

Protein purification and chromatography: *Solutions*

1. A protein is found to have an isoelectric point $pI = 8.0$. Is *anion* or *cation* exchange chromatography likely to be more effective for purification? What kind of gradient can be used for elution?

$pI = 8.0$, so protein has no net charge at that pH. Lowering the pH causes protonation, so the protein will become more +ve charged at pH 7 and -ve charged at pH 9.0. With no other knowledge of the properties of this protein, the most sensible approach is to choose pH 7. ∴ **Choose a cation exchanger, such as CM-cellulose.** The +ve charged proteins can be eluted by a salt gradient (typically, 0 to 0.1 M NaCl). An alternative is to run a pH gradient from pH 7 towards pH 8.

2. Arthur Kornberg has an adage: “When you are purifying an enzyme from an extract, you should not rest until you have recovered 200% of the original activity in your purified preparation!” How can this ever be so?

Sometimes, the total amount of activity will increase during purification. The most likely explanation is the removal, during purification, of inhibitory factors present in the crude preparation.

3. According to my sequence analysis software, the amino acid composition of the (single polypeptide chain) protein *bovine serum albumin* (BSA) is: A, 48; R, 26; N, 14; D, 40; C, 35; E, 59; Q, 20; G, 17; H, 17; I, 15; L, 65; K, 60; M, 5; F, 30; P, 28; S, 32; T, 34; W, 3; Y, 21; V, 38. (a) *In what order* is the above list of amino acids given? (b) What is the approximate molar mass (MW) of BSA? (c) What is the approximate net charge on this protein at pH 7? (d) The reagent *cyanogen bromide* (CNBr) cleaves the peptide bonds of polypeptide chains following *methionine* residues. BSA is treated with excess CNBr, and the resulting peptides are analyzed by cation-exchange chromatography. The elution of the peptides from the column is accomplished with a pH gradient from pH 2 to pH 8. The eluted peptides are detected by monitoring A_{280} , using a UV detector. Discuss the appearance of the chromatogram.

a) Alphabetical

b) 607 residues @ approx. 110 per residue = approx. 67 kDa

c) Acidic residues 40 D + 59 E = **99 -ve charges**
Basic residues 26 R + 60 K = **86 +ve charges**.

Histidines: 17; side-chain $pK_a = 6.0$. At pH 6.0, His is 50% protonated; at pH 7, it is only slightly protonated. Michaelis equation: $K_a = 10^{-6}$ $\alpha = K_a / ([H^+] + K_a) = 10^{-6} / (10^{-7} + 10^{-6}) = 10/11$
= 0.9; so His is about 10% protonated at pH 7, i.e., about 0.1 +ve charge per His). $17 \times 0.1 \approx 2$

Total net charge is $86 + (\approx 2) - 99 = -11$

d) CnBr cuts at methionines. A maximum of six peptide fragments are formed by cleavage at the five M residues; some fragments could be very small, depending on the distribution of M's. Probably all fragments will be +ve at pH 2, and probably all will be -ve at pH 8, since the overall protein is -ve at pH 7. So we should hope to get six distinct peaks from the column. However, at most three of these will have strong A_{280} , since *there are only three W residues*. So we expect at most *three large peaks* and a few small peaks (some absorbance due to Y and other residues).

4. We have developed a procedure to separate mg quantities of enzyme Y by gel chromatography. You have recently cloned and over-expressed enzyme Y in *Escherichia coli*, so you repeat the procedure with a new crude sample which has twenty times higher activity per μL . Instead of getting the usual nice separation, you find all the activity running off the column in the void volume. What has happened? What can you do about it?

Probably, the enzyme has aggregated (to form multimers) at the higher concentration obtained in the new sample. Switch to a gel with a higher MW range. Or, you could try diluting the enzyme in buffer, before chromatography.

5. The MW of the rod-shaped protein fibrinogen has been determined accurately by sequence analysis. However, when fibrinogen is analysed on a gel-filtration column, its MW is seriously overestimated, *i.e.*, the protein elutes much sooner than would be expected (see Voet and Voet, Biochemistry, 3rd edn., 2004; vol. 1, Fig. 6-10). (a) Explain why this is so. (b) For most proteins, V_e is not affected by the flow rate. However, in the case of fibrinogen, V_e is larger when the gel filtration column is run very slowly (Meredith, S.C. and Nathans, G.R., Gel permeation chromatography of asymmetric proteins, *Anal. Biochem.* 121: 234-243, 1982); see figure. Explain the mechanism of this effect.

a) Rapidly tumbling in solution, the molecule behaves as though it fills a volume much greater than its true molecular volume (just as a rapidly-turning propeller looks like a solid disc). So its apparent molecular volume and molar mass are greater than the true values.

b) Most of the collisions between the rod-shaped molecule and the matrix occur on trajectories that do not allow the protein to enter the gel pores. Rare collisions are "end-on", and allow entry. When the column is run very slowly, these collisions have sufficient time to occur, but when it is run quickly, they do not.

6. After partially purifying a protein by "salting-out", we now have a sample (0.3 mg) of protein dissolved in 1.4 M ammonium sulfate solution (25 μL volume). We wish to recover the protein in a small volume of 10 mM "Tris" buffer (because our next step will be ion-exchange). Briefly explain how you would do this.

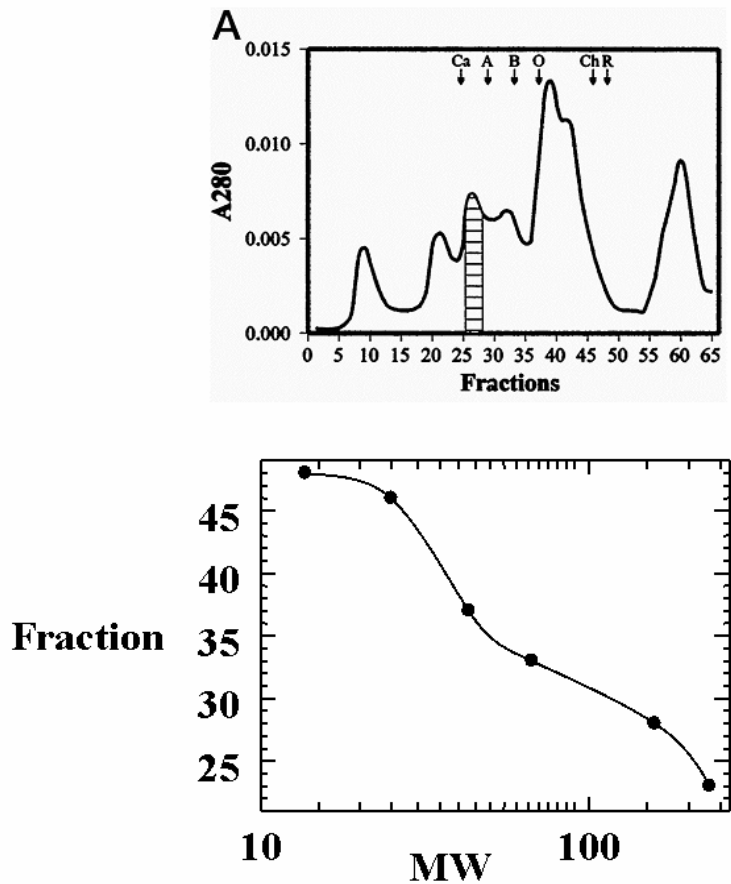
Use a desalting column (low-MW-cutoff gel filtration column). Keep the column short to minimize the volume needed for elution (V_0). Apply the protein sample and then elute with the smallest volume of Tris buffer.

7. An example of the application of gel-filtration chromatography is given in the study by

Le Dantec *et al.*, shown in the lecture notes (slide #60). Plot a calibration curve for their Superose 12 column, using the data for their MW standards. (Plot $\log(\text{MW})$ versus elution volume.) What is the MW_r (relative molecular weight) of the protein fraction they collected (the hatched region in the chromatogram)?

See figure.

The authors stated that the MW_r was approximately 180 KDa. They should probably have used a gel with a larger pore size to get a more accurate determination.



8. Ion exchangers are most commonly used as column chromatography packings. An alternative method of ion exchange is *batch separation*. This method involves mixing and stirring the equilibrated exchanger directly with the solute mixture to be separated. Why is this approach sometimes used in ion exchange, but never used in gel filtration?

Ion exchangers bind proteins, so, after centrifugation or filtration to remove the exchanger and bound proteins, the unbound proteins can simply be poured off. The bound proteins are then eluted with a different buffer.

In gel chromatography, there is no binding of proteins to the matrix; if the buffer is separated from the beads, all of the proteins, both large and small, will be found in the buffer.

9. You are purifying enzyme X. You apply 30 units of preparation to an ion-exchange column, and elute fifteen fractions. Frustratingly, none of the fractions is active. You pour all the fractions into a big beaker, prior to chucking it down the sink and going home to watch a video. But your supervisor stops you, and suggests that you should assay the mix in the beaker. To your surprise, you find a total of 27 units of activity. What has happened?

Probably, the enzyme activity required two or more proteins (possibly complexed), which were separated into distinct fractions on the column, but re-united in the final mix. Or, the enzyme may have been separated from a necessary cofactor or activator.

(10 and 11; to be done in the next tutorial.)