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Review

Excluded Cosolvent in Chromatography

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ABSTRACT

The concept of cosolvent exclusion was developed by a group of Timasheff's laboratory in 1970-1990 and is currently used widely to explain the effects of a variety of cosolvents on the stability and solubility of macromolecules. Not surprisingly, these concepts have had substantial influence in the fields of formulation, protein folding and unfolding, but they have perhaps more surprisingly found their way into the field of chromatography. A variety of excluded cosolvents have been used to enhance binding and resolution of proteins and other macromolecules in ion exchange, hydroxyapatite, affinity, and hydrophobic interaction chromatography. These cosolvents include salting-out salts, amino acids and polymers, and frequently polyethylene glycol (PEG). A new mode of chromatography, termed "steric exclusion chromatography," was recently introduced. It employs hydroxylated solid phase surfaces. Steric exclusion of the PEG stabilizes the association of macromolecules with the solid phase. Elution is achieved by reducing the PEG concentration. Magnetic particles are also used in this chromatography. This review summarizes the concepts of preferential cosolvent exclusion and its applications in column chromatography.

Introduction

The theoretical foundation and supporting data on protein solvent interactions were developed primarily by pioneering work conducted in Timasheff's laboratory. Early studies focused on the mechanisms of protein denaturants, such as organic solvents and guanidine hydrochloride or urea, as they were found to play a critical role in understanding the unfolding and refolding pathways of proteins.[1-6] These additives (cosolvents) exerted their effects at very high concentrations, where conventional stoichiometric binding measurements were neither practical nor meaningful. Timasheff's group employed equilibrium experiments, for example, dialysis, which measured differences in cosolvent concentration between the vicinity (inside the dialysis bag) of protein molecule and the bulk phase (dialyzing solvent).[7] At high cosolvent concentrations, many different interactions between the protein surface and cosolvent were found to occur.[8-11] Water molecules, which were at high concentration in aqueous solution, were also bound to the protein or other macromolecules.[12,13] All these interactions could cause difference in cosolvent concentration around the protein surface.

These experiments provided a wealth of information about the effects of cosolvents on protein denaturation and stability in aqueous solution. Protein denaturants, for example, organic solvents and urea, were observed to bind to the proteins in the denatured, unfolded state at high cosolvent concentration, leading to stabilization of the denatured structure.[1-6] Protein stabilizers, for example, sugars and polyols, were found to show lower cosolvent concentration inside the dialysis membrane than the dialyzing solvent.[14-22] This showed that they were excluded from protein surfaces. Carpenter and Crowe and others[23-27] demonstrated that these excluded cosolvents also stabilized proteins against freeze-thaw stresses, and they have since been shown to prevent proteolysis.[28]

Other important applications of preferentially excluded cosolvents are found in the field of chromatography. Chromatography plays an essential role in purification of biopharmaceuticals such as proteins, peptides, nucleic acids, and viruses. Affinity and hydrophobic interaction chromatography (HIC) permit the application of various excluded salts to modulate binding and elution. Anion and cation exchange chromatography are less tolerant of salts but invite application of nonionic and zwitterionic excluded cosolvents such as polyethylene glycol (PEG) and glycine. Many of these applications have been found particularly to enhance fractionation of native proteins from fragments and aggregates.

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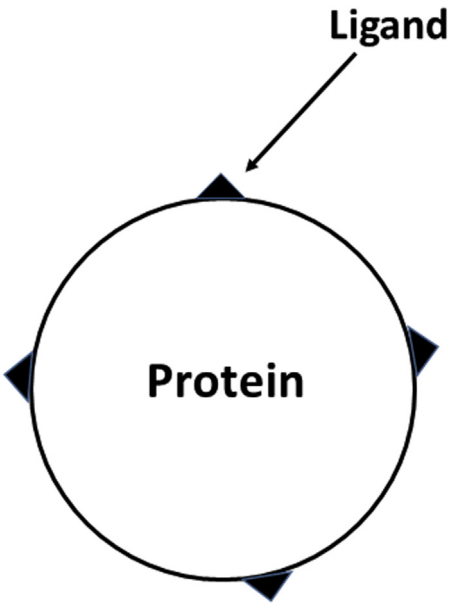

Figure 1. Specific ligand binding.

Excluded salts permit protein and virus species to be retained even on chemically unmodified solid phases such as size exclusion chromatography (SEC) media, which are generally considered not to be chemically interactive with biomolecules. These methods tend to be discussed in connection with HIC because of their use of precipitating salts, but they more properly belong in a classification that could be referred to as "preferential exclusion chromatography."

PEG has been found capable of promoting retention of large proteins and virus species even on solid phases that lack an active binding chemistry; no charge, no hydrophobicity, only hydroxylated surfaces. The effect is mediated by cosolvent exclusion of the polymer. This body of methods has been termed steric exclusion chromatography (SXC) in reference to the mechanism underlying cosolvent exclusion of polymers such as PEG from protein surfaces.[29-31] The cosolvent is excluded from the solid phase and the biomolecules. Instead of associating with each other, the biomolecules preferentially associate with the solid phase and remain there as long as the PEG concentration remains adequate.

This review describes the lifelong pioneering study of cosolvent exclusion by Professor Timasheff and the transition from academic research to practical applications in the field of biopharmaceutical chromatography.

Preferential Interaction

Preferential interaction is in principle different from conventional ligand binding. Stoichiometric ligand binding is normally composed of small number of high-affinity binding, as schematically depicted in Figure 1. Such tight binding may make isolation of ligand/protein complex possible, influence the spectroscopic properties of the proteins (the magnitude of changes in spectroscopic properties increases with binding), or generate sufficient heat (the binding enthalpy increases with binding). On the contrary, preferential interaction ranges from weak, transient binding to strong binding of ligand (here called cosolvent) and water with the protein, all of which can occur at high concentration: it should be emphasized that in aqueous solution water is always at high concentration.

Figure 2 depicts preferential cosolvent interaction, where cosolvent molecules are represented by black circles. At high concentrations, cosolvent molecules show not only strong binding as shown in Figure 1, but also weak, transient binding as shown in Figure 2. Such a widely different binding can be measured from the difference in ligand (cosolvent) concentration between protein solution (m_L^P) and bulk phase (m_L^B), that is, $m_L^P - m_L^B$, which are in equilibrium achieved by dialysis or gel filtration. The value, thus, obtained is called "preferential cosolvent interaction" to indicate whether the cosolvents are bound or excluded. It is easier to understand this concept using an illustration in Figure 2a, where there is no preference of protein molecule for cosolvent or water (no preferential cosolvent binding). Figure 2b shows preferential cosolvent binding where there is excess of cosolvent molecules inside the dialysis membrane (dotted line), namely in the vicinity of protein molecules, relative to its concentration outside the membrane (in bulk phase). This situation is called preferential cosolvent interaction. Figure 2c depicts the opposite case, where the protein surface is depleted of cosolvent and surrounded by water molecules. A typical example of this case is salting-out salts, which are preferentially excluded from the protein surface. Why are they excluded from the surface? There appear to be 3 different mechanisms proposed. Obviously, strong hydration can lead to a negative cosolvent binding. Such hydration may not allow cosolvent molecules to penetrate the hydration layer, which should create excess water or deficient cosolvent concentration at the protein surface.

The second mechanism is exclusion of salts, sugars, amino acids, and polyols, which increase the surface tension of water. Traube[32] demonstrated the importance of surface tension in cosolvent effects on protein solubility. Gibbs[33] has shown that those solutes that raise the surface tension of water are depleted in the air-water interface. This can be explained from the hydration of salt ions. Hydrated solutes are more stable and more likely to remain in the

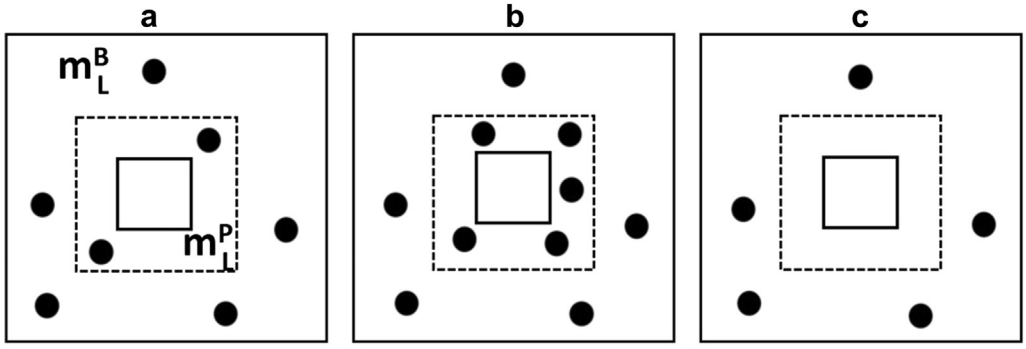

Figure 2. Preferential cosolvent interaction. (a) No preferential cosolvent binding; (b) preferential cosolvent binding; (c) preferential cosolvent exclusion. Square, protein molecule; dashed square, dialysis membrane; circles, cosolvent molecules.