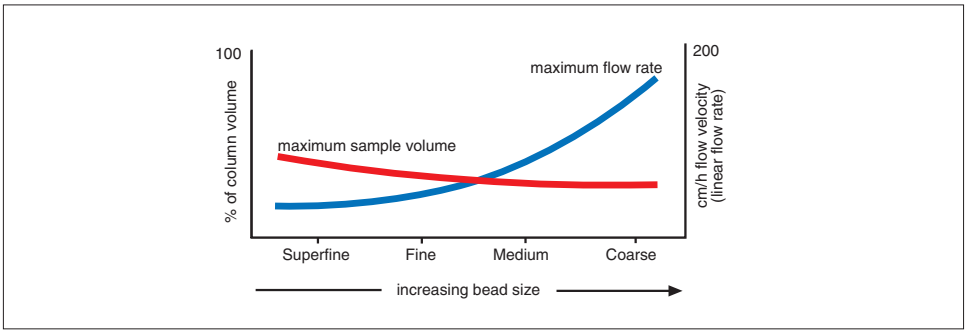


Fig 29. Sephadex G-25: sample volume and flow rate varies with bead size.

Fractional precipitation

For extraction and clarification at laboratory scale
Partially purifies sample, may also concentrate
Use before the first chromatographic step

Most precipitation techniques are not suitable for large scale preparations.

Precipitation techniques are affected by temperature, pH and sample concentration. These parameters must be controlled to ensure reproducible results. Precipitation can be used in three different ways, as shown in Figure 30.

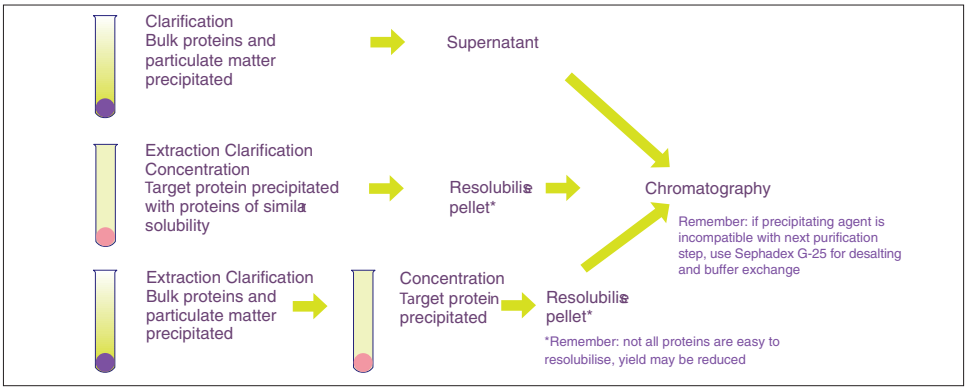


Fig 30. Three ways to use precipitation.

Precipitation techniques are reviewed in Table 11 and the two common methods are described in more detail.

Table 11. Examples of precipitation techniques.

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	as described	>1mg/ml proteins especially immuno-globulins	stabilizes proteins, no denaturation, super-natant can go directly to HIC
Dextran sulfate	as described	samples with high levels of lipoprotein, e.g ascites	precipitates lipoprotein
Polyvinylpyrrolidine	Add 3% (w/v), stir 4 hours, centrifuge, discard pellet	"	alternative to dextran sulfate
Polyethylene glycol (PEG, M.W. >4000)	up to 20% wt/vol	plasma proteins	no denaturation, super-natant goes direct to IEX or AC. Complete removal may be difficult
Acetone	up to 80% vol/vol at 0 °C	useful for peptide precipitation or con-centration of sample for electrophoresis	may denature protein irreversibly
Polyethyleneimine	0.1% w/v		precipitates aggregated nucleoproteins
Protamine sulfate	1%		"
Streptomycin sulfate	1%		precipitation of nucleic acids

Details taken from Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer., Protein Purification, Principles, High Resolution Methods and Applications, *J-C. Janson and L. Rydén,* 1998, 2nd ed. Wiley VCH and other sources

Ammonium sulfate precipitation

Materials

Saturated ammonium sulfate solution
Add 100 g ammonium sulfate to 100 ml distilled water, stir to dissolve
1 M Tris-HCl pH 8.0
Buffer for first chromatographic purification step

Procedure

1. Filter (0.45µm) or centrifuge (refrigerated, 10000 g) sample.
2. Add 1 part 1 M Tris-HCl pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulfate solution, drop by drop (solution becomes milky at about 20% saturation). Add up to 50% saturation*. Stir for 1 hour.
4. Centrifuge 20 minutes at 10000g.
5. Discard supernatant. Wash pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e. a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the chromatographic buffer.

7. Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex G-25 or during hydrophobic interaction separations.

*The % saturation can be adjusted either to precipitate a target molecule or to precipitate contaminants.

The quantity of ammonium sulfate required to reach given degrees of saturation varies according to temperature. Table 12 shows the quantities required at 20 °C.

Table 12. Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C.

Values calculated according to Protein Purification, R. K. Scopes (Springer-Verlag, New York),Third Edition, p. 346, 1993.

	Final percent saturation to be obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting percent saturation	**Amount of ammonium sulfate to add (grams) per liter of solution at 20 °C**																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

For further details showing variation of % saturation versus temperature and a review of precipitation techniques see Guide to Protein Purification, Methods in Enzymology, Vol. 182, p. 291 Academic Press 1990.

Dextran sulfate precipitation

Materials
10% dextran sulfate
1 M calcium chloride
Buffer for first chromatographic purification step.

Procedure
1. Add 0.04 ml dextran sulfate solution and 1 ml calcium chloride solution to every 1 ml of sample. Mix 15 minutes.
2. Centrifuge (10000 g, 10 minutes), discard precipitate.

Dextran sulfate is removed during a clarification/buffer exchange Sephadex G-25 step.

Resolubilisation of protein precipitates

Many proteins are easily resolubilised in a small amount of the buffer to be used in the next chromatographic step. However, an agent, selected from Table 13, may be required for less soluble proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete re-folding of the protein and to maximise recovery of mass and activity.

A chromatographic step often removes a denaturant during purification.

Table 13. Examples of denaturing agents.

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2–8 M	remove using Sephadex G-25
Guanidine hydrochloride	3–8 M	remove using Sephadex G-25 or during IEX
Triton X-100	2%	”
Sarcosyl	1.5%	”
N-octyl glucoside	2%	”
Sodium dodecyl sulfate	0.1–0.5%	exchange for non-ionic detergent during first chromatographic step, avoid anion exchange chromatography
alkaline pH	> pH 9, NaOH	may need to adjust pH during chromatography to maintain solubility

Details taken from Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer., Protein Purification, Principles, High Resolution Methods and Applications, *J-C. Janson and L. Rydén,* 1998, 2nd ed. Wiley VCH and other sources

Expanded bed adsorption (STREAMLINE)

For large scale recombinant protein and monoclonal antibody purification. The technique requires no sample clean up and enables clarification, concentration and capture in a single step.

EBA can be regarded as a technique in which sample preparation and capture are combined in a single step. Crude sample is applied to an expanded bed of STREAMLINE media, target proteins are captured whilst cell debris, cells, particulate matter, whole cells, and contaminants pass through. Flow is reversed and the target proteins are desorbed in the elution buffer.

Chapter 9

Principles and standard conditions for purification techniques

Ion Exchange (IEX) Chromatography

IEX separates proteins with differences in charge to give a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. This elution is usually performed by increases in salt concentration or changes in pH. Changes are made stepwise or with a continuous gradient. Most commonly, samples are eluted with salt (NaCl), using a gradient elution (Figure 31). Target proteins are concentrated during binding and collected in a purified, concen-trated form.

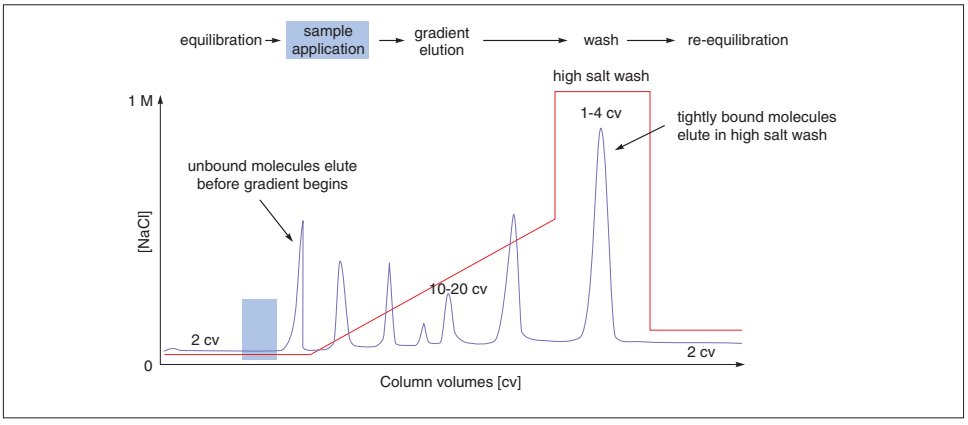


Fig 31. Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein will bind to an anion exchanger, when below its pI a protein will behind to a cation exchanger. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities if required. IEX can be repeated at different pH values to separate several proteins which have distinctly different charge properties, as shown in Figure 32. This can be used to advantage during a multistep purification, as shown in the example on page 24.

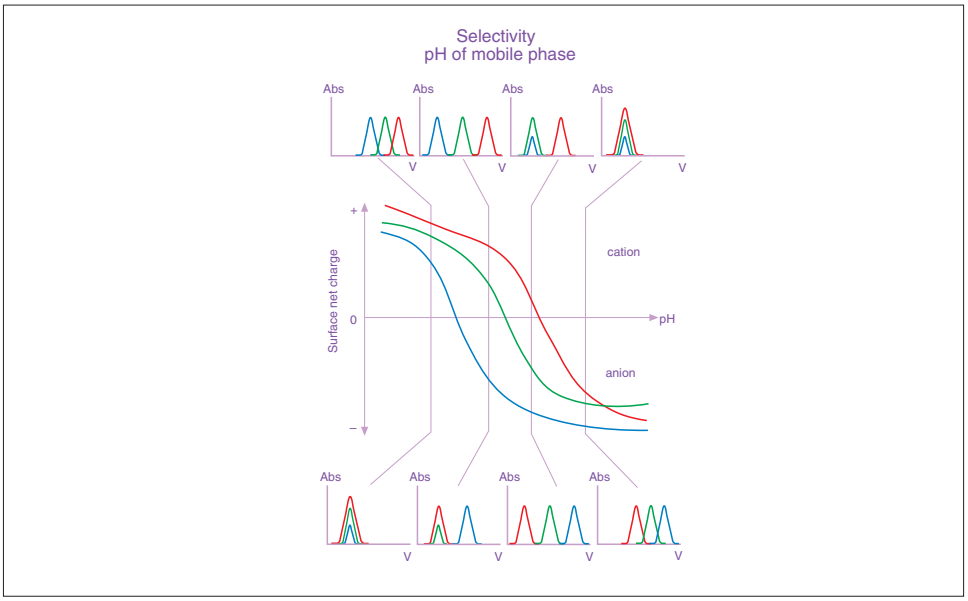


Fig 32. Effect of pH on protein elution patterns.

Choice of ion exchanger

For most purification steps it is recommended to begin with a strong exchanger, allowing work over a broad pH range during method development. Use a strong anion exchanger (Q) to bind the target if the isoelectric point is below pH 7.0 or unknown.

Strong ion exchangers

Q (anion exchange), S and SP (cation exchange) are fully charged over a broad pH range (pH 2–12).

Weak ion exchangers

DEAE (anion exchange) and CM (cation exchange) are fully charged over a narrower pH range (pH 2–9 and pH 6–10, respectively), but give alternative selectivities for separations.

Sample volume and capacity

Ion exchange chromatography is a binding technique, independent of sample volume provided that the ionic strength of the sample is low and the target molecule is highly charged. The total amount of protein which is loaded and binds to the column should not exceed the total binding capacity of the column. For optimal separations when performing gradient elution, use approximately one fifth of the total binding capacity of the column.

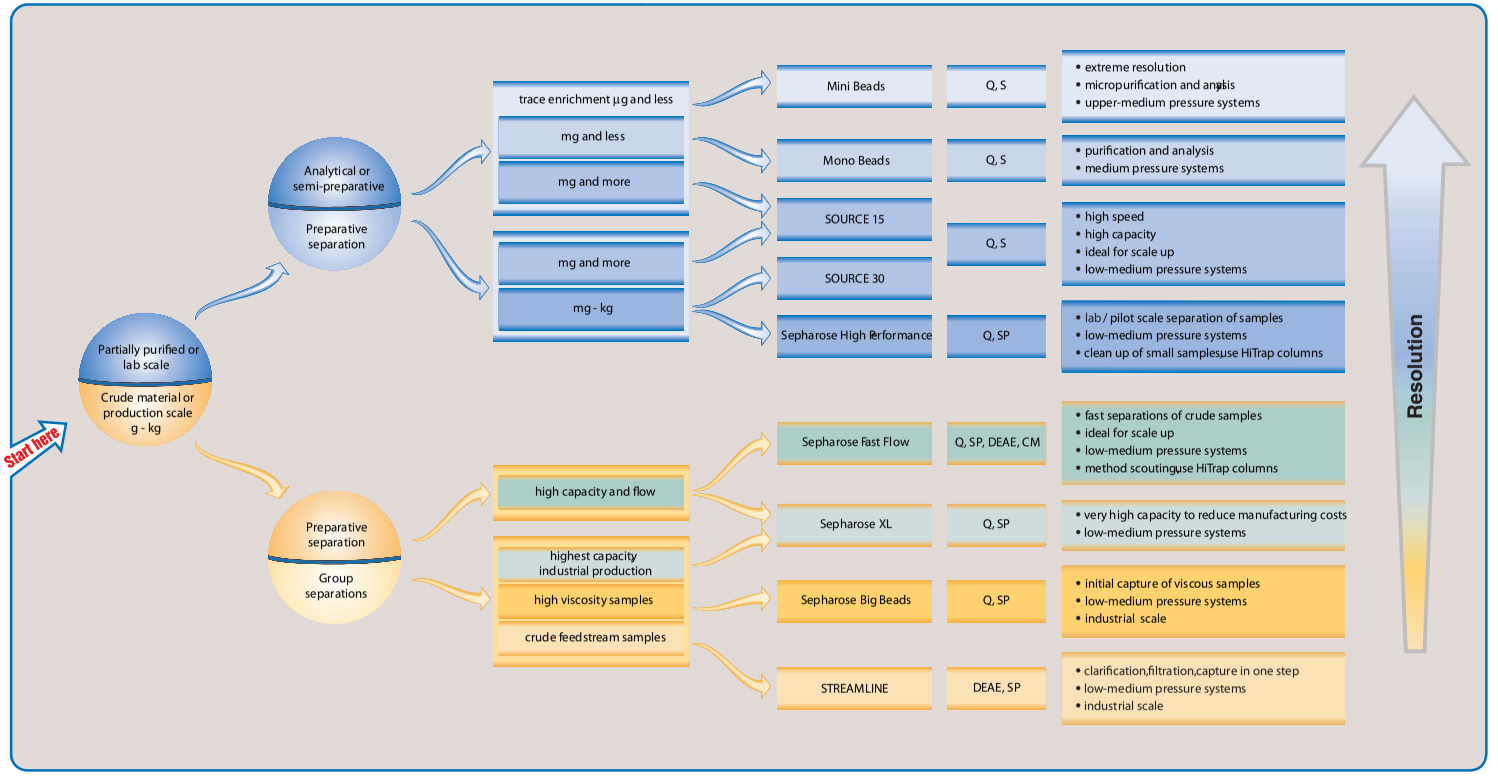


Fig 33. Ion exchange media selection guide. (Code no. 18-1127-31)

Media selection

Parameters such as scale of purification, resolution required, speed of separation, sample stability and media binding capacity, should be considered when selecting a chromatographic medium. Figure 33 on page 75 shows a guide to selecting ion exchange media.

Sample Preparation

Correct sample preparation ensures good resolution and extends the life of the column. To ensure efficient binding during sample application samples should be at the same pH and ionic strength as the starting buffer. Samples must be free from particulate matter, particularly when working with bead sizes of 34 µm or less (see page 65 for details of sample clarification procedures).

Column Preparation

Prepacked columns

To increase speed and efficiency in method development, use small prepacked columns for media scouting and method optimisation. HiTrap IEX Test Kit is ideal for this type of work, as shown in Figure 34.

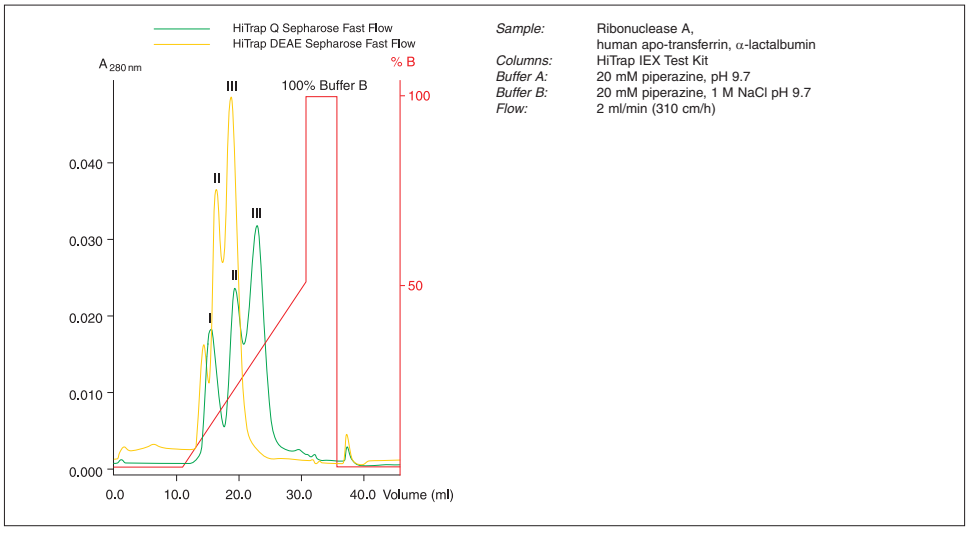


Fig 34. Media selection using 2 columns from HiTrap IEX Test Kit.

Using prepacked columns at any scale will ensure reproducible results and high performance.

Column packing

The following guidelines apply at all scales of operation:

Column dimensions = typically 5 - 15 cm bed height.

Quantity of gel = estimate amount of gel required to bind the sample, use five times this amount to pack a column.

See individual product packing instructions for more detailed information on a specific medium.

Buffer Preparation

Buffering ions should have the same charge as the selected medium, with a pKa within 0.6 pH units of the working pH. Buffer concentration should be sufficient to maintain buffering capacity and constant pH during sample application and while an increase in salt concentration is applied.

When working with a sample of unknown charge characteristics, try these conditions first:

Anion Exchange

Gradient: 0-100% elution buffer B in 10 - 20 column volumes
Start buffer A: 20 mM Tris-HCl, pH 8.0
Elution buffer B: 20 mM Tris-HCl + 1 M NaCl, pH 8.0

Cation Exchange

Gradient: 0-100% elution buffer B in 10 - 20 column volumes
Start buffer A: 20 mM $Na_2HPO_4.2H_2O$, pH 6.8
Elution buffer B: 20 mM $Na_2HPO_4.2H_2O$ + 1 M NaCl, pH 6.8

Method Development (in priority order)

1. Select the optimum ion exchanger using small columns such as prepacked HiTrap columns to save time and sample.
2. Scout for optimum pH. Begin 0.5-1 pH unit away from the isoelectric point of the target protein if known (see Figure 32 showing changes in elution versus pH).
3. Select the steepest gradient to give acceptable resolution at the selected pH.
4. Select the highest flow rate which maintains resolution and minimises separation time. Check recommended flow rates for the specific medium.
5. For large scale purification and to reduce separation times and buffer consumption, transfer to step elution after method optimisation as shown in Figure 35. It is often possible to increase sample loading when using step elution.

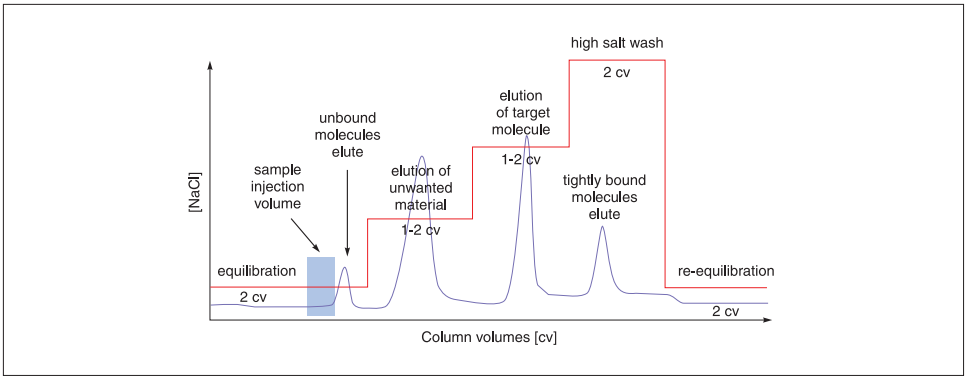


Fig 35. Step elution.

Cleaning, sanitisation and sterilisation

Procedures vary according to type of sample and medium. Guidelines are supplied with the medium or prepacked column.

Storage of media and columns

Recommended conditions for storage are supplied with the medium or prepacked column.

Further information

Ion Exchange Chromatography: Principles and Methods Code no. 18-1114-21.

Hydrophobic Interaction Chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The technique is ideal for the capture or intermediate steps in a purification. The separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatographic medium. This interaction is enhanced by high ionic strength buffer which makes HIC an ideal 'next step' after precipitation with ammonium sulfate or elution in high salt during IEX. Samples in high ionic strength solution (e.g. 1.5 M ammonium sulphate) bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially. Elution is usually performed by decreases in salt concentration (Figure 36). Changes are made stepwise or with a continuous decreasing salt gradient. Most commonly, samples are eluted with a decreasing gradient of ammonium sulphate. Target proteins are concentrated during binding and collected in a purified, concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropic species (urea, guanidine hydrochloride) or detergents, changing pH or temperature.

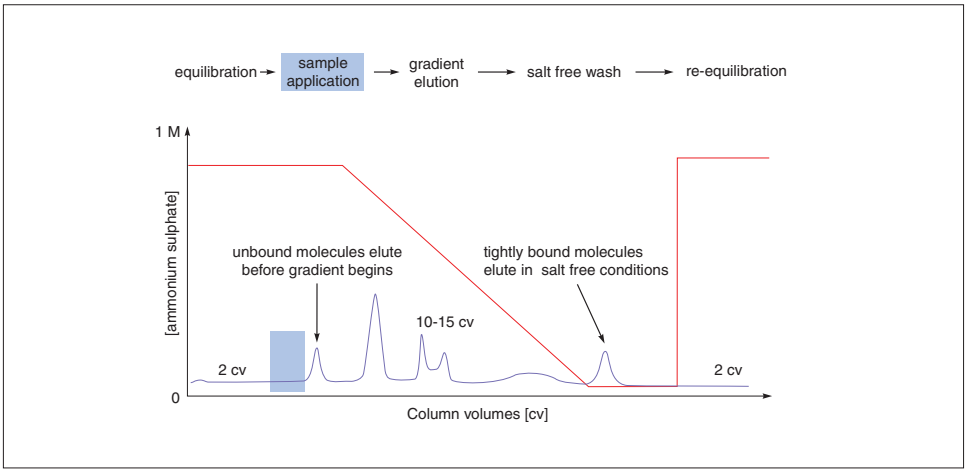


Fig 36. Typical HIC gradient elution

Choice of hydrophobic ligand

Very hydrophobic proteins bind tightly to very hydrophobic ligands and may require extreme elution conditions, e.g. chaotropic agents or detergents, for the target protein or contaminants. To avoid this problem it is recommended to screen several hydrophobic media, using HiTrap HIC Test Kit or RESOURCE HIC Test Kit. Begin with a medium of low hydrophobicity if the sample has very hydrophobic components. Select the medium which gives the best resolution and loading capacity at a reasonably low salt concentration. Typically the strength of binding of a ligand to a protein increases in the order: ether, isopropyl, butyl, octyl, phenyl. However, the nature of the binding, both the selectivity and the binding strength, can vary and must be tested in individual cases.

Sample volume and capacity

HIC is a binding technique and therefore rather independent of sample volume, provided that conditions are chosen to bind the target protein strongly.
The total amount of protein which is loaded and binds to the column should not exceed the total binding capacity of the column. For optimal separations when performing gradient elution, use approximately one fifth of the total binding capacity of the column

Media selection

In HIC the characteristics of the chromatographic matrix as well as the hydrophobic ligand affect the selectivity of the medium. This should be considered, together with parameters such as sample solubility, required resolution, scale of purification and availability of the medium at the scale intended. Figure 37 on page 81 shows a guide to selecting HIC media.

Sample Preparation

Correct sample preparation ensures good resolution and extends the life of the column. To ensure efficient binding during sample application samples should be at the same pH as the starting buffer and in high ionic strength solution (e.g. 1.5 M ammonium sulphate or 4 M NaCl). Samples must be free from particulate matter, particularly when working with bead sizes of 34 µm or less (see page 65 for details of sample clarification procedures).

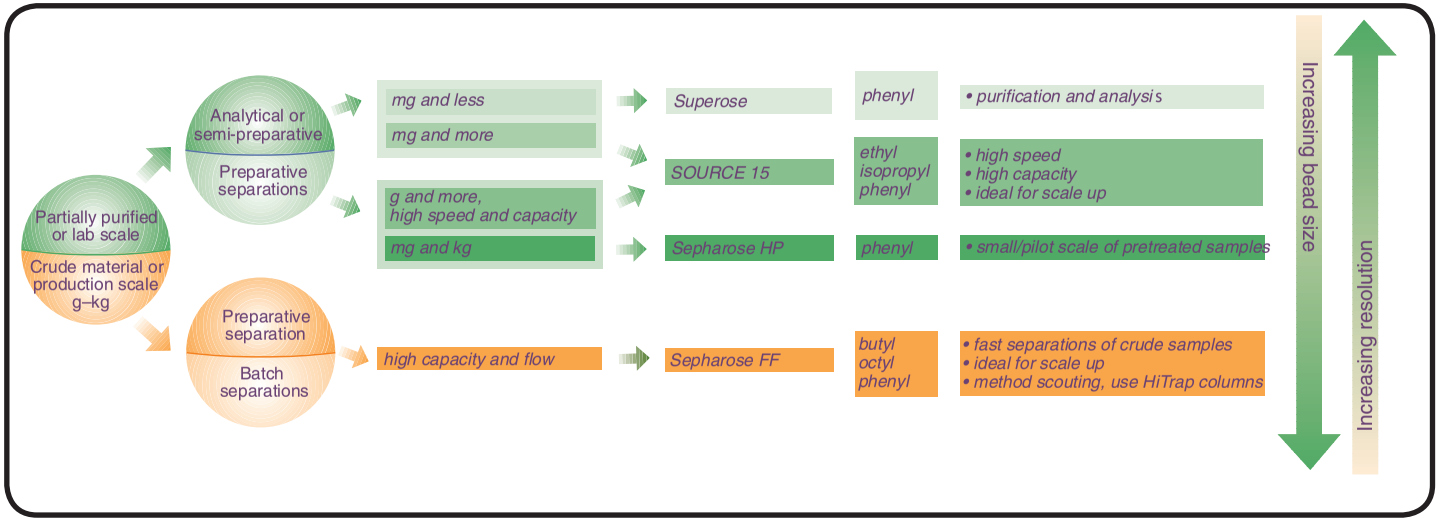
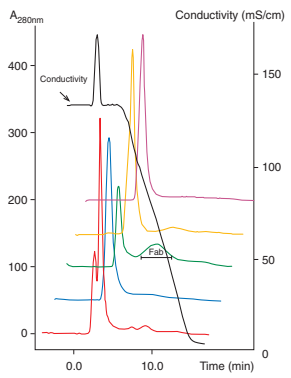


Fig 37. Hydrophobic interaction media selection guide.

Column Preparation

Prepacked columns

To increase speed and efficiency in method development use small Prepacked columns for media scouting and method optimisation. HiTrap HIC Test Kit and RESOURCE HIC Test Kit are ideal for this work. Using Prepacked columns at any scale will ensure reproducible results and high performance. Figure 38 shows an example of media screening with HiTrap HIC Test Kit



System: ÄKTAexplorer
Sample: Fab fraction from STREAMLINE SP, 2 ml
Columns: HiTrap HIC Test Kit (1 ml columns),
Phenyl Sepharose High Performance,
Phenyl Sepharose 6 Fast Flow (low sub),
Phenyl Sepharose 6 (high sub),
Butyl Sepharose 4 Fast Flow, Octyl Sepharose 4 Fast Flow
Buffer A: 1 ml $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Buffer B: 50 mM NaAc, pH 5.0
Gradient: 20 column volumes
Flow: 2 ml/min (300 cm/hr)

Fig 38. Media screening with HiTrap HIC Test Kit.

Column packing

The following guidelines apply at all scales of operation:
Column dimensions = typically 5–15 cm bed height.
Quantity of gel = estimate amount of gel required to bind the sample, use five times this amount to pack a column.
See individual product packing instructions for more detailed information on a specific medium.

Buffer Preparation

Buffering ion selection is not critical for hydrophobic interaction. Select a pH compatible with protein stability and activity. Buffer concentration must be sufficient to maintain pH during sample application and changes in salt concentration.

When working with a sample of unknown hydrophobic characteristics, try these conditions first:

Gradient: 0–100% elution buffer B in 10–20 column volumes
Start buffer A: 50 mM sodium phosphate pH 7.0 + 1–1.5 M ammonium sulphate
Elution buffer B: 50 mM sodium phosphate pH 7.0

Method Development (in priority order)

1. The hydrophobic behavior of a protein is difficult to predict and binding conditions mst be studied carefully. Use a HiTrap HIC Text Kit or a RESOURCE HIC Test Kit to select the medium which gives optimum binding and elution over the required range of salt concentration. For proteins with unknown hydrophobic properties begin with 0-100%B (0%B=1 M ammonium sulphate).
2. Select the gradient which gives acceptable resolution.
3. Select the highest flow rate which maintains resolution and minimises separation time. Check recommended flow rates for the specific medium.
4. For large scale purification and to reduce separation times and buffer consumption, transfer to a step elution after method optimisation, as shown in Figure 39. It is often possible to increase sample loading when using step elution, an additional benefit for large scale purification.

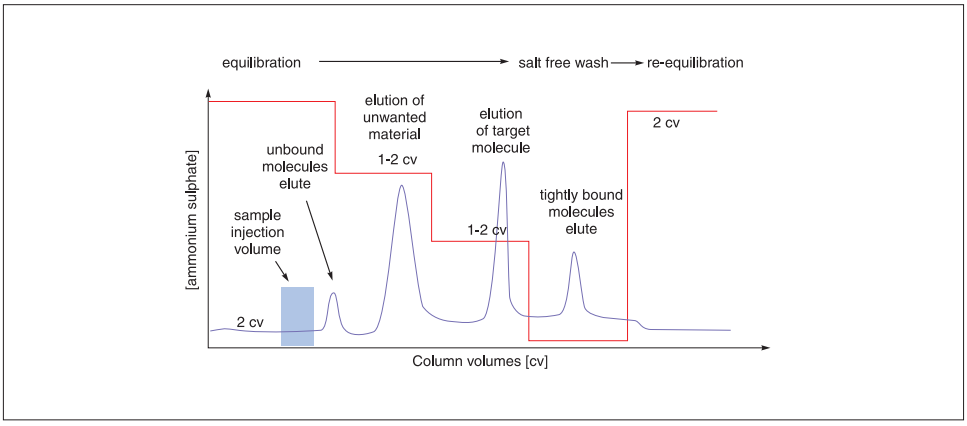


Fig 39. Step elution.

5. If samples adsorb strongly to a gel then conditions which cause conformational changes, such as pH, temperature, chaotropic ions or organic solvents can be altered. Conformational changes caused by these agents are specific to each protein. Use screening procedures to investigate the effects of these agents. Alternatively, change to a less hydrophobic medium.

Cleaning, sanitisation and sterilisation

Procedures vary according to type of sample and medium. Guidelines are supplied with the medium or prepacked column.

Storage of media and columns

Recommended conditions for storage are supplied with the medium or prepacked column.

Further information

Hydrophobic Interaction Chromatography: Principles and Methods
Code no. 18-1020-90

Affinity Chromatography (AC)

AC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand attached to a chromatographic matrix. The technique is ideal for a capture or intermediate step and can be used whenever a suitable ligand is available for the protein(s) of interest. AC offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest.

The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favour specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Desorption is performed specifically, using a competitive ligand, or non specifically, by changing the pH, ionic strength or polarity. Samples are concentrated during binding and protein is collected in purified, concentrated form. The key stages in a separation are shown in Figure 40. Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose 6B removes serine proteases.

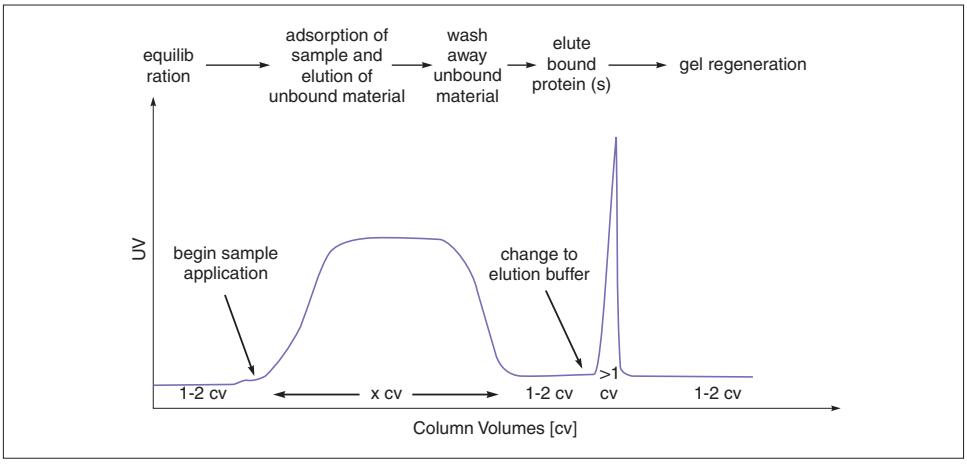


Fig 40. Typical affinity separation.

Sample volume and capacity

AC is a binding technique, independent of sample volume provided that conditions are chosen to bind the target protein strongly.

Media selection

Parameters such as scale of purification and commercial availability of affinity matrices should be considered when selecting affinity media. To save time and ensure reproducibility, use prepacked columns for method development or small scale purification. HiTrap affinity columns are ideal for this work. Table 6 on page 34 shows examples of prepacked affinity columns. Specific affinity media are prepared by coupling a ligand to a selected gel matrix, following recommended coupling procedures.

Further details on other affinity media are available in the Affinity Chromatography Product Profile (Code No. 18-1121-86).

Sample Preparation

Correct sample preparation ensures efficient binding and extends the life of a column. Removal of contaminants which may bind non-specifically to the column, such as lipids, is crucial. Stringent washing procedures may damage the ligand of an affinity medium, destroying the binding capacity of the column. Samples must be free from particulate matter (see Chapter 8 for details of sample clarification procedures).

Column Preparation

Prepacked columns

Prepacked columns ensure reproducible results and highest performance.

Column packing

The following guidelines apply at all scales of operation:

Column dimensions = short and wide.

Quantity of gel = calculate according to known binding capacity of medium, use 2-5 times excess capacity.

See individual product packing instructions for more detailed information on a specific medium.

Buffer Preparation

Binding, elution and regeneration buffers are specific to each affinity medium. Follow instructions supplied with the medium or column.

1. Select the correct specificity for the target protein. Follow the manufacturer's instructions for binding or elution conditions and check recommended flow rates for the specific medium.
2. Select optimum flow rate to achieve efficient binding
3. Select optimum flow rate for elution to maximise recovery.
4. Select maximum flow rate for column regeneration to minimise run times.

Cleaning, sanitisation and sterilisation

Procedures vary according to type of sample and medium. Guidelines are supplied with the medium or prepacked column.

Storage of media and columns

Follow the manufacturer's instructions.

Further information

Affinity Chromatography: Principles and Methods Code no. 18-1022-29.

Gel Filtration (GF)

GF separates proteins with differences in molecular size. The technique is ideal for the final polishing steps in a purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient Figure 41). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in purified form in the chosen buffer.

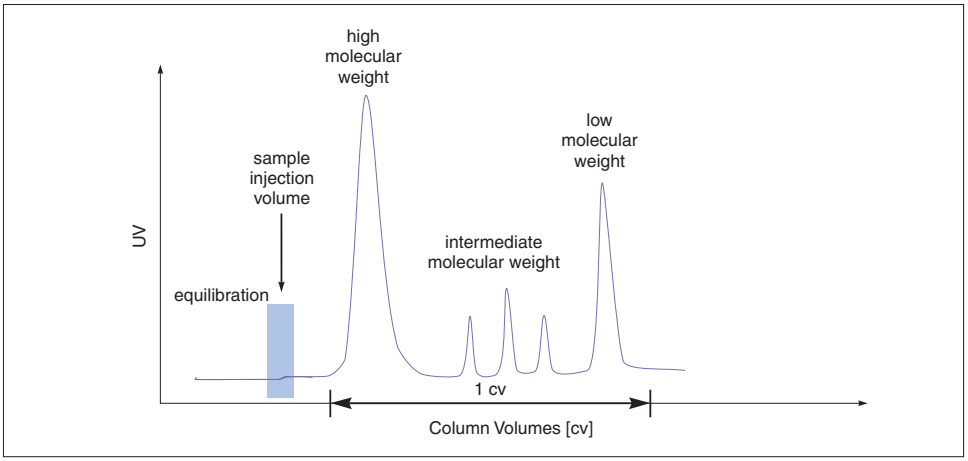


Fig 41. Typical GF elution.

Sample volume and capacity

To achieve the highest resolution the sample volume must not exceed 5% of the total column volume. Gel filtration is independent of sample concentration, although above 50 mg/ml protein viscosity effects may cause 'fingering'. Extremely viscous samples should be diluted.

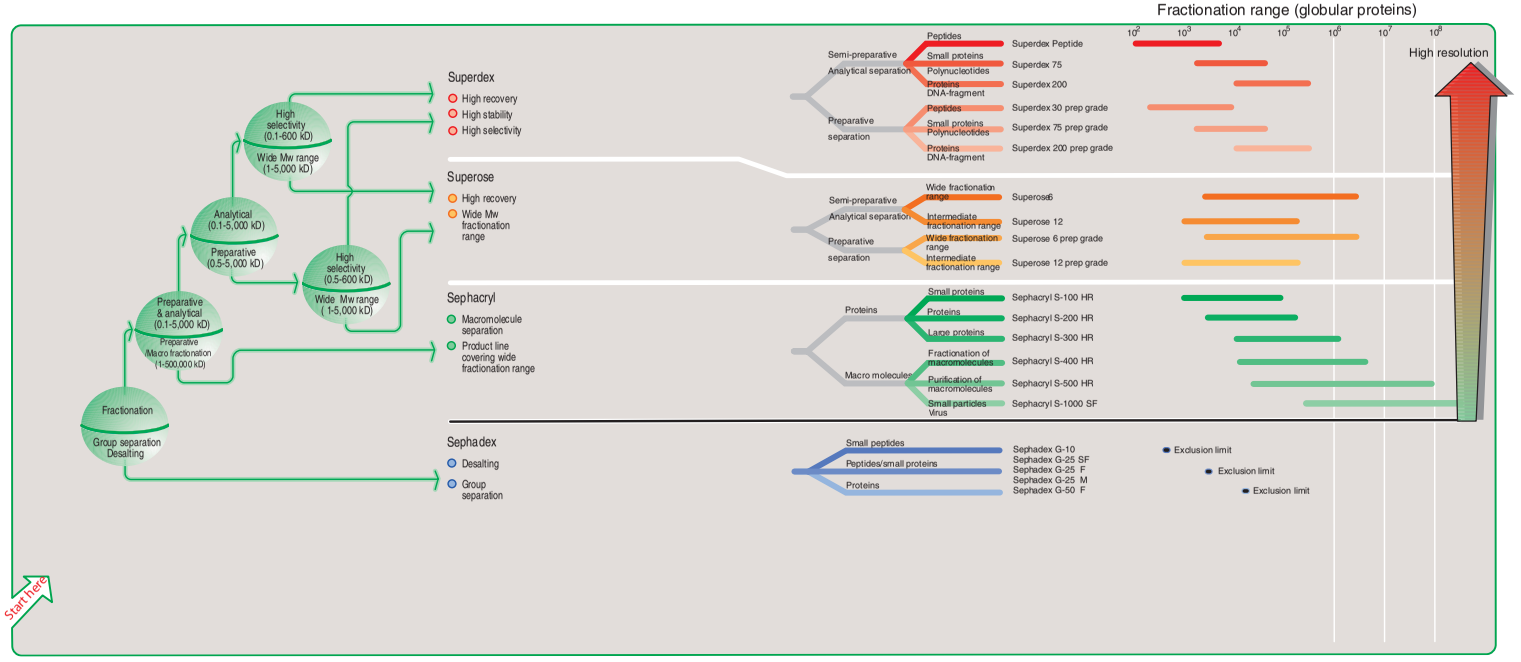


Fig 42. Gel filtration media selection guide Code: 18-1124-19.

Media selection

Parameters such as molecular weight of target proteins and contaminants, resolution required, scale of purification should be considered when selecting gel filtration media. Figure 42 on page 89 shows a guide to selecting of GF media.

Sample Preparation.

Correct sample preparation ensures good resolution and extends the life of the column. Sample buffer composition does not directly affect resolution. During separation the sample buffer is exchanged with buffer in the column. Viscous samples, which could cause an increase in back pressure and affect column packing, should be diluted. Samples must be free from particulate matter, particularly when working with bead sizes of 34 µm or less (see page 65 for details of sample clarification procedures)

Column Preparation

Prepacked columns

Prepacked columns ensure reproducible results and highest performance.

Column packing

In gel filtration good column packing is essential. The resolution between two separated zones increases as the square root of column length. The following guidelines apply:

Column dimensions: =	minimum 50 cm bed height (Sephacryl™) minimum 30 cm bed height (Superdex, Superose)
Bed volume =	depending on sample volume per run (up to 5% of bed volume)

See individual product packing instructions for more detailed information on a specific medium.

Buffer Preparation

Selection of buffering ion does not directly affect resolution. Select a buffer in which the purified product should be collected and which is compatible with protein stability and activity.
Buffer concentration must be sufficient to maintain buffering capacity and constant pH.
Ionic strength can be up to 150 mM NaCl in the buffer, to avoid non-specific ionic interactions with the matrix (shown by delays in peak elution).

When working with a new sample try these conditions first
Buffer: 50 mM sodium phosphate, pH 7.0 + 0.15 M NaCl
or select the buffer in which the sample should be eluted for the next step

Method Development (in priority order)

1. Select the medium which gives the best separation of target proteins from contaminants.
2. Select the highest flow rate which maintains resolution and minimises separation time. Check recommended flow rates for the specific medium. Lower flow rates improve resolution of high molecular weight components, faster flow rates may improve resolution of low molecular weight components
3. Determine the maximum sample volume which can be loaded without reducing resolution (sample volume should be 0.5-5% of total column volume).
4. To further improve resolution increase column length by connecting two columns in series

Cleaning, sanitisation and sterilisation

Procedures vary according to type of sample and medium. Guidelines are supplied with the medium or prepacked column.

Storage of media and columns

Recommended conditions for storage are supplied with the medium or prepacked column.

Further information

Gel Filtration: Principles and Methods Code no. 18-1022-18.

Reversed Phase Chromatography (RPC)

RPC separates proteins and peptides with differing hydrophobicity based on their reversible interaction with the hydrophobic surface of a chromatographic medium. Samples bind as they are loaded onto a column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrices, the binding is usually very strong and requires the use of organic solvents and other additives (ion pairing agents) for elution. Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile.

Samples, which are concentrated during the binding and separation process, are collected in a purified, concentrated form. The key stages in a separation are shown in Figure 43.

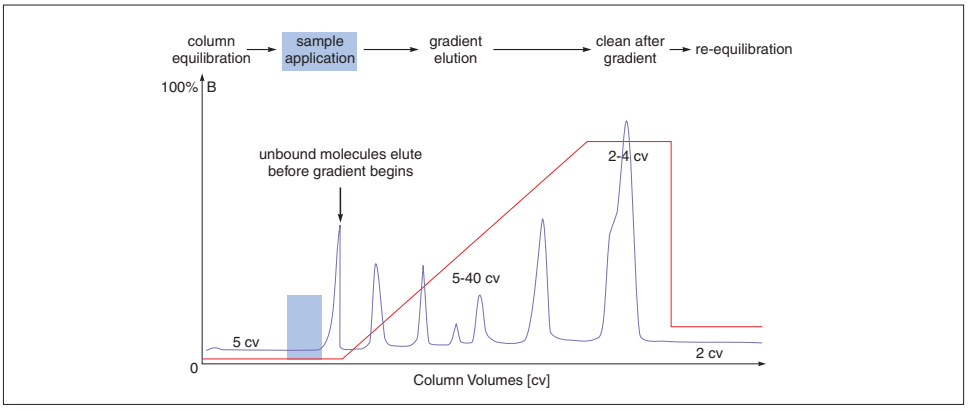


Fig 43. Typical RPC gradient elution.

RPC is often used in the final polishing of oligonucleotides and peptides and is ideal for analytical separations, such as peptide mapping.

RPC is not recommended for protein purification if recovery of activity and return to a correct tertiary structure are required, since many proteins are denatured in the presence of organic solvents.

Choice of hydrophobic ligand

Select hydrocarbon ligands according to the degree of hydrophobicity required. Highly hydrophobic molecules bind tightly to highly hydrophobic ligands, e.g. C18.
Screen several RPC media. Begin with a medium of low hydrophobicity, if the sample has very hydrophobic components (more likely with larger biomolecules, such as proteins). Select the medium which gives the best resolution and loading capacity. A polymer based medium such as SOURCE RPC can offer significant advantages over silica based media as it can be used across the pH range 1-14 providing not only an alternative selectivity to silica but also a wider working pH range for method optimisation.

Sample volume and capacity

RPC is a binding technique, often independent of sample volume. Total capacity is strongly dependent upon experimental conditions and the properties of the gel and sample. For optimal conditions during gradient elution, screen for a sample loading which does not reduce resolution.

Media selection

In RPC the chromatographic medium as well as the hydrophobic ligand affect selectivity. Screening of different RPC media is recommended.

Sample Preparation

Samples should be free from particulate matter and, when possible, dissolved in the start buffer.

Column Preparation

Reversed phase columns should be 'conditioned' for first time use, after long term storage or when changing buffer systems.

Buffer Preparation

Try these conditions first when sample characteristics are unknown:

Gradient: 2-80% elution buffer B in 20 column volumes
Start buffer A: 0.065% TFA (trifluoroacetic acid) in water
Elution buffer B: 0.05% TFA in acetonitrile

Method Development

1. Select medium from screening results.
2. Select gradient to give acceptable resolution. For unknown samples begin 0–100%B.
3. Select the highest flow rate which maintains resolution and minimises separation time.
4. For large scale purification transfer to a step elution.
5. Samples which adsorb strongly to a gel are more easily eluted from a less hydrophobic medium.

Cleaning, sanitisation and sterilisation

Procedures vary according to type of sample and medium. Guidelines are supplied with the medium or prepacked column.

Storage of media and columns

Recommended conditions for storage are supplied with the medium or prepacked column.

Further information

Visit www.gelifesciences.com

Expanded Bed Adsorption (EBA)

EBA is a single pass operation in which target proteins are purified from crude sample, without the need for separate clarification, concentration and initial purification to remove particulate matter. Crude sample is applied to an expanded bed of STREAMLINE adsorbent particles within a specifically designed STREAMLINE column. Target proteins are captured on the adsorbent.
Cell debris, particulate matter, whole cells, and contaminants pass through and target proteins are then eluted.
Figure 44a shows the steps involved in an EBA purification and Figure 44b shows a typical EBA elution pattern.

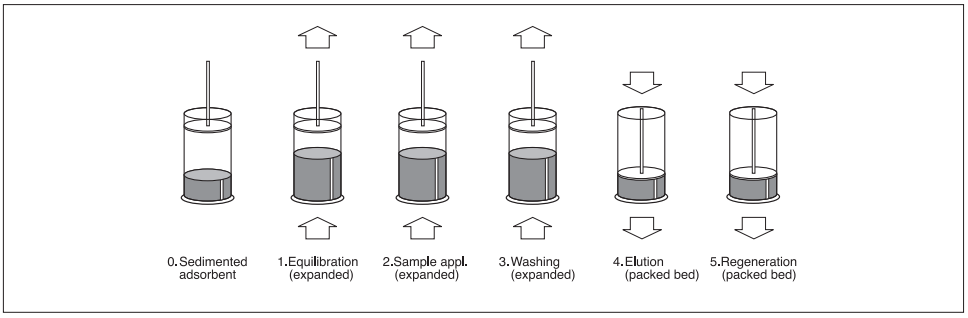


Fig 44a. Steps in an EBA purification process.

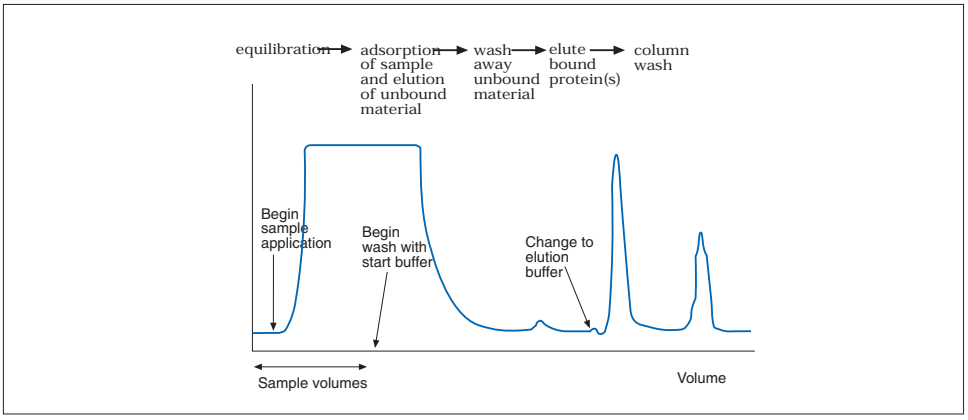


Fig 44b. Typical EBA elution.

Selection of STREAMLINE adsorbent

Selection of adsorbent is based on the same principles that are used for chromatography. Select the medium with the strongest binding to the target protein and which binds as few of the contaminants as possible, i.e. the medium with the highest selectivity and/or capacity for the protein of interest.

Sample volume and capacity

All STREAMLINE media are binding techniques, independent of sample volume. The total amount of protein which is loaded should not exceed the total binding capacity of the column.

Sample Preparation

STREAMLINE is able to handle crude, particulate feedstock, reducing the need for significant sample preparation steps. Adjustment of pH or ionic strength may be required according to the separation principle being used (IEX, AC, HIC)

Column Preparation

For preliminary method scouting STREAMLINE media is used in packed bed mode in an XK 16 or XK 26 chromatography column. When used in expanded bed mode the media must be packed in specially designed STREAMLINE columns, following the manufacturer's instructions.

Buffer Preparation

Buffer preparation will depend upon the chosen separation principle.

Method Development

1. Select suitable ligand to bind the target protein.
2. Scout for optimal binding and elution conditions using clarified material in a packed column (0.02 - 0.15 litres bed volume of media). Gradient elutions may be used during scouting, but the goal is to develop a step elution.
3. Optimise binding, elution, wash and cleaning-in-place procedures using unclarified sample in expanded mode at small scale (0.02 - 0.15 litres bed volume of media)
4. Begin scale up process at pilot scale (0.2 - 0.9 litres bed volume of media)
5. Full scale production (up to several hundred litres bed volume of media)

Cleaning, sanitisation and sterilisation

Guidelines are supplied with each STREAMLINE adsorbent.

Storage of STREAMLINE adsorbents and columns

Recommended conditions for storage are supplied with STREAMLINE adsorbents and columns.

Further Information

Expanded Bed Adsorption: Principles and Methods Code No. 18-1124-26

BioProcess Media- for large scale production

Specific BioProcess Media have been designed for each chromatographic stage in a process from Capture to Polishing. Large capacity production integrated with clear ordering and delivery routines ensure that BioProcess media are available in the right quantity, at the right place, at the right time. GE Healthcare can assure future supplies of BioProcess Media, making them a safe investment for long term production. The media are produced following validated methods and tested under strict control to fulfil high performance specifications. A certificate of analysis is available with each order.

Regulatory support files contain details of performance, stability, extractable compounds and analytical methods. The essential information in these files gives an invaluable starting point for process validation, as well as providing support for submissions to regulatory authorities. Using BioProcess Media for every stage results in an easily validated process. High flow rates, high capacity and high recovery contribute to the overall economy of an industrial process.
All BioProcess Media have chemical stability to allow efficient cleaning and sanitisation procedures. Packing methods are established for a wide range of scales and compatible large scale columns and equipment are available.