Fine-Tuning Selectivity on Ion Exchangers

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In a previous column concerning selection of chromatography media for industrial process applications, we recommended limiting selections to media that can be shown to conform to certain minimum specifications, the first and foremost being lot-to-lot reproducibility (Validated Biosystems, 2(1) 1-14 (1997)). This recommendation places fairly stringent limitations on the scope of choices; not all of the chromatography products on the market embody all the characteristics that make an ideal manufacturing ion exchanger. On the one hand, the restriction is a blessing. Even under the best circumstances, process developers never have time to evaluate *all* the products on the market. On the other hand, the limitation is a curse. Even modest experience with a few different products quickly reveals significant differences in selectivity and resolution. The gels most qualified for manufacturing applications may not support the best selectivity in your particular application. In fact, their selectivity may be substantially inferior to a less well suited column. Process developers face this Catch-22 all too often. And, all too often, the seductiveness of selectivity results in a media choice that causes manufacturing problems down the road.

This impasse can fortunately be avoided. There are a number of simple buffer additives that can be used to modulate selectivity in ways that allow you to reproduce results between different exchangers even though their respective selectivities in any given buffer system are significantly different. This allows you to focus your process development efforts on chromatography media with a proven manufacturing track record. The specific benefits include (1) not having to validate new materials, (2) not having to qualify new vendors, and (3) by using established materials for new processes and thereby adding to the volume purchase of a given medium, you may be able to obtain better volume discounts. Even if your house-favorite manufacturing ion exchanger gives you the best selectivity and performance among media options, simple buffer additives may still enhance the quality of the separation. At the same time, if you wish to upgrade an old process because of a major improvement in gel technology -- for example higher flow rate or capacity -- buffer additives can help ensure that the overall selectivity of the method will be preserved, regardless of selectivity differences that may exist between the old and new media.

The ability of buffer additives to modulate selectivity resides in ion exchange being *not* the sole chemical interaction between proteins and exchangers, but merely the *dominant* interaction. Proteins embody many properties other than charge, and so do ion exchangers. This provides nearly unlimited opportunities for secondary interactions to modify separation performance: hydrophobic interactions, hydrophilic interactions, allosteric interactions, even affinity interactions. This translates into an equivalent opportunity to control these secondary interactions directly with buffer additives, which allows you to bring the selectivities of different ion exchangers into phase.

Urea binds to the amide backbone and hydrophobic residues of proteins, conferring its high solubility on these otherwise insoluble sites, and to the protein overall. It also binds to hydrophobic surfaces on the gel matrix, thereby reducing the strength of matrix:protein hydrophobic interactions. To the extent that hydrophobicity is a significant contributor to the selectivity obtained with a particular sample on a particular exchanger, urea will either

relax or suspend that component of the interaction. Retention of strongly hydrophobic proteins will be left-shifted in the elution gradient more than weakly hydrophobic proteins. Hydrophobic proteins are also likely to elute in peaks that are sharper than those observed in the absence of the additive.

Urea is also a strong hydrogen donor and acceptor, making it an effective agent for breaking hydrogen bonds. Hydrogen bonding of proteins with ion exchangers has not been convincingly demonstrated in the literature, but to the extent that loss of intraprotein hydrogen bonds may alter protein conformation, its effects on selectivity could still be significant. Urea can generally be used at concentrations up to 2M without risk of significant conformational alteration of your product. It can usually be used up to 4M without risk of permanent alteration. Start at the higher end, adding equivalent urea concentrations to both your binding and elution buffers. If you obtain useful results, back the urea concentration down as far as you can without losing the desired selectivity. Residual urea is not a great concern to regulatory authorities and can be easily removed downstream in any case. Be aware that high concentrations of residual urea may interfere with downstream product binding if the next process step is affinity or hydrophobic interaction chromatography (HIC).

Ethylene glycol acts on ion exchange systems by a completely different combination of mechansims. First, it is a polarity reductant, so it outcompetes the protein for hydrophobic interactions with the matrix in the same way that eluting salts outcompete ion exchange interactions. This can yield results similar to urea: earlier elution and sharper peaks for hydrophobic proteins; but there are other mechanisms operating than can give completely different selectivities. As a polarity reductant, ethylene glycol reduces the interactivity of matrix- and protein-charge groups with the mobile phase. This increases the strength of their interactions with one another. Given that hydrophobic interactions are not a major contributor to the retention characteristics of a given protein, expect to see it elute later in the gradient.

Ethylene glycol is also of interest because it is protein-stabilizing. This results from it being excluded (repelled) from protein surfaces at concentrations up to 50%. This leaves a pure water hydration sheath around the protein. The discontinuity of the high ethylene glycol concentration in the bulk mobile phase, with the pure-water hydration sheath around the protein, exerts an exclusionary pressure on the protein which tends to conserve its native conformation. From a selectivity standpoint, the interesting thing is that the additive is likewise repelled from the matrix. This leaves the matrix, like the protein, preferentially hydrated and thermodynamically favors their association. Like polarity reduction, this should cause proteins to elute later in the gradient, but the mechanistic difference will affect different proteins to different degrees. Protein hydration is roughly proportional to protein size. The greater the degree of hydration, the more thermodynamically favorable its association with the hydrated exchanger surface. Binding of larger proteins will therefore be enhanced more than smaller ones.

Start by adding 50% ethylene glycol to both your binding and elution buffers. If you obtain useful results, back the additive concentration down as far as you can without losing the desired selectivity. Residual ethylene glycol is easy to remove, but be prepared to validate that removal if you are purifying an injectable. It is toxic when administered internally. Also be aware that high residual concentrations of ethylene glycol may interfere with product binding if the next purification step is affinity or HIC. A final point to watch out for with ethylene glycol is that the polarity reduction may reduce protein solubility, which may in turn limit the concentration of product you can elute in sharp peaks. In other words, it may reduce your usable binding capacity.

Polyethylene glycol (PEG) is another protein-stabilizing additive. As with ethylene gly-

col, the stabilizing effect arises from it being strongly excluded from protein surfaces. Likewise, it enhances association of preferentially hydrated surfaces, such as those of proteins with an ion exchanger. However, the effect is much stronger. As noted previously, protein hydration correlates roughly with size. All other factors being equal, given a pair of proteins that coelute on an ion exchanger, one twice the size of the other, PEG will preferentially increase the retention of the larger one. This effect is described in more detail in a poster from our electronic library (A method for obtaining unique selectivities in ion exchange...). Retention of protein dimers and higher oligomers can be enhanced to the point of supporting baseline separation from the monomeric form. Likewise, retention of intact monomers can be enhanced enough to substantially improve their separation from otherwise coeluting fragments.

PEG is viscous and will increase operating backpressures. This may require reduction of flow rate. The increased viscosity will also decrease diffusivity. This will cause some degree of peak broadening and it can can cause a reduction in binding capacity, although in practice this occurs only with proteins less than 15kD. For larger proteins, the binding enhancement overcompensates for the loss of diffusivity, and binding capacities are actually increased, sometimes by a factor of 2 or more. The most difficult aspect of working with PEG is that it strongly reduces protein solubility. After all, its primary application is for precipitating proteins. This limits the practical PEG concentration range over which a sample can be pre-equilibrated. Try starting at 10% PEG-6000 in both the binding and elution buffers. If sample precipitation is a problem, reduce it in 1% increments until you get beneath the level of the problem. If you obtain a useful result then continue to reduce the PEG concentration as much as possible.

Glycine is another protein-stabilizing additive that also effects ion exchange selectivity in a variety of ways. In direct contrast to ethylene glycol, it is a strong polarity enhancer. The dielectric constant of water is about 80. 1M glycine raises it to about 100; 2M glycine to about 120. This represents a 50% increase in the electrostatic interactivity of the solvent. Electrostatic interactions become stronger between the exchanger and the solvent, and between the proteins and the solvent; but they become weaker between the proteins and the exchanger. This suggests that there should be a reduction in ion exchange retention, but there is also a compensatory mechanism in operation. Glycine is strongly excluded from protein surfaces and hydrated surfaces (like ion exchange supports). This favors their association, but as with ethylene glycol and PEG there is a size dependency to this effect. Only large proteins are likely to exhibit a significant increase in retention. Small proteins, affected mainly by the increase in polarity, are likely to exhibit reduced retention.

Glycine is zwitterionic between pH 3.5 and 8.5, and does not contribute to conductivity. Consequently, it is perfectly compatible with ion exchange separations, even at high concentrations. Start at 2M in both the binding and elution buffers. If you obtain a useful result, then reduce the concentration as much as you can while conserving that result. Glycine, to the relief of regulatory professionals, is injectable. It is in fact a frequent component of lyophilization formulations, so its removal is not a disproportionate concern. Neither does it interfere with most other purification methods, the main exception being some immobilized metal affinity systems.

Nonionic and zwitterionic detergents provide another ion exchange-compatible means of relaxing hydrophobic interactions, but they tend to be protein destabilizing and they can be very difficult to remove. If you know that your final product formulation will employ a given detergent and that that detergent will not affect any purification steps downstream from the ion exchange step, then use that detergent. Otherwise detergents are probably best avoided. Organic solvents can be used as polarity reductants like ethylene glycol.

However, the majority of such solvents are strongly protein-destabilizing. Like detergents, these additives are generally best avoided. The exception might be with peptides and small very stable proteins, but even with these be mindful that polarity reduction may also reduce solubility.

Ultimately there is no way to predict how buffer additives will affect your system, or which ones will give you the result you want. You just have to try them and see what you get. This raises an important point. Buffer additives are not a substitute for thorough systematic evaluation of operating pH and gradient configuration. Begin there, and continue with additives to the extent that they give you the results you seek. And have some fun while you're at it. The results you'll observe will teach you a world of things about protein chemistry that you'll never find in books.

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