

BL/CH401 -- Biochemistry I -- Fall 1995

Protein Purification Summary

Requirements for Protein Purification:

Need a way to keep track of the protein you are purifying: if it is an enzyme then use its enzyme activity; or if it is colored protein, then monitor its specific wavelength of absorbance. Must maintain biological conditions of pH, salt concentration and temperature to keep protein from denaturing and losing its biological activity.

General Protein Purification Strategy:

To purify the protein of interest (ie separate it from other proteins in a mixture) take advantage of its general and specific properties: its native surface charge using ion exchange chromatography, its unique shape and size using gel filtration column chromatography, and its biological activity using affinity chromatography. These steps are sometimes applied in succession: first ion exchange to separate other proteins that have a different charge from the protein of interest, next gel filtration to separate all other proteins with a different size/shape than the protein of interest, and finally affinity chromatography to separate based on biological activity, which is usually highly specific for the protein/enzyme of interest. In some cases, an enzyme may be purified to a homogeneous state (ie completely purified) using affinity chromatography alone since it is so effective at separating a specific enzyme from all other proteins in a mixture.

Details of the 3 Key Purification Methods:

Surface Charge Properties can be used in ion exchange chromatography, where the charge on the protein depends on its pI and the pH of buffer used. Negatively charged proteins are separated using anion exchange chromatography on a support like DEAE Cellulose (positively charged groups extend out from the cellulose particles). Positively charged proteins are separated using cation exchange chromatography on a support like CM Cellulose (negatively charged groups extend out from the cellulose particles).

Gel filtration takes advantage of protein size and shape to separate it from other proteins.

The gel is a molecular sieve where large proteins exit from the column first, followed by medium size proteins and finally small proteins elute. If the gel filtration column is calibrated with proteins of known native molecular weight, then one can estimate the protein's native molecular weight by comparison to the standard proteins using a calibration curve.

Affinity chromatography takes advantage of the biological activity of proteins for separation.

Make a solid support (gel) by covalently linking the substrate of the enzyme (the substrate is the small molecule or metabolite the enzyme normally acts on to catalyze the reaction specific to the enzyme & the substrate binds in the enzyme's active site). Put the substrate-gel (sometimes called the affinity material or affinity gel) in a column and allow the enzyme of interest to bind, then wash away all unbound proteins by passing a lot of buffer over

the column. Next elute the enzyme by adding a buffer to the column containing the substrate. The free substrate in solution binds more tightly to the enzyme than the substrate covalently linked to the gel. The enzyme-substrate complex can be dialyzed to remove the substrate or the complex can be passed over a gel filtration column where the substrate will elute after the enzyme.

Protein Purity Testing:

To test the purity of a protein after each separation step use a **Native PAGE gel (PAGE = polyacrylamide gel electrophoresis)**. This method separates proteins based on charge density while maintaining biological activity. The proteins separated on the Native PAGE gel will retain their biological activity or enzyme activity and this can be detected by using an appropriate activity stain. The proteins will also bind dyes which recognize all proteins and so even non-enzyme proteins on the gel can be identified by a total protein stain. Thus, the specific enzyme of interest can be revealed by an activity stain and by comparing its position on the gel to the all the proteins revealed by the total protein stain, it can be determined if the enzyme/protein of interest is pure or not. If you find you have only one protein on the gel and it stains for both enzyme activity and protein, then you have homogeneous protein and do not need to do more purification.

Determination of the Biochemical Properties:

Amino Acid Composition and Amino Acid Sequence can be determined as described in Lecture 5. Enzyme Kinetic Property determination will be described in second part of the course. Subunit composition determination is done by comparing the native molecular weight of a protein to the molecular weight of its subunits after the protein is denatured. **Determination of the subunit molecular weight is done by SDS-PAGE or denaturing PAGE.**

SDS-PAGE:

To determine the molecular weight of the polypeptide chain (subunit size) of the protein you use denaturing or SDS-PAGE. In this method, the protein is denatured by heating in a detergent (SDS = sodium dodecylsulfate) and a thiol reductant (like 2-mercaptoethanol) which breaks the disulfide bonds (ie Cys-S-S- Cys). The SDS binds to the backbone of the protein and gives it a uniform negative charge. Since all the proteins will have the same charge density in SDS-PAGE, then during electrophoresis, the proteins separate by size due to the sieving effect of the gel. Thus, the high molecular weight polypeptides (ie denatured subunits of the protein) stay near the top of the gel (ie do not move very far during the gel electrophoresis) while smaller polypeptides move down the gel. So when standard proteins with known molecular weight for their subunits are also run in the SDS-PAGE gel, the size of the subunit of the unknown protein can be determined using a calibration plot.

Protein Subunit Composition:

Protein Subunit Composition is determined by dividing the native molecular weight by the subunit molecular weight. Native molecular weight is determined by gel filtration as described above in the section on using gel filtration for purification. If the native molecular weight = 80,000 Daltons and subunit molecular weight = 38,000 Daltons, then the protein is a dimer (ie $80,000/38,000 = \sim 2$). If a protein is a monomer, its native molecular weight should be the same

as its subunit molecular weight. Some proteins are composed of 2 different subunits and therefore two polypeptides are found when they are analyzed by SDS-PAGE. Of course, a pure protein must be used to do SDS-PAGE in order to avoid confusion when analyzing the results.

Note Well:

There are two types of PAGE used:

- 1) Native PAGE used for purity determination; and**
- 2) SDS-PAGE used for determining subunit molecular weight.**

There are also two types of Molecular Weight (MW):

- 1) Native MW determined by gel filtration; and**
- 2) Subunit MW determined by SDS-PAGE.**

Subunit Composition of a Protein (ie the number of subunits in a protein) is determined by dividing the Native MW by the Subunit MW, which should be rounded to the nearest whole number.