Ligand–Protein Coprecipitative Isolation by Matrix Stacking and Entanglement

REX LOVRIEN,* CHARLES WU, and DALJMANTAS MATULIS

BIOCHEMISTRY DEPARTMENT
UNIVERSITY OF MINNESOTA
1479 GORTNER AVENUE, ST. PAUL, MINNESOTA 55108, USA

ABSTRACT

Ligands are being developed for the upstream isolation-purification of sought-for proteins from dilute crude extracts by ligand–protein coprecipitation. The ligands are alkane-substituted azoaromatic anions (dyes) with sulfonate heads. Overall coprecipitation is comprised of two main reactions. Ligands first bind electrostatically to protein molecule cationic side chains in solution, approximately to a point where the protein net charge \( Z_H \) is ion-pair titrated with organic anion ligand heads. Organic tail groups cover a sizable portion of the protein molecular surface, triggering the second category of reactions: matrix formation and coprecipitation. Organic tails stack and hydrophobically associate, pulling the complexes together in a host lattice or matrix, enclosing protein molecule guests. Protein molecule structural determinants for coprecipitation of a sought-for protein are protein cationic charge density and location (governed by pH, amino acid composition, and Scatchard–Black reactions). Ligand structural determinants for forcing coprecipitation using \( 10^{-5} \) to \( 10^{-3} \) M ligands depend on the ion pairing capacity of the ligands (which determines the stoichiometry) and the details and size of the organic moiety of the ligands. Binding ligands to the target protein in solution contributes the initial part of the overall coprecipitation. However ligand–ligand interactions, in conjunction with ligand placement on proteins to build the host lattice, contribute a large part of the overall coprecipitation. They are sharply dependent on the foregoing factors and on the topology of each lattice to determine the selectivity of matrix ligand coprecipitation. An example is presented of direct coprecipitation of two lectins out of their crude extracts. Very strongly acting ligands that sweep most proteins and polypeptides out of solution are available. However, use of the maximal coprecipi-
tating power is not necessarily the best strategy. Rather, there needs he struck a balance between coprecipitating power, selectivity, and reversibility for later release of the sought-for protein.

**INTRODUCTION**

Precipitation and coprecipitation of proteins are principal means for the large-scale processing of crudes: Fermentation and cell cultures, tissue extracts, etc. About a dozen practical methods for protein precipitation are currently used in large-scale applications. Rothstein reviewed their molecular basis (1). Ammonium sulfate salting out is the workhorse, dominant technique for protein precipitation using sulfate's Hofmeister kosmotropy. The technique has been analyzed by Collins and Washabaugh (2). Inorganic sulfate binds and ion pairs with protein molecules. The mechanics of what inorganic sulfate docs to protein molecules in bioseparations no longer appears to be exclusively an "ionic strength effect," and probably not exclusively a Hofmeister kosmotropic force (3). Inorganic sulfate exerts most of its precipitating power as an exclusion-crowding agent and as a kosmotrope, but it requires large concentrations (0.3 to 3 M) to function in this way. Inorganic sulfate need not necessarily bind to discrete sites of proteins to act as a kosmotrope. In contrast to inorganic sulfate, large organic sulfonate and sulfate anions, herein called matrix and entanglement ligands, are fully dependent on binding to discrete protein molecule sites to promote coprecipitation, and do so in far lower concentrations, ca. \(10^{-4}\) to \(10^{-3}\) M ligands.

Precipitation and coprecipitation are not near-synonyms. They do not mean approximately the same thing in implementation, function; nor molecular mechanism. Coprecipitation here mainly refers to the use of organic anions as ligands to drive it. Inorganic sulfate on occasion plays a hybrid role as both a precipitating (kosmotropic) agent and coprecipitating (restricted binding) agent, as in cosolvent partitioning for protein isolation (3). However \(\text{SO}_4^{2-}\)'s lack of hydrophobic character makes it very unlike the organic sulfonate ligands described below in precipitating power requiring site binding and in concentration ranges over which it functions. Inorganic sulfate in large concentrations, especially when acting as a Hofmeister kosmotrope, raises the chemical potentials of protein molecules in solution in a positive direction (4). This is called "pushing" because proteins (or any other solute) react under such a stress to relieve chemical potential increases by precipitating ("salting out") kosmotropy. Hofmeister kosmotropy and osmophobic exclusion-crowding agent-based techniques are foremost examples of pushing. Pulling protein molecules out of solution is achieved by the use of ligands which are described next.
ENERGETICS BASIS FOR LIGAND–PROTEIN COPRECIPITATION

Coverage of protein molecules with organic ligands by ligands' large tail groups indicated in Fig. I convert protein hydrophilic surfaces to hydrophobic nonpolar surfaces if several ligands become attached. Thereafter, protein–ligand complexes substantially coated with alkane-azoaromatic tails attract one another and coprecipitate. Ligand heads and sulfonate anions are ion paired with protein cationic side chains. Ligands equipped for this are capable of coprecipitating even very water-soluble proteins from 0.01 to 0.2% proteins in solution (5), although, as stated above, optimal ligands in best practical use are not necessarily the most strongly coprecipitating ligands. After coprecipitation and removal of solvent and unwanted proteins, each ligand needs he removed to release the sought-for protein hack into solution.

Attraction between tails to draw protein–ligand complexes together and form a matrix which coprecipitates is referred to as "pulling." Pushing and pulling carry both a mechanical and a thermodynamic meaning. Pushing is a result of bombardment or exclusion of protein molecules by a large concentration of Hofmeister kosmotropes, especially sulfate which has a very large hydration shell (6). Pushing is expressed not only by precipitation of proteins, which such compounds cannot enter, but also by contraction of the macromolecule hydrodynamic volume if the macromolecule had been conformationally expanded and penetrated by water beforehand (5).

Fig. 1 Organic anion structures of a matrix stacking ligand, an azoaromatic sulfonate, and an entangling ligand: alkane sulfate: dodecyl sulfate. Alkane substituents, t-butyl and ethyl, on azoaromatic moieties reinforce tail-tail stacking and displace water of protein surface hydration.
Pulling follows from the action of ligand organic tails drawing together to form complexes as in Fig. 2. This involves a kind of intermolecular hydrophobic glue from which some portion of the hydrate water has been squeezed out to make a host lattice of ligand tails in a matrix. Pushing and pulling are both capable of protecting protein molecules from chemical and thermal stresses, sometimes severe stresses. A recent paper described the protective capabilities of matrix ligands for enzymes stressed by acid and heat denaturing conditions (7).

Organic anion ligands that first hind to macromolecules in solution and then into a lattice function in two sequential reactions presented here in abbreviated form by Reactions (1) and (2). The notation is $P = \text{protein}$, $L = \text{ligand}$, $v = \text{total moles ligand added/protein}$, and $v_{\text{Ligand}} = \text{moles ligand bound/protein}$ molecule. $L_{v1}P$ and $L_{v2}P$ are complexes between ligands and targeted proteins in solution and in matrix coprecipitates, respectively. $Z_{H^+} = \text{net charge on protein molecules (pH dependent)}$ and $\varepsilon = \text{total cationic charge born on a protein molecule from lys H}^+, \text{his } H^+, \text{and arg}^+$ side chains.

**Binding reaction in homogeneous solution:**

$$y_1 L_{\text{soln.}} + P_{\text{soln.}} \rightarrow L_{v1}P_{\text{soln.}} \quad (1)$$

**Bound complex precursor to coprecipitation:**

$$y_2 L_{\text{soln.}} + P_{\text{soln.}} \rightarrow L_{v2}P_{\text{soln.}} \rightarrow L_{v2}P_{\text{coppt.}} \quad (2)$$

Reaction (1) can be pushed from the left by simply increasing the concentrations of the reactants $L_{\text{soln.}}$ and $P_{\text{soln.}}$. However, within Reaction (1) there is no perceptible interaction between the $L_{v1}P_{\text{soln.}}$ complexes remaining in solution. Reaction (2), on the other hand, may derive a large part of its overall Gibbs negative free energy from the overt coprecipitation phase change. It is polled by the stability of $L_{v2}P_{\text{coppt.}}$, a product of lattice and aggregative forces between these complexes after the initial binding reaction in the solution denoted by Eq. (1). In Reaction (1), which concerns the protein in solution before coprecipitation, the stoichiometry or binding level denoted $v_1$ is set primarily by ion pairing between ligand anionic head groups and the net protein cationic charge. $Z_{H^+}$. Building up coverage of the surface of protein molecules to level $v_2$ substantially covers the protein surface with organic tail groups. Then previously hydrophilic surfaces, such as those on proteins like very water-soluble serum albumin, become converted to hydrophobic surfaces (5). Reaction (2) is promoted through tail–tail stacking or entanglement and the hydrophobic stacking interaction between rails to form the coprecipitating matrix. Protein molecules are guests inside the hydrophobic matrix host. Thus organic anion ligands of these kinds bind to each other through their tails besides binding to charged protein molecules.
the strong pulling nature of Reaction (2) due to lattice/aggregative forces forms coprecipitates of constant composition and binding isotherms for such coprecipitates. (see below).

The coprecipitative process needs to be selective, reversible, high yielding and scavenging, concentrative, and protective with suitable ligands. The thermodynamic nature of protein coprecipitative reactions and ligand structural design are mutual determinants. Sulfate salting out partly achieves those goals in some cases. although inorganic sulfate does its job largely by pushing (crowding, exclusion). By attaching an organic group to sulfate, another opportunity and another thermodynamic means can be added to amplify what sulfate can partially do as a protein conformation tightener (5) and protein separation!, agent. Organic groups, large hydrocarbons, and azoaromatics displace some water near cationic sites to which their $-\text{OSO}_3^-$ (sulfate) and $-\text{SO}_3^-$ (sulfonate) anion head group binds. The organic groups lower the dielectric constant in the vicinity (probably unsymmetrically) and increase the electrostatic attraction in the ion pair. If the organic tails are large enough and of the right kind, they will associate among themselves to pull the ligand–protein complexes together, as signified in Reaction (2) by $\mathbf{L}_{\nu_2} \cdot \mathbf{P}_{\text{coppt}}$. 

FIG. 2 Matrix ligands associate with one another through their tail groups, reinforced by alkane–alkane attraction between substituents. Placement of ion-pairing ligand heads (sulfonate anions) and number of such ligands depends on the number and topology of protein cationic groups. Tail–tail interactions pull ligand–protein complexes together and coprecipitate.
Figure 1 shows two kinds of organic anion ligands. These two kinds of ligands are capable of being coprecipitating agents for many proteins when used in optimal concentrations. Such ligands pull proteins out of solution by aggregation in a mechanical sense, as indicated in Fig. 2. They also pull in a thermodynamic sense by stabilizing each coprecipitate. The formation of very stable, insoluble coprecipitates, protein–ligand complexes, yields products of constant composition within a suitable range of added ligand, denoted y. The achievement of constant composition is equivalent to purification and is a leading criteria of purification. Foreign components unable to fit inside stable coprecipitate lattices are excluded.

Formation of ion pairs between ligand anion heads and protein cationic sidechains require his H\(^+\), lys H\(^+\), and Arg\(^+\) groups for initial binding in solution (Reaction 1). Accordingly, the amino acid composition together with the pH controlled H\(^+\) titration properties of the sidechains (which determine protein overall charge \(Z_{\text{pH}}\)) are the basis for much of the pH dependency of binding followed by coprecipitation (Reactions 1 and 2) for each protein. The topology of where his H\(^+\), lys H\(^+\), and arg\(^+\) are located on protein molecules direct where incoming ligands take up their positions around protein molecules by acting as anchor points. Each protein's cationic group topology, which prescribe the docking points for ligand anion beads, followed by tail–tail mutual adhesion, determines how the lattice of ligands is constructed around the target protein molecule (as in Fig. 2). These primary formative steps frequently also involve and even trigger large conformation changes by the macromolecule as seen by hydrodynamic measurements. Macromolecular shrinkage and displacement of some hydrate water.

Stacking ligands are synthesized to carry structural features to promote efficient coprecipitating and protecting capacity for proteins. Stacking is the ability of conjugated aromatic and azaaromatic ring molecules to associate face-to-face using their pi electron assays by \(\pi-\pi\) stacking between tails and dispersion forces. \(\pi\) face stacking is routinely used to stabilize many meso-, supramolecular, and container complexes (8, 9). Alkane substituents around the peripheries of aromatic rings add to their van der Waal and nonpolar attractive forces, reinforcing the \(71-71\) stacking illustrated in Fig. 2 with \(7\)-butyl groups. Methyl, ethyl, propyl alkanes, and extra benzene rings are also reinforcing substituents for \(\pi-\pi\) stacking if they can be maneuvered into place when ligand tails come together in Reaction (2) to build the host matrix. Hydroxyl groups ortho to the azo linkage contribute to stacking because 11-atom tautomerism and internal hydrazone formation in conjugated azaaromatics reinforce their conjugative coplanarity (10). Ring system rigidification and stacking plus hydrophobic alkane reinforcing substituents all function to pull
ligand tails together and provide the tail–tail stacking indicated in Fig. 2. The ligand tails are tethered to protein molecules through their $-$SO$_3$ ion pairing head groups.

The sheer bulk and water displacement capacity of ligand tail organic groups, especially those hearing alkane substituents, lowers the average dielectric constant in the critical region of ion-pair formation between ligand head anions and protein cationic sites. This enhancement of attractive electrostatic forces coupled with the H$^+$ titration properties and amino acid composition of target proteins determine ligand–protein stoichiometry by the number of ligands bound/protein molecule, $v_\text{Ligand}$ in Reaction (1). But the slacking of hydrophobic tails of the ligands determines the tail–tail interactive forces and most of the structural detail of the host matrix enclosing the proteins in Reaction (2). Ion-pair formation between polarizable organic anions, dyes of these kinds, and protein cationic sidechains is generally exothermic. Such ion pairs generate $-5$ to $-10$ kcal enthalpy/mol of anion upon complex formation in solution up to the near-coprecipitation endpoint (11).

Accordingly the overall (integral) calorimetric enthalpies are also expressions of the number of lysine, histidine, and arginine sidechains available to ion pair with such ligands. A strong dependency of $v_\text{Ligand}$ marks a range or interval where $v$ approximates $Z_{\text{H}^+}$ ($v \approx Z_\text{H}^+$) at the coprecipitative endpoint. Fifteen proteins; proteases such as papain, hemoglobin, trypsin, and chymotrypsin; and other classes of enzymes such as lysozyme and ribonuclease exhibit this simple relationship (12). The $v$ values of successful ligands that bring down these proteins as coprecipitates are closely in register with each protein’s $Z_{\text{H}^+}$ parameters within the optimal pH range for each protein’s coprecipitation.

**PRACTICAL USES**

The overall concentrations of ligands necessary for the capture of target proteins from crude mixtures, as in the isolation of lectins from raw crudes (13), often are required to be comparatively large ($10^{-4}$ to $10^{-3}$ M) if foreign proteins bind many ligands and coprecipitate out first. Optimization of isolation procedures for a target protein out of a crude ("toning") is usually carried out by bracketing ca. $10^{-4}$ M overall ligand concentrations and varying the pH in a range 2 to 4 units below the isoelectric point (if known). The aim is to capture the maximal bioactivity of the target protein after redissolving coprecipitates and trapping the ligand. This is generally performed by treating the coprecipitate with a buffer 3 to 5 pH units above the coprecipitative pH and scavenging the ligand with an anion-exchange resin (Dowex-1 in Cl$^-$ form, 6–8% crosslinked) (13). Approximately a 20-fold excess of exchanger equivalents (moles) relative to the amount of ligand to be trapped (moles) suffices to remove the ligands from coprecipitated proteins quantitatively. A
lower degree of resin crosslinking is not advisable because oligopeptides and very small proteins may enter the resin beads.

The amounts of ligand bound, referred to as \( v_{\text{coprep}} \), for azoaromatic (dye) ligands as in Fig. 1 are simple to analyze. They have intense absorption spectra in the visible range (450–600 nm) with molar absorption coefficients of 20,000 to 40,000 \( \text{M}^{-1} \cdot \text{cm}^{-1} \). A sample of the coprecipitate is dissolved in alkali, and the matrix or stacking ligands are easily quantitated by conventional spectrophotometry (12).

Specific activities of proteins released under nondestructive conditions are obtained from appropriate bioassays (5, 7, 13). Protein molecular weights in coprecipitates are measured (from tiny quantities of coprecipitates) by MALDI-electrospray mass spectrometry, often within 1 dalton, as further identification and confirmation. Such ligands are noncovalently bound to their coprecipitated proteins, and therefore the complexes fly apart in the mass spectrometer. Hence ligand molecular weights do not add to the parent protein molecular weight in MALDI spectrometry.

The pH often shifts upward appreciably as ligands bind. 0.1 to as much as 1 pH unit depending on solvent buffering capacity, when a neutral amine sidechain is forced to the ammonium form by ligands. This is the Scatchard–Black effect (14), often observed with related organic sulfonate ligands (15). It is easily monitored with a pH meter. It is indicative of the strength and number of the association–ion pairing reactions. When an organic sulfonate ligand has the potential to form a particularly stable ion pair it may do so with the help of a water molecule, starting from a neutral amino side chain. The Scatchard–Black reaction may be represented as

\[
\text{Protein} - \text{NH}_2 + \text{Ligand} - \text{SO}_3^- + \text{H}_2\text{O} \rightarrow \\
\text{Protein} - \text{NH}_2^+ - \text{O}_3\text{S} - \text{Ligand (ion pair)} + \text{OH}^-,
\]

\[
\Delta \text{pH} \quad (3)
\]

If ion pairing pulls this equilibrium far to the right, it derives a proton from a water molecule to convert an amine sidechain to the ammonium form. The remnant \( \text{OH}^- \) from the water molecule shifts the pH upward, producing a measurable \( \Delta \text{pH} \), as much as 0.5 to 1 pH unit. The compound 1-anilino-8-naphthalene sulfonate (ANS), a fluorogenic probe anion, likewise is an able matrix coprecipitant for proteins, an ion-pair forming agent, and a pH shift agent by virtue of the Scatchard–Black effect (11). Another factor governs the ion pairing, the Scatchard–Black hydrolytic reactions, and the \( \Delta \text{pH} \) production: sulfonate groups are rather "hard" anions, in the Pearson sense (16). If an amine group is converted to an ammonium group, a rather hard cation immediately becomes available. Mutually hard ions are best able to associate relative to mismatched situations in which hard ions interact with soft counterions (17). Water molecules are also of comparable "hardness" and of comparable polarizability. These basic factors underlying chemical reactivity operate lo
promote Reaction (3), ion pairing by the ligand, and the accompanying matrix formation and coprecipitation.

Entanglement ligands are closely related to matrix ligands in their head groups, sulfate, or sulfonate anions. However, entanglement ligands have flexible alkane chains, unlike the rather rigid slacking ligands. Figure 3 illustrates protein molecule surface coverage with dodecyl sulfate (DDS⁻): ion-pair formation and organic tail coverage or near coverage. The alkane tails cover hydrophilic protein molecules with very hydrophobic coats that mutually associate, bring complexes together, displacing water, and hence coprecipitating analogous to Fig. 2 and as illustrated in a previous paper (5). Dodecyl sulfate is an efficient entangling and protein coprecipitating ligand for many otherwise very water soluble hydrophilic proteins, including serum albumin (18). This may seem surprising inasmuch as DDS is a detergent normally used to solubilize and unfold proteins rather than coprecipitating and protecting. It is the relative concentrations of DDS⁻ and proteins and the overall concentration of DDS⁻ which defines this striking dichotomous behavior. The detergent is an entangling/coprecipitating ligand (and conformation tightening agent) in relatively low concentrations, $10^{-3}$ to $10^{-1}$ M, where $\gamma_{\text{DDS}^-}$ and $\gamma_{\text{DDS}^-}$ are less than or equal to $Z_H$. The D D S ligand is also a protein cocrystallizing agent, e.g., for β-lactoglobulin (19) and lysozyme (20), when it is correctly deployed.

In concentrations ca. $10^{-3}$ to $10^{-2}$ M DDS⁻, where the bulk of detergent-protein research is centered and D D S approaches the c.m.c. (critical micelle

**FIG. 3** Alkane anions form hydrophobic patches on protein molecules, and they also interact and entangle with one another through their hydrocarbon tail groups to promote agglomeration and coprecipitation of a ligand host and a protein molecule guest.
concentration), it behaves as conventionally expected: Solubilizing, unfolding, denaturing. This remarkable low versus high concentration behavior by DDS$^-$ toward proteins will be reviewed elsewhere. Here, it clearly expresses its entanglement-coprecipitation abilities, e.g., with insulin (21), by virtue of ion pairing through its sulfate anion up to the endpoint for coprecipitation, $\nu_{\text{DDS}^-} = Z_H$. Flexible C$_{12}$ alkane chains entwine themselves to form a matrix host with protein molecules as guests. There exists a chain length dependency for such alkane sulfates. Protein molecule conformation tightening, protection, and coprecipitation are all optimal in the C$_{10}$ to C$_{14}$ range, centered on a C$_{12}$ detergent, dodecyl sulfate (22). Significantly, strong anion detergents are used in submicellar concentrations in all such examples to coprecipitate target proteins and to protect them. "Entanglement" ligands like dodecyl sulfate are of low toxicity at low levels as reviewed by Potokar (23). Azoaromatic ligands likely are toxic but are thoroughly removed by the above described resin exchange method.

Coprecipitation by anionic ligands can be modulated and optimized by the addition of small quantities of divalent cations, especially Zn$^{2+}$. The Zn$^{2+}$ cation coordinates with protein carboxylate anions to neutralize some of the protein's negative charge, in effect increasing the protein's overall cationic charge, Z$_{1+}$. This reinforces matrix ligand anion binding in neutral pH ranges. Zinc cations are not nearly so prooxidative as most transition metal cations, e.g., Cu$^{2+}$, Ni$^{2+}$, and Fe$^{n}$, toward sensitive disulfide-sulfhydryl side chains.

Zn$^{2+}$ facilitated the isolation of peanut lectin via matrix ligands (13) and saw similar use in coprecipitation–cocystallization of hemoglobin (24) and insulin. Because Zn$^{2+}$ has a negative specific volume, $\bar{v} \approx -0.33 \text{ cm}^3/\text{g}$, it helps densify coprecipitates, thus aiding in centrifugation–filtration unit operations.

**CONSTANT COMPOSITION CRITERIA AND PERFORMANCE**

The purification powers of coprecipitative isolation of a sought-for protein from crudes are tellingly expressed by the extent to which the composition of such coprecipitates remains constant. That is, the composition remains constant over variations in the amount of ligand/protein added, which we call $\nu_{\text{ligand}}$ (moles/mole). The constant composition of ligand–protein coprecipitate complexes is expressed by the $\nu_{\text{ligand}}$ quantity in the coprecipitate. Figure 4 shows an example of a coprecipitate composition plot: $\nu_{\text{ligand}}$ in a binding isotherm for coprecipitating a protein with its ligand versus widely varying $y$ values. The composition of the coprecipitate. chymotrypsin coprecipitated by Razoarback Red ligand, is nearly invariant as shown by the nearly flat plot.

Consider the point in Fig. 4 where $y = 2$. Analysis of the coprecipitate (by dissolving in alkali and measuring the ligand spectrophotometrically shows
LIGAND–PROTEIN COPRECIPITATIVE ISOLATION

FIG. 4 Binding-compositional isotherm for a matrix ligand–enzyme system. Razorback Red and α-chymotrypsin in 0.002 N HCl, 25°C Abscissa: Level of addition of ligand to protein. Left ordinate: Composition of coprecipitate, moles ligands bound/mole protein. Right ordinate (pertains only to the intercept): $Z_{H^+} = 11$, the formal H$^+$ titration charge on the enzyme. Plateau of the plot, near flatness, indicates nearly constant composition of the coprecipitate over relatively wide variation of the ligand addition. $y$.

that at $y = 2$, $\nu_{\text{coppt.}} = 11$ to 12 moles ligand bound/mole protein in the coprecipitate. A large portion of protein is left in the supernate, but practically no ligand. As the $y$ level is increased ($y = 4, 8, 10, \text{etc.}$), successively more target protein is coprecipitated as a brightly colored complex with near constant composition and a virtually flat plot as shown. The endpoint is sharply clearly seen by the intense color of excess ligand left in the supernate after all target protein is brought down. A pictorial representation of such a coprecipitative titration is shown in Ref. 5.

Such titrations and analyses are quite simple to perform. They are analogous to coprecipitative titration of an inorganic cation with a suitable ligand (25) or the coprecipitative titration of an antibody with a suitable antigen (immunoprecipitation, which produces a specific lattice). Numbers of protein–matrix ligand coprecipitates hervsc similarly with strongly acting ligands (5.22).

The intercept on the right ordinate of Fig. 4 shows the stoichiometric nature of ligand–protein in the coprecipitated lattice in an ion pairing between strong anions donated by the ligand (a disulfonate dye) and the cationic charge on the protein molecule, chymotrypsin. At the pH used, 3.2, chymotrypsin has $Z_{H^+} = +25$ (26). Because the disulfonate ligand bears two negative charges, interception of the plot on $Z_{H^+}/2 = 12$ is in good register with simple electrostatic ion pairing equivalency that determines ligand–protein stoichiometry,
in the coprecipitate. Strong organic anions like sulfonate dye ligands, and also other types of sulfonated anions able to coprecipitate cationic proteins (11), do the same thing; intercept at \( n_{\text{coppt}} = Z_{H^+} \) at optimum pH.

**SELECTIVITY**

Constant composition of ligand–protein coprecipitates and the sharp cooperativity of their formation spanning the range of ligand addition between the beginning and the endpoint of coprecipitation are expressions of selectivity of the process. The basis for these aspects of selectivity may be visualized by the structural nature of coprecipitate lattices. The determinants of selectivity in coprecipitation are also determinants in cocrystallization of ligand–protein complexes (12). A foreign or flawed protein in the presence of a coprecipitate (or cocystal of a sought-for protein) should fail to fit the lattice of the desired host. Entry of flawed/foreign protein proteins upsets cooperativity and diverts the coprecipitate from constant composition. Hence the flawed/foreign protein shall be rejected if it cannot fit, nor generate the negative free energy (pulling) comparable to that generated by the native protein molecule which does fit. The rather large, space-filling organic tails, with differing topologies of distribution on surfaces of the various proteins, plus any stereochemical demand of tail–tail interaction in case of stacking ligands, decisively narrow the choices of macromolecule accommodation in host lattices. Sizes of ligand tails and their detailed structural features (Fig. 1) enable them to stack or to entangle, amplifying the selectivity represented in Fig. 2. For example, if two different protein molecules have different \( Z_{H^+} \) charge values, protein molecular shapes, and different topologies of cationic charge around their surfaces, it is quite unlikely that both would fit well into the same lattice. One of them will become rejected even if it hindered, (in solution) some of the same ligands good at selecting the other protein. However, if one dwarfs the mixture with a large excess of ligand, they may all come down together in an amorphous, useless mixture. Practical work with crude protein mixtures requires restrain! from adding large amounts of ligand, such as \( 10^{-2} \) M. in one step, especially with quite strongly acting ligands.

Selectivity is also fostered by using rather dilute crudes, 0.01 to 0.20% total protein (5). Accordingly, the general method is also quite "volume reducing." Water, foreign compounds, and impurities are removed as supernates by using low speed centrifugation or "low technology" filtration.

**Matrix Coprecipitation of Lectins from Crudes**

An example of matrix ligand coprecipitative purification of lectin proteins directly from their crudes is summarized in Fig. 5. Four separate lectins were captured in good yields and specific activities from their parent raw material.
Phaseolus vulgaris Lectin (PHA)
Little Rock Orange Coprecipitates From Crude

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>M.W. Sigma Markers</th>
<th>Crude Y=25</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/mg dry wt.</td>
<td>64,000</td>
<td>1,738</td>
</tr>
<tr>
<td>Protein Load in μg</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

66K →
29K →
17.5K →

Wheat Germ Agglutinin (WGA)
Little Rock Orange Coprecipitates From Crude

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>M.W. Sigma</th>
<th>Crude Y=23</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/mg protein</td>
<td>3,200</td>
<td>17</td>
</tr>
<tr>
<td>Protein Load in μg</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

66K →
29K →
17.5K →

FIG. 5 SDS-PAGE electrophorctograms of two lectins isolated from their crude by Little Rock Orange, LRO, ligand coprecipitation. Sigma lane is affinity chromatographed lectin; LRO lanes are results from use of the technique in one step from particulate-free extract. Specific activities (13) are based on standard human erythrocyte agglutination assays. Protein loads specify amounts of total protein injected in each lane.
extracts in one or two short steps by using the stacking ligand Little Rock Orange, LRO. It is 1-(2-hydroxy-6-t-butyl-1-naphthylazo)-4-benzene sulfonate, guanidine salt, synthesized by Timothy Richardson (27). LRO is an azoaromatic sulfonate anion substituted with a t-butyl alkane on the naphthyl moiety. In Fig. 5 an SDS-PAGE gel reports human red cell agglutinative specific activities for two of the four lectins, Kidney Bean (PHA) and Wheat Germ Agglutinin (WGA), together with the protein loads in each electrophoretic lane, from LRO coprecipitation from crudes. Comparison is made with standard molecular weight markers and with commercially available (Sigma Co.) affinity chromatographed PHA and WGA lectins. The SDS-PAGE electrophoreses display one strong hand for the PHA lectin via LRO coprecipitation and reversal, and elimination of large amounts of foreign proteins on comparison with the crudes before the LRO coprecipitation step. There appears to be apparent heterogeneity for the WGA lectin out of LRO coprecipitation, showing five bands with good specific activities in hemagglutination. The multihand state of WGA occurs from this protein's isolectin character (28).

Use of LRO ligand (as with other classes of proteins) required finding the best pH ranges for coprecipitation from each crude, which usually are about half of a pH unit (13). Coprecipitating conditions with respect to $y_{LRO}$ optimization and amounts of LRO added (moles) per nominal mole of protein in crudes (average MW assumed equal to that of purified lectins) were found close to $y_{LRO} \approx 25$ for both lectins. In general, coprecipitation of the lectin class of proteins (which are diverse), and also many proteases and nucleases like ribonuclease, are high yielding practical techniques for coprecipitative isolation using matrix stacking ligands such as LRO.

**CONCLUSIONS**

Matrix stacking ligands and “entanglement” ligands provide an interesting means for isolating specific proteins from dilute crudes via upstream coprecipitation. Practical concerns and opportunities may be summarized as: Denaturing, graininess, hydrate water displacement by large ligands, stabilization or protection against a number of stresses during isolation. scalability, volume reduction, reversibility, interfacing with downstream processes. scavenging, and cost.

Ligands resembling those reported here have been richly studied in their binding to protein molecules in homogeneous solution in the sense of Reaction (1) (29). The homogeneous solution stage has its initiation and a stoichiometry ($P_{ligand}$) triggered largely through ion pairing by strong anion (sulfonate) attraction to cationic counterions on protein molecules. Conventionally, the appearance of precipitates or coprecipitates is seen as a hindrance, not as an opportunity, in many venues. But production of the kinds of coprecipitates discussed above, outlined in Reaction (2), is an ex-
pression of tail–tail interaction with a large free energy drop of its own. This second category of reaction is also a ligand hinding event, but the ligands in the Reaction (2) stage mutually bind to one another. Their organic tails form a lattice or host. Some protein molecules have surface patches that are hydrophobic (30), promoting protein–protein contacts that should tend to make them insoluble, but if no ligands were involved, the process would be precipitation, not coprecipitation. The process often centers around the protein isoelectric point. Exclusively (nearly exclusively) hydrophilic surfaced and water penetrated proteins such as serum albumin require organic ligands of these kinds to force coprecipitation (18), whereas homogeneous precipitation fails. The amounts of ligands necessary to bring about coprecipitatively precipitate protein molecules, nearly always fall in the \( v_{\text{ligand}} \sim Z_{\text{H}^+} \) range. Reaction category (2), tipping the system into an overt phase change, is predominantly an expression of nonpolar (hydrophobic) forces provided by the tail–tail interaction. As the bound ligand levels, \( v_{\text{ligand}} \) builds up even when starting from the most hydrophilic, water penetrated, and water-soluble proteins, serum albumin, many enzymes and lectins, as water is displaced. Target proteins become substantially coated with alkane azoaromatic or hydrocarbonaceous tails that displace the water, as in Fig. 2 and Reaction (2). Coprecipitation becomes quite dependent on the detailed organic structure of such ligands because the host lattice is sharply dependent on the same thing. However, the binding stoichiometry is largely set by the anion heads. The sulfonate, or sulfate ion pairing abilities of the ligands.

The "practical concerns and opportunities" listed above issue from these structural factors and the intermolecular forces they promote. The origins of selectivity were described above. The scavenging ability of chosen ligands for the coprecipitative isolation of sought-for proteins from 0.01 to 0.20% protein in starting crude (see Refs. 5, 12, and 18 for examples) is the basis of scalability of such systems. When sought-for proteins are coprecipitated, they centrifuge and/or filter out with "low technology" filtration practically eliminating the need for expensive "dewatering" lyophilization or concentration by pressure membrane filtration.

The partial specific volumes of such ligands, \( \bar{v} = 0.6 \) to 0.7 cm\(^3\)/g, are fairly low. Hence their effective densities \( (1/\bar{v}) \) are considerably above that of water itself or of most hydrated proteins (12). In addition their water-displacing capacities, both because of the intrinsic volumes of the tail groups and their marked abilities for tightening the conformation of extensively water-penetrated protein molecules as seen by hydrodynamic measurements (22, 31), all function to ease centrifugation and/or filtration. Such coprecipitates are grainy besides being dense and are quite convenient to handle in contrast to protein products which are colloidal and gummy because they are quite hydrated. Accordingly, when the general process works it generally interfaces well with other unit processes, including downstream chromatography, that may be re-
quired. The role of water in penetrating protein molecules and promoting unfolding is well known (32, 33). Such ligands do not exclusively react toward protein molecules. They help with the "water problem" which is the source, or partly the source, of several denatured and unit process difficulties.

Many kinds of ligands can be synthesized, and that enables many kinds of coprecipitative lattices to be built and new hydrophilic surfaces to be laid down over hydrophilic protein molecules. Variation in hydrocarbon substituents on matrix ligands and variation in anionic valencies provide an enlarged scope for development of protein isolation technique in the crude, dilute, upstream stage.

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REFERENCES


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