Ammonium sulfate fractionation

The solubility of proteins varies according to the ionic strength and hence according to the salt concentration of the solution. Two distinct effects are observed. At low concentrations of salt, the solubility of the protein increases with salt concentration. This phenomenon is called 'salting-in'. However, as the salt concentration (ionic strength) is increased still further, the solubility of the protein begins to decrease. At sufficiently high ionic strength the protein solubility will have decreased to the point where the protein will be almost completely precipitated from solution - an effect called 'salting-out'. The theoretical basis of salting-out is complex but one factor is probably the competition between the protein and salt ions for available water molecules. At high salt concentrations, insufficient water molecules are available for full solvation of the protein so that protein protein interactions become predominant over protein-water interactions, and precipitation occurs.

Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein. Indeed, enzyme purification schemes almost invariably include such a step. In practice, ammonium sulfate is the salt commonly used since it is highly water-soluble, relatively cheap and available at high purity. Furthermore it has no adverse effects upon enzyme activity. Typically a small-scale experiment is conducted initially to determine the optimal ammonium sulfate concentration to use. This procedure is typically carried out at 0 - 4ºC to maximize protein stability. Great care must be taken to ensure that the salt concentration of the whole solution increases uniformly without the occurrence of local high concentrations which could precipitate the protein of interest along with the undesired proteins. Therefore the solution is stirred continuously as small aliquots of crushed, solid ammonium sulfate (or preferably saturated ammonium sulfate solution) are added. After each addition, the ammonium sulfate is allowed to disperse fully before the next addition. Once the required ammonium sulfate concentration is reached, incubation at 0 - 4ºC is continued for a brief period to allow protein precipitation to occur, and the precipitated protein is then recovered by centrifugation. The concentration of ammonium sulfate in the solution is thus increased stepwise, recovering the precipitated protein at each stage. Each protein precipitate is dissolved individually in fresh buffer and assayed for both total protein content and the activity of the desired enzyme. Based on these data one can then plan a successful ammonium sulfate fractionation procedure for the bulk purification of the enzyme. An ammonium sulfate concentration is chosen which will precipitate the maximum proportion of undesired protein whilst leaving most of the enzyme still in solution. This precipitated protein is removed by centrifugation and then the ammonium sulfate concentration of the remaining solution is increased to a value that will precipitate most of the enzyme whilst leaving the maximum amount of residual protein contaminants still in solution. The precipitated enzyme is recovered by centrifugation and dissolved in fresh buffer for the next stage of purification. Residual ammonium sulfate will be present in this enzyme solution and may need to be removed by gel filtration or dialysis before the next purification step can be attempted.

In addition to its role as an extremely useful and universally applicable purification step, ammonium sulfate purification is often employed again at later stages of purification simply to concentrate the protein from dilute solution after procedures such as gel filtration.

Heat treatment

Protein purification procedures are usually carried out at low temperature (0 - 4ºC) since most proteins are stable at low temperature. As the temperature increases from 0ºC to 37 - 40ºC their stability decreases significantly. Above 40ºC or so, most proteins become increasingly unstable and denature, and at neutral pH, the denatured proteins usually precipitate.

Individual proteins differ in their heat sensitivity and so this can be used for purification purposes. The temperature stability of the desired enzyme is determined by a trial experiment following enzyme activity in the cell extract after incubation at different temperatures for a set period of time. The minimum temperature at which gross inactivation occurs is noted. Once this temperature is known, less stable

proteins can be preferentially inactivated by incubating the cell extract at a temperature 5 - 10ºC below this value for 15 - 30 min. The denatured precipitated protein is then removed by centrifugation.

It should be noted that the heat stability of the desired enzyme may be increased during heat denaturation by the presence of its substrate, product or a competitive inhibitor which bind to the active site and help stabilize the protein conformation. Since denaturation of cell proteins occurs to some extent at all temperatures and simply increases with increasing temperature, the total activity of the desired enzyme usually falls to some extent after a heat denaturation step. This procedure is therefore rather crude. However, it may be a useful early step for the purification of rather more heat-stable proteins.

Two-dimensional gel electrophoresis

Gel electrophoretic separation of polypeptides can be achieved on the basis of size and charge (gel electrophoresis under non-dissociating conditions), size alone (SDS-PAGE) or charge alone (isoelectric focusing, IEF). Irrespective of the method chosen, there is a real possibility that two or more polypeptides may co-migrate as a single band, particularly if the original sample is a complex mixture of proteins. This co-migration of proteins will mask the complexity of a protein mixture and so can lead to incorrect conclusions as to the purity of protein samples.

Two-dimensional gel electrophoretic methods have been devised which separate proteins on the basis of charge in one dimension followed by separation on the basis of size in a second dimension. The first-dimensional separation on the basis of charge is carried out by electrophoresis of the protein sample in a rod polyacrylamide gel, usually by IEF. After electrophoresis, this rod gel is arranged to lie horizontally against the top edge of the stacking gel of a slab gel prepared for SDS-PAGE. During this second dimensional electrophoresis, the polypeptides separated by charge in the rod gel during the first stage electrophoresis now migrate into the slab gel where they separate on the basis of size. Either uniform-concentration polyacrylamide slab gels or concentration-gradient slab gels may be used for the second dimensional SDS-PAGE step. The separated proteins appear as spots after staining. If one measures the pH gradient in the first-dimensional rod gel and also co-electrophoreses polypeptides of known molecular weight down the side of the slab gel during the second-dimensional SDS-PAGE step, one can estimate both the isoelectric point and the molecular weight of any sample polypeptide of interest simply by noting its horizontal position and vertical position respectively.

Because of the two-stage separation involved, two-dimensional polyacrylamide gel electrophoresis is the method of highest resolution of all the protein electrophoretic methods in current use. As such it is an extremely valuable test of purity of a protein sample. Thus, although SDS-PAGE or IEF may well be used to monitor the purity of a protein sample at several stages of a purification scheme, two-dimensional gel electrophoresis should be used as the final test when the protein of interest appears to be pure. Note, however, that there is no guarantee that a single spot after two-dimensional gel electrophoresis is only a single polypeptide; the use of two-dimensional gel electrophoresis simply minimises the possibility of co-migration of polypeptides. Indeed there are occasions when two or more polypeptides may well co-migrate, not because they have identical size and charge, but because they are interacting with each other and so migrate as an aggregate rather than individually. For example, proteins isolated from membranes may form very stable aggregates that resist the normal conditions of dissociation employed by IEF and SDS-PAGE. In these cases one may need to include additional reagents during sample preparation and electrophoresis, such as urea, to ensure complete dissociation of the proteins.

Isoelectric focusing

Isoelectric focusing, IEF, (often called electrofocusing) is a method for separating molecules which differ in their charge characteristics. For IEF of proteins, the protein mixture is subjected to an electric field in an inert support in which a stable pH gradient has previously been generated. The anode region is at a lower pH than the cathode and the pH range is chosen such that the proteins to be separated have their isoelectric points within this range. A protein which is in a pH region below its pI will be positively charged

and so will migrate towards the cathode. However, as it migrates, so the pH will decrease until the protein reaches a pH which is its pI. At this point it has no net charge and so migration ceases. Should it overshoot this point, it will enter a region of pH above its pI and so become negatively charged. It will then reverse its direction of migration and now migrate towards the anode. Therefore proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands.

The stable pH gradient between the electrodes is formed by including a mixture of low molecular weight 'carrier ampholytes' in the inert support. These are synthetic, aliphatic polyaminopolycarboxylic acids available commercially whose individual pI values cover a preselected pH range. Thus one can purchase carrier ampholytes spanning either a wide pH range (e.g. pH 3 - 10) or a narrow range (e.g. pH 7 - 8). Commercial ampholytes include Ampholine, BioLyte and Pharmalyte.

The exact format for IEF depends on the nature of the inert support which is used. Early applications of IEF used water-cooled vertical glass columns filled with carrier ampholytes in a sucrose density gradient. The sucrose density gradient was the inert support, the density gradient helping to stabilize the liquid column against convective mixing due to heat generated during electrophoresis. The upper electrode (anode) was connected to a reservoir of acid (e.g. phosphoric acid) and the lower electrode to a reservoir containing an alkaline solution (e.g. NaOH). After a period of electrophoresis to establish the pH gradient, the protein sample was introduced into the column and electrophoresis continued until the proteins had reached their pI's. The individual protein bands were then recovered by draining the column from its base into a series of fractions. This procedure is still used for some applications of preparative IEF although it is not widespread due to the high cost of the ampholytes used. Preparative IEF can also be carried out on a somewhat smaller scale (but still capable of producing milligram amounts of pure proteins) using horizontal flat beds of a gel bead matrix, such as Sephadex, in cooled glass troughs. After electrophoresis the gel is subdivided into fractions from which the proteins can be eluted with buffer.

Despite the above preparative approaches, IEF is mainly used as an analytical technique to assess the complexity or purity of protein samples. As with SDS-PAGE, however, if the amount of protein required in pure form is only of the order of a few micrograms, even analytical scale electrofocusing may be sufficient to prepare it. Analytical IEF is carried out either in vertical polyacrylamide rod gels (especially as the first-dimensional separation of two-dimensional gel electrophoresis) or in horizontal polyacrylamide or agarose slab gels.

Horizontal slab gel IEF is now the most widely used format for electrofocusing. Thin sheets of polyacrylamide or agarose gel mounted on glass or plastic plates and containing carrier ampholytes chosen to give the correct pH range can be purchased or prepared in the laboratory. It is important to avoid molecular sieving effects so that the protein separation occurs solely on the basis of charge. Hence agarose gels would be chosen in preference to polyacrylamide gels for the separation of larger proteins because of their larger pore size. If polyacrylamide gel is used, the gel concentration is chosen so as to give a large pore gel and so minimize molecular sieving. Gel thicknesses of 1 - 2 mm can be used but increasingly ultra-thin gels only 0.1 - 0.25 mm thick are being used since these are cheaper (carrier ampholytes are expensive) and allow shorter running times (since higher voltages can be used) and improved resolution.

The horizontal gels on their glass or plastic sheets are arranged on water-cooled plates since this allows the heat generated by electrophoresis to be readily dissipated and so avoid distortion of the separating protein bands. As with vertical polyacrylamide slab gels, multiple samples can be analysed side by side. Only a few micrograms of proteins are required for analysis. A simple procedure to load the samples onto the gel is to dip a small piece of filter paper into the sample and then lay this on the gel surface at the origin. A short period of electrophoresis now allows the proteins to enter the gel, the filter paper is removed and electrophoresis is continued for several hours to allow IEF to occur. The profile of separated proteins can be visualized after electrophoresis by fixing and staining the protein bands.

Unlike SDS-PAGE, IEF gives no information about the molecular weight of the separated polypeptides since separation occurs on the basis of charge alone. However, by measuring the pH gradient across the gel after electrophoresis using a surface electrode and recording the position of the separated protein of interest, one can estimate the pI of this protein; information which could be invaluable in planning any large-scale ion-exchange chromatography step in a purification scheme.

Gel filtration

A wide range of biological molecules can be separated on the basis of differences in their size and shape which lead to differences in their ability to penetrate porous matrices. This procedure is known as molecular sieve chromatography or molecular exclusion chromatography. A variety of porous matrices can be employed depending on the nature of the molecules to be fractionated. For protein purification purposes, the matrix typically consists of porous beads of an inert, highly hydrated gel and so the process is often referred to as gel filtration.

Common commercial gel matrices are Sephadex (dextran beads), Sepharose and Bio-Gel A (agarose) and Bio-Gel P (polyacrylamide), whilst other materials such as polyacryloylmorpholine and various polystyrenes have also been used. The dextran, agarose and polyacrylamide gel beads can all be manufactured with different degrees of porosity and so will fractionate different size ranges of proteins. The Sephadex dextran gels and Bio-Gel P polyacrylamide gels allow fractionation of globular proteins up to about 800,000 molecular weight whereas agarose gels, because of their greater porosity, can separate protein molecules and macromolecular complexes up to several million molecular weight. More recently, cross-linked dextran gels (Sephacryl) have been marketed which are exceptionally rigid, stable matrices which can fractionate proteins of molecular weights up to 8 million. The rigidity and stability of polyacrylamide gels can be improved by including agarose in the gel matrix. Polyacrylamide/agarose gels of this type are also available commercially (Ultrogel). New types of commercial gel are constantly being developed. The table below summarizes the properties of the matrices used in this program.

Characteristics of gel filtration media used in this simulation.

Matrix name	Bead type	Approximate fractionation range for peptides and globular proteins (molecular weight)
Sephadex G-50[1]	dextran	1500 - 30000
Sephadex G-100[1]	dextran	4000 - 150000
Sephacryl S-200 HR[1]	dextran/acrylamide	5000 - 250000
Ultrogel AcA 54[2]	polyacrylamide/agarose	6000 - 70000
Ultrogel AcA 44[2]	polyacrylamide/agarose	12000 - 130000
Ultrogel AcA 34[2]	polyacrylamide/agarose	20000 - 400000
Bio-Gel P-60[3]	polyacrylamide	3000 - 60000
Bio Gel P-150[3]	polyacrylamide	15000 - 150000
Bio-Gel P-300[3]	polyacrylamide	60000 - 400000

[1] Sephadex® and Sephacryl® are registered trademarks of GE Healthcare Bio-Sciences AB.

[2] Ultrogel® is a registered trademark of BF Biotechnics, Inc.

[3] Bio Gel® is a registered trademark of Bio-Rad Laboratories, Inc.

Fractionation of a protein mixture by gel filtration is typically carried out as follows. The gel beads, present as a slurry in the chosen buffer, are poured into a glass or plastic chromatography column of suitable dimensions and allowed to settle by gravity. After washing and equilibration of the column with buffer alone, the protein mixture in buffer is applied to the top of the column and the eluate is collected at the column base in a series of fractions. As the proteins pass down the column they penetrate the pores of the gel beads to different extents and so travel down the column at different rates. All proteins which exceed the maximum size of the pores will be unable to enter the beads. These proteins will therefore distribute only in the solution between the beads and elute from the column first in the so-called 'exclusion volume' (or void volume). All proteins below the minimum size of the pores will equilibrate completely with the buffer inside and outside the gel beads and so spend a proportion of their time inside the beads. These proteins will therefore move more slowly through the column and will be eluted last, together with any other small molecules present in the original protein mixture such as salt ions. The total volume of the column is called the bed volume. Therefore these proteins will elute in a volume of buffer very close to the bed volume of the column (although somewhat less due to the volume physically occupied by the gel matrix itself). The pores in the beads are not of exactly identical sizes but rather span a narrow range of sizes. Some proteins will have sizes very similar to the size range of the pores and so be excluded from some pores whilst entering others. These proteins of intermediate size will therefore be partially excluded from the beads to an extent that depends on their size and shape. They will elute from the column in order of molecular weight with the largest proteins eluting first and the smaller proteins later.

Careful choice of gel type is important for the optimal fractionation of complex protein mixtures. Use of an inappropriate gel type may lead to the protein of interest either being totally excluded from the gel beads and so eluting with many other proteins in the void volume or, conversely, it may be able to equilibrate with the whole gel volume and so elute with many other proteins in the salt fraction near the bed volume. In both situations the resolution of the fractionation is poor. Ideally the gel pore size should be such that the desired protein is partially excluded from the gel beads, a condition which leads to the greatest degree of fractionation from other protein species. For partially excluded proteins, a plot of log(protein molecular weight) versus K_{av}, leads to a straight line relationship, where

$$K_{av} = \frac{\text{elution volume - void volume}}{\text{total volume of the packed matrix - void volume}}$$

Linear relationship between K_{av} and log(molecular weight)

Thus a column can be calibrated with standard proteins of known molecular weight, noting their elution volumes, and these data used to draw such a graph. Subsequently, during fractionation of the sample protein mixture on the same column, measurement of the elution volume of the protein of interest allows its molecular weight to be deduced by reference to the standard curve. However, it should be noted that the shape of protein molecules also plays an important role in gel filtration. Long, extended polypeptides and proteins tend to behave as though they were larger, globular protein molecules. Therefore a calibration curve is only as accurate as the nature of the protein standards used to construct it will allow.

When first applying gel filtration for any particular protein purification, it is good practice to fractionate the protein mixture on several gel matrices of different porosity, collect fractions and assay each for protein content and enzyme activity. Empirically one can then choose a matrix that gives good resolution of the desired protein from other proteins whilst also yielding some information about its molecular weight - information which may prove extremely useful in subsequent purification steps.

Ion-exchange chromatography

An ion-exchange resin consists of an insoluble matrix with charge groups covalently attached. Both positively charged and negatively charged ion exchangers are commercially available. Negatively charged exchangers bind positively charged ions (cations). They can bind one type of cation but, when presented with a second type of cation, this may displace, or exchange with, the first. Hence these resins are called

cation-exchange resins. Similarly anion-exchange resins are positively charged and bind (and exchange) negatively charged ions (anions).

A Cation exchange resin with bound positive counterions

B Anion exchange resin with bound negative counterions

Several side-chain groups of the amino acid residues in proteins are ionizable (e.g. lysine or glutamic acid) as are the N-terminal amino and C-terminal carboxyl groups. Therefore proteins are charged molecules. This characteristic can be used to separate different proteins by ion-exchange chromatography. The two most commonly used resins for ion-exchange chromatography of proteins are carboxymethyl-cellulose (CM-cellulose) and diethylaminoethyl-cellulose (DEAE-cellulose). These are granular celluloses which have been chemically modified. CM-cellulose is a resin where the —CH_2OH groups of the carbohydrate have been converted to —CH_2OCH_2COOH groups. At neutral pH this group is ionized as —$CH_2OCH_2COO^-$ so that CM-cellulose is negatively charged, i.e. it is a cation exchanger. DEAE-cellulose contains an ionizable tertiary amine group instead. It is positively charged at neutral pH and so DEAE-cellulose is an anion exchanger. Closely related to these ion-exchange celluloses are DEAE-Sephadex and CM-Sephadex, containing the same ionizable groups covalently attached to a Sephadex bead matrix, and the covalently cross-linked agarose bead ion exchangers DEAE-Sepharose and CM-Sepharose. DEAE-cellulose is now itself also available in a bead form called DEAE-Sephacel. The Sephadex and Sepharose types are particularly useful for the separation of high molecular weight proteins. In practice, since these matrices are very similar to those used for gel filtration some molecular sieving may accompany the ion-exchange process. This may either enhance or reduce the effectiveness of the fractionation compared to using an ion-exchange cellulose. For simplicity in the computer simulation, molecular sieving is assumed not to occur during ion-exchange chromatography. Other ion-exchange resins using different ionizable groups from those described here are also commercially available. This computer simulation includes the following ion-exchange resins: CM-cellulose, DEAE-cellulose, Q-Sepharose Fast Flow and S-Sepharose Fast Flow. The charged group of Q-Sepharose is a quarternary amine which carries a non-titratable positive charge. Therefore this matrix can be used at alkaline pH values at which the positive charge of the DEAE group would have been titrated. The charged group of S-Sepharose is the sulphonyl group (—SO_3^-).

The fractionation of proteins by ion-exchange chromatography depends upon differences in the charge of different proteins. The charge of a protein depends upon the number and type of ionizable amino acid side chain groups. Lysine residues, for example, have a positively charged side chain group when ionized, whereas glutamic acid residues are negatively charged when ionized. Each ionizable side chain group has a distinct pK_a; that is, the pH at which it is half dissociated. Therefore the overall number of charges on a particular protein at a particular pH will depend on the number and type of ionizable amino acid side chain groups it contains. Since, by definition, different proteins have different amino acid compositions, they will tend to have different charges at a given pH and so can be fractionated on this basis.

For any one protein there will be a pH at which the overall number of negative charges equals the number of positive charges and so it has no net charge. This is its isoelectric point (pI), or more strictly speaking its isoionic point. At this pH the protein will not bind to any ion-exchange resin. Below this pH the protein will have a net positive charge and will bind to a cation exchanger, whilst above this pH it will have a net negative charge and bind to an anion exchanger. In principle, therefore, one could choose to use either a cation exchanger or an anion exchanger to bind the protein of interest. However, proteins are usually stable (and functionally active) only within a fairly narrow pH range so that the choice of ion exchanger is often dictated by the pH stability of the desired protein. If the protein is most stable at pH values below its pI, a cation exchanger should be used whereas if it is most stable at pH values above its pI, an anion exchanger would be chosen. Clearly, if it is stable over a wide pH range, use of either type of resin can be attempted and that which gives the best fractionation is selected.

When ion-exchange resins are purchased, the ions bound to the charged groups on the resin are called 'counterions'. For CM-cellulose the counterion is usually Na^+ and for DEAE-cellulose the counterion is

normally Cl^-. After choice of the appropriate resin, it is mixed with buffer to form a slurry which is poured into a suitable chromatography column. The pH of this starting buffer is crucial since it will determine the charge on the proteins to be separated. The starting buffer pH should be at least one pH unit above or below the pI of the protein to be bound to the resin to ensure adequate binding. However, bear in mind that CM-cellulose and DEAE-cellulose are examples of weak ion exchangers. A weak ion exchanger is one which is ionized over only a limited pH range. Thus DEAE-cellulose begins to lose its charge above pH 9 whilst CM-cellulose begins to lose its charge below about pH 5. The term 'weak' does not refer to the strength of binding of ions to the resin nor to the physical strength of the resin itself. With these points in mind then, the effective starting pH range when using DEAE-cellulose or CM-cellulose is only about pH 5 - 9. In addition to correct choice of the pH of the starting buffer, one should take care that its ionic strength is reasonably low since the affinity of proteins for ion-exchange resins decreases as ionic strength increases. Indeed this property is used in one method of eluting the bound proteins (see below).

Once poured into the column, the bed of ion-exchange resin is washed well with starting buffer and then the protein mixture is applied. Proteins which are oppositely charged to the resin at the starting pH will bind to it, so displacing the counterions. Proteins with the same charge as the resin or with no net charge will not bind and so will flow straight through the column. The different proteins bound to the column, one of which will be the protein of interest, will have different affinities for the ion exchanger due to differences in their net charge. These affinities can be altered by varying either the pH or the ionic strength of the column buffer. Consider a set of proteins bound to the anion exchanger, DEAE-cellulose. As the pH is lowered, $—COO^-$ groups on the protein begin to become protonated and so lose their charge. Thus the overall negative charge of the protein will decrease and therefore so will its affinity for the resin. Different proteins will elute from the resin at different pH values as their number of negative charges decreases to a critical value. Therefore one can resolve the proteins bound to the column by slowly reducing the pH using a buffer pH gradient and collecting fractions, each of which will contain different proteins eluted at different pH values. Conversely, when using a cation exchanger, the pH gradient would be arranged to increase to elute the bound proteins.

Instead of changing pH to elute proteins bound to an ion exchanger, one can increase the ionic strength of the column buffer instead. At low ionic strength, competition between the buffer ions and proteins for charged groups on the ion exchanger is minimal and so the proteins bind strongly. However, once the proteins are bound, increasing the ionic strength increases the competition and so reduces the interaction between the ion exchanger and proteins, causing the proteins to elute. One can elute the bound proteins by increasing ionic strength irrespective of whether an anion or cation exchanger was used. This is usually accomplished by incorporating a linear concentration gradient of NaCl in the column buffer while keeping the pH constant.

Use of either a continuous pH gradient or a continuous salt (ionic strength) gradient will result in a high degree of protein fractionation based on protein charge. In practice one can also use stepwise elution, i.e. alter the pH or ionic strength in discrete known steps. Although technically simpler to carry out on occasions, stepwise gradients typically give poorer resolution than continuous gradient elution and so are not modelled in this program.

Hydrophobic interaction chromatography

This technique fractionates proteins on the basis of their binding to and elution from a hydrophobic matrix, commonly octyl- or phenyl-agarose. Binding of the proteins is often carried out at high salt concentration to favour hydrophobic interactions. Some proteins may precipitate at this high ionic strength and so need to be removed by centrifugation prior to loading the protein mixture onto the column. Selective elution of bound proteins is then carried out by applying a decreasing salt gradient. Proteins that fail to elute at low salt concentration may be eluted by washing the column with aqueous ethylene glycol, ethanol or certain chaotropic agents such as urea, but this is not simulated in this program. This technique is therefore like other general chromatographic procedures such as ion-exchange chromatography and gel filtration in that

it relies on differences in a particular physical property of the protein molecules being separated.

Affinity chromatography

Affinity chromatography is far more specific than other purification techniques. It relies on the preparation of a matrix to which the protein of interest, and preferably only this protein, will bind reversibly. The matrix is usually beaded agarose (Sepharose or BioGel A), polyacrylamide (e.g. BioGel P) or cross-linked dextran (e.g. Sephacryl) to which a ligand has been covalently attached. The chemical nature of the ligand is determined by the known biological specificity of the protein to be purified. In practice, affinity chromatography has been used to purify proteins such as immunoglobulins, membrane receptors, enzymes, and hormones as well as nucleic acids and even whole cells. In the case of an enzyme, the ligand chosen would probably be a substrate or a reversible inhibitor or activator. One could attempt to use a ligand which is absolutely specific in that it will bind only that enzyme but, failing this, one can use a 'group-specific' ligand. For example, if 5' AMP is used as a ligand, its structural similarity to NAD^+ causes it to bind to many NAD^+-dependent dehydrogenases which are therefore co-purified from other proteins.

The procedure for affinity chromatography of proteins is similar to that for the other types of liquid chromatography. The matrix is packed into a column in a buffer that will be optimal for enzyme-ligand binding. Thus the buffer must contain any co-factors such a metal ions that are needed for binding. Usually the buffer has a fairly high ionic strength to minimize non-specific binding of other proteins to the ligand. The sample is applied and washed through the column. Ideally, only the enzyme of interest should bind. It can then be eluted specifically by the addition of a relatively high concentration of substrate or competitive inhibitor or, failing this, by changing the pH and/or ionic strength to disrupt enzyme-ligand interaction.

An alternative protocol can be used if a specific antibody to the protein of interest is available. This procedure is applicable to all proteins irrespective of their functional activities. The antibody is covalently coupled to a suitable matrix and then poured into a column and exposed to the sample protein mixture as described above. Only the required protein will bind to the antibody and can then be eluted by procedures which weaken the antibody antigen interaction.

Affinity chromatography is theoretically capable of purifying a single protein from a complex mixture in a single step. However, even if this theoretical ideal is not achieved, the degree of purification is commonly very good indeed. As such, it is perhaps the most powerful protein purification procedure currently available. However, affinity chromatography can be applied only when the functional activity of the required protein is known and a suitable ligand is available or when a specific antibody to the protein has already been obtained. Unfortunately, in many cases neither condition is satisfied and so protein purification must rely upon more general procedures.

In this simulation, three types of immobilised monoclonal antibody are available for each protein in the mixture. These differ in their affinities for the protein. Low affinity antibodies will not bind the protein sufficiently tightly to prevent its passage through the column. High affinity antibodies will bind the protein so tightly that it cannot be eluted in an active form. So you will need to use the medium-affinity antibodies. You will need to carry out some experiments to find out which they are! An immobilised polyclonal IgG preparation is also available and, for some proteins, an immobilised competitive inhibitor.