Estimation of Recombinant Bovine Somatotropin Solubility by Excluded-Volume Interaction with Polyethylene Glycols

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Purpose. The potential to estimate protein solubilities, with limited protein, by excluded-volume interactions was evaluated using polyethylene glycols (PEG) and recombinant bovine Somatotropin (rbSt).

Methods. Solutions of rbSt were prepared at concentrations significantly below saturation solubility. Subsequently, varying amounts of PEG were added to force protein precipitation. Following centrifugation, the protein concentration in the supernatant was assayed by reversed-phase HPLC, where a logarithmic relationship between solubility and % PEG was observed.

Results. An apparent protein solubility in the absence of PEG was determined by extrapolation and compared well with values measured by conventional approaches. Slopes of log solubility versus % PEG curves were consistent with excluded-volume principles and depended on the molecular weight of the PEG used. Furthermore, the precipitation process proved to be reversible, allowing for recovery of intact protein. Solubility-pH profiles obtained in the presence of PEG greatly reduced the quantities of protein needed and compared favorably with profiles in the absence of PEG.

Conclusions. Thus, it appears feasible and practical, with certain limitations, to obtain solubility estimates of proteins by volume-exclusion methods with limited supplies of protein.

KEY WORDS: recombinant bovine somatotropin; protein; growth hormone; polyethylene glycol; volume exclusion; solubility.

INTRODUCTION

The need for solubility information on proteins at very early stages of preformulation and formulation development can be crucial. The availability of bulk protein at early stages of drug development is limited and an efficient method for estimating protein solubility with minimal material is needed. Typical methods for measuring solubility include ultrafiltration, dialysis, dissolution of solid protein (phase/solubility), and abrupt pH changes. Alternately, macromolecules have been used to induce phase separation of a solid protein via principles of volume exclusion. Some of the potential pitfalls and uses of these solubility methods have been previously discussed (1).

Excluded-volume interactions of proteins with other macromolecules has been used to describe protein fractionation (2–4), partitioning of proteins in gel chromatography (5), protein aggregation (6,7) and protein solubility (1,8–13). The use of volume-exclusion principles to determine apparent thermodynamic activities of proteins in saturated protein systems has also been investigated (14). It has been demonstrated that precipitation by volume-exclusion does not denature the protein (2,8,10), and recovery of the protein for further studies is possible. Additionally, the conformational stabilization of proteins through volume-exclusion principles may allow solubility measurements to be obtained on proteins, which would otherwise be difficult to obtain due to limited conformational stability (10).

Volume-exclusion involves the addition of an inert macromolecule, such as polyethylene glycol (PEG), to a protein solution until precipitation of an amorphous protein phase occurs (4). A log-linear relationship between protein solubility and weight percent PEG has been demonstrated, where the intercept indicates protein solubility (1,3,8,9,14–16). The practical use of this method for obtaining preformulation information on recombinantly-derived bovine Somatotropin (rbSt) was evaluated (11,17).

METHODS AND MATERIALS

Materials

PEG was obtained from the following suppliers: PEG 8000 from Sigma (St. Louis, MO), PEG 3350 from Sentry Carbowax (Danbury, CT) and PEG's 900 and 300 from Dow Chemical (Midland, MI). Sodium phosphate was obtained from Baker (Phillipsburg, NJ), and sodium acetate, sodium carbonate and NaCl were obtained from Mallinckrodt (St. Louis, MO). Acetonitrile was from Fisher Scientific (Pittsburg, PA) and trifluoroacetic acid was obtained from Aldrich (Milwaukee, WI).

Buffer Preparation

To avoid pH changes due to alteration of ionization of buffer species with the addition of PEG, the PEG solutions were prepared as 40% PEG (%w/v) in 0.05 M acidic component of buffer, and 40% PEG (%w/v) in 0.05 M basic component of buffer system. Subsequently, the PEG stock solutions were combined to attain the desired pH. The ionic strength of each buffered master solution was adjusted with NaCl to either 0.12, 0.2 or 0.3.

Solubility Methods

Constant [rbSt]/Varying [PEG]. Initially, the rbSt concentration was kept constant and only PEG concentration was varied. An rbSt stock solution/suspension (2 mg/ml or 100 mg/ml) was prepared in 0.05 M phosphate, pH 7.4, μ = 0.12, and 1 ml aliquotted into 10 vials. The vials were then diluted to 2 ml with varying ratios of 40% PEG stock solution with buffer to give a final rbSt concentration of 1 mg/ml or 50 mg/ml, and up to 20% final PEG concentration. The vials were equilibrated for 15 minutes, centrifuged and the supernatant was assayed by reversed-phase HPLC. Samples were also pulled immediately after precipitation with PEG and at 15 minute intervals for 2 hours to evaluate suspension equilibration times. It was determined that PEG induced precipitation of rbSt came to equilibrium within 15 minutes.

Varying [rbSt]/Varying [PEG]. Sequential aliquots of

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PEG were added to a single rbSt solution, minimizing protein material consumption. A 2 ml rbSt stock solution/suspension (0.1 mg/ml-50 mg/ml), in 0.05 M phosphate, pH 7.4, μ = 0.12, was prepared. Serial 400 µl aliquots of 40% PEG buffered stock solution were added to the protein solution until precipitation was initiated. The suspension was allowed to equilibrate for 15 minutes before a 400 µl aliquot of suspension was pulled, centrifuged and the supernatant assayed by HPLC. A subsequent 400 µl aliquot of 40% PEG was then added to the suspension to cause further precipitation and the procedure was repeated up to eight times. The pH was monitored after each serial addition of PEG.

Reversibility. Solubilization studies were performed by precipitating >90% of the rbSt present in solution and then adding serial 400 µl aliquots of buffer to reduce the PEG concentration and resolubilize the protein. The supernatant was then assayed by pulling 400 µl aliquots of the suspension, centrifuging and assaying by HPLC. The process was repeated up to eight times.

Conventional Solubility Measurements. Two conventional methods were used to confirm our volume-exclusion values. These methods included 1) assaying the supernatants of saturated rbSt solutions at varying pH obtained by addition of excess solid and 2) dialyzing (<10kD cutoff) concentrated rbSt solutions in water against buffer to induce precipitation and assaying the supernatant of the retentate by HPLC.

Reversed-phase Liquid Chromatography

RbSt samples were assayed on a reserved-phase HPLC system consisting of a RP 300 Aquapore MPLC (4.6 mm × 3 cm) 10 µm particle size guard column from Brownlee, a RP 300 Aquapore MPLC (4.6 mm × 10 cm) 10 µm particle size analytical column from Brownlee, a Varian Vista 5500 pump system, a Perkin Elmer ISS 100 autoinjector, a Kratos Spectroflow 783 detector, 214 nm, and a Varian DS601 integrator. A gradient method from 46%–57% B at 0.5%/min was used, where mobile phase A was 0.1% trifluoroacetic acid in water (v/v) and mobile phase B was 0.1% trifluoroacetic acid in acetonitrile (v/v). Concentrations were determined using external standards.

RESULTS

Effect of rbSt Concentration

The effect of holding rbSt concentration constant during precipitation versus allowing the rbSt concentration to be diluted upon addition of PEG was explored. Precipitation curves were prepared at an initial rbSt concentration exceeding the saturation solubility (50 mg/ml). The rbSt concentration was allowed to decrease with increasing % PEG in one sample, while the rbSt concentration was maintained at 50 mg/ml in a second sample. A log-linear relationship between rbSt solubility and % PEG was determined for each of these samples and permitted estimation of rbSt solubility in the absence of PEG (Table 1) (Figure 1).

Secondly, a precipitation curve was prepared at an initial rbSt concentration approximately 10-fold less than saturation solubility (1 mg/ml). The protein concentration was kept constant with increasing PEG concentration. The intercept of the 1 mg/ml rbSt solubility curve was seen to be approximately one half that obtained from the 50 mg/ml curve (Table 1) (Figure 1), indicating that lower initial protein concentration may underestimate the solubility. When rbSt concentration was allowed to vary with increasing PEG, the extrapolated solubilities increased as the initial rbSt concentration was increased, indicating that the most accurate estimates can be obtained as the initial rbSt concentration approaches the saturation solubility. Similar to the 50 mg/ml rbSt results, the extrapolated solubility from the 1 mg/ml rbSt solution maintained at constant protein concentration agreed quite well with the 1 mg/ml varying rbSt concentration method (Table 1).

Table 1. Solubility of rbSt by Phase Solubility, Dialysis and Volume Exclusion Methods at Four Initial rbSt Concentrations (pH 7.4, μ = 0.12, 25°C)

Method	Initial rbSt concentration (mg/ml)			
	0.1	1.0	10.0	50.0
Phase solubility[a]			8.0 ± 0.4	12.1 ± 0.2
Dialysis			8.8 ± 0.5	10.8 ± 0.4
Constant [rbSt]		5.7 ± 0.4		9.1 ± 0.7
Varying [rbSt]	2.4 ± 0.3	4.1 ± 0.4	8.4 ± 1.0	11.6 ± 0.4
Vary, precipitate		4.6 ± 0.1	8.4 ± 0.3	
Vary, resolubilize		3.8 ± 0.3	11.5 ± 0.9	

[a] (initial [rbSt] = 100 mg/ml gave 14.3 ± 0.7 mg/ml; initial [rbSt] = 150 mg/ml gave 15.8 ± 0.6 mg/ml).

Reversibility

Solubilization curves were performed as well as precipitation curves for several reasons. First, to determine that volume-exclusion met the criteria for a reversible solubility determination. Secondly, to minimize material consumption

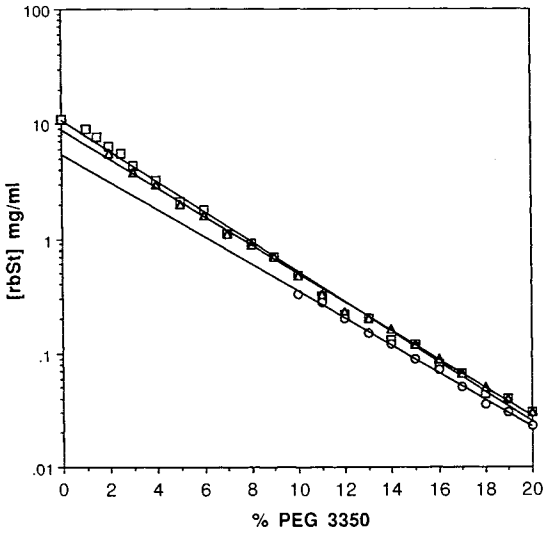

Fig. 1. Log-linear response of rbSt concentration (mg/ml) versus % PEG where (□) indicates an initial rbSt concentration of 50 mg/ml which decreased with increasing aliquots of PEG, (△) indicates 50 mg/ml rbSt and (○) indicates 1 mg/ml rbSt which were maintained at their respective total protein concentration irrespective of PEG concentration (25°C, pH 7.4, μ = 0.12).

by using the same protein solution for multiple solubility determinations. Studies were performed to determine if saturation solubility values could be reliably obtained from precipitate resolubilization curves as well as from volume-exclusion curves. RbSt precipitated with increasing concentrations of PEG or resolubilized by dilution of a precipitated rbSt suspension gave similar solubility estimates, demonstrating the reversibility of PEG precipitation on rbSt (Table 1). Furthermore, estimation of rbSt solubility via extrapolation from volume-exclusion data, using the varying [rbSt] method, compared well with more conventional techniques such as phase solubility and dialysis (Table 1). Conventional methods gave solubility values dependent on the initial rbSt concentration as well, indicating that this effect is not specific to volume-exclusion methods.

Effect of PEG

RbSt solubility was extrapolated using varying molecular weights of PEG in order to determine the effectiveness of volume-exclusion with increasing PEG size (Figure 2). Using a model which tests for difference in slope and intercepts (18), it was shown that the slopes were different (ρ = 0.0001), but the intercepts were not different (ρ = 0.15). Subsequent regression analysis using a common intercept model (18) gave an estimated solubility of 8.3 ± 0.4 mg/ml, in good agreement with other values determined from 10 mg/ml stock solutions (Figure 2) (Table 1).

Effect of Ionic Strength

Varying concentrations of PEG 3350 were used to precipitate rbSt (initial [rbSt] = 0.1 mg/ml) from solutions of varying ionic strength. Solubility estimates based on regressions of the individual lines were 1.2 ± 0.1, 2.1 ± 0.2 and 7.0 ± 1.9 mg/ml for ionic strengths of 0.12, 0.2 and 0.3, respectively. A model which uses the data from all three ionic strengths was used to test for equivalent slopes and intercepts (18). With such treatment, differences in slopes (ρ = 0.0054) and intercepts (ρ = 0.029) indicated that not only was the extrapolated solubility impacted by ionic strength, the effectiveness of PEG as a precipitant may also vary with changes in ionic strength. The effect of ionic strength on extrapolated and measured solubilities are shown in Figure 3.

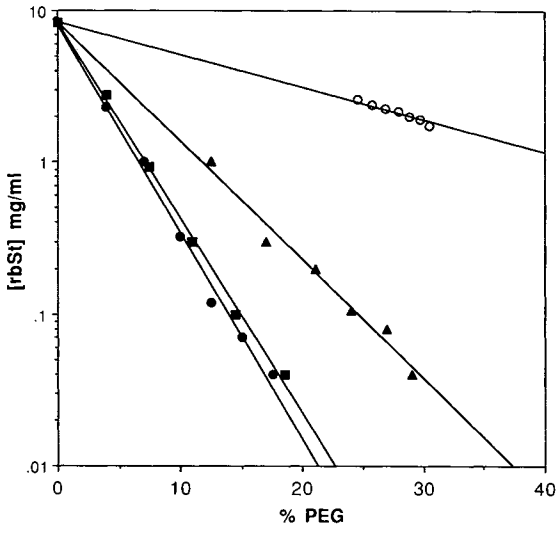

Fig. 2. Effect of PEG molecular weight on solubility of rbSt where (○) PEG 300, (▲) PEG 900, (□) PEG 3350 and (●) PEG 8000 (25°C, pH 7.4, μ = 0.12, initial [rbSt] = 10 mg/ml). Regression lines result from the use of a common intercept model.

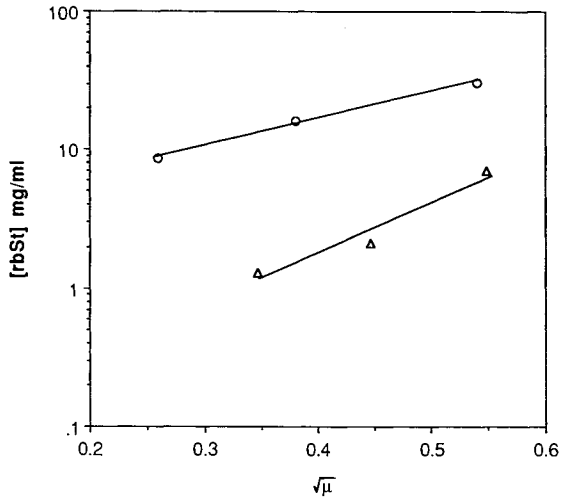

Fig. 3. Effect of ionic strength on rbSt solubility obtained (○) in the absence of PEG or by (△) extrapolated solubility values obtained from PEG induced precipitation of a 0.1 mg/ml rbSt solution (25°C, pH 7.4).

Effect of pH

The effect of PEG on solubility-pH profiles was explored (Figure 4), where rbSt solubility determined from a saturated rbSt solution (0% PEG) was compared to rbSt solubility curves in 1% and 5% PEG. The 1% and 5% PEG curves were similar in shape to the 0% PEG curve and occurred directly below one another, i.e., at a lower solubility without a significant shift of the curve along the pH axis.

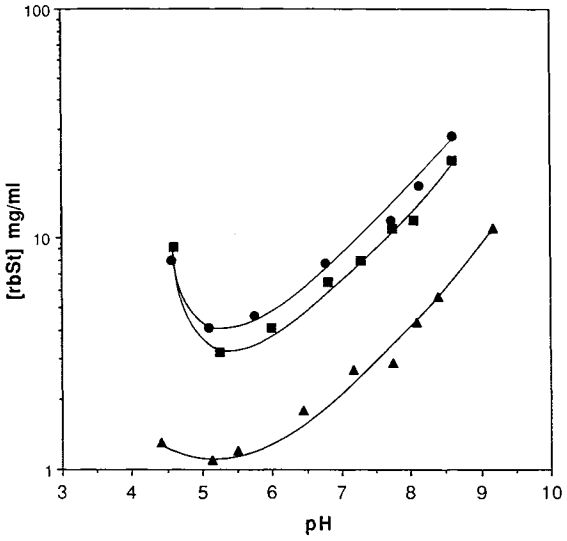

Fig. 4. Effect of PEG 3350 on pH profile of rbSt solubility where (●) 0% PEG, (■) 1% PEG and (▲) 5% PEG (25°C, μ = 0.12).

DISCUSSION

The use of volume-exclusion methods for estimating the solubility of proteins may be best described in Figure 5. The precipitation curve depicted by a 1 mg/ml protein solution (▲) represents a delineation between solubilized and precipitated protein. To the right of the line, protein has precipitated out of solution and to the left of the line, all of the protein remains in solution.

The amount of protein necessary can be minimized by using a method where successive aliquots of PEG solution (40% PEG) in precipitation studies, or buffer solution (0% PEG) in solubilization studies are added to a protein-PEG system. In precipitation studies (△), where PEG aliquots were added, the concentration of protein decreased from its initial concentration (e.g. 1 mg/ml), **a**, until the formation of the second phase, **b**, occurs. Upon further addition of PEG aliquots, the concentration of the protein in the solution phase will be indicated by the phase solubility line **b** through **c** to **e**, whereas the summed or total protein concentration in both phases will go from **b** to **d**. As long as the total amount of protein in the system exceeds a concentration dictated by the phase solubility line (▲), **b** to **e**, this aliquot addition method will allow estimates with minimal drug.

An alternative method, solubilization, can be used by adding a sufficiently large aliquot of PEG solution to result in a definite solid phase separation and is indicated by a change in total protein concentration from **a** to **d** with the concentration in the solution phase at that point being **e**. Additional aliquots of buffer (0% PEG) result in dilutions of the overall concentration of protein in the system from **d** to **c** as the concentration in the supernatant increases from **e** to **c**. With further additions of buffer, from **c** to **f** the total amount of protein becomes resolubilized. In either case, it is the aliquots of supernatant pulled and assayed from the two-phase system which define the phase solubility line and allow for extrapolation to 0% PEG, **g**, yielding an estimated solubility in the absence of PEG.

The importance of dilutions on the total concentration of protein in the system should always be considered to insure that aliquot additions do not over-dilute the protein concentration and prevent it from ever reaching a point of phase separation. For example, if a 5% PEG stock solution were used, the dilution of protein per aliquot addition versus the increase in % PEG would be such that the phase separation would not be obtained, for example, **a** to **h**.

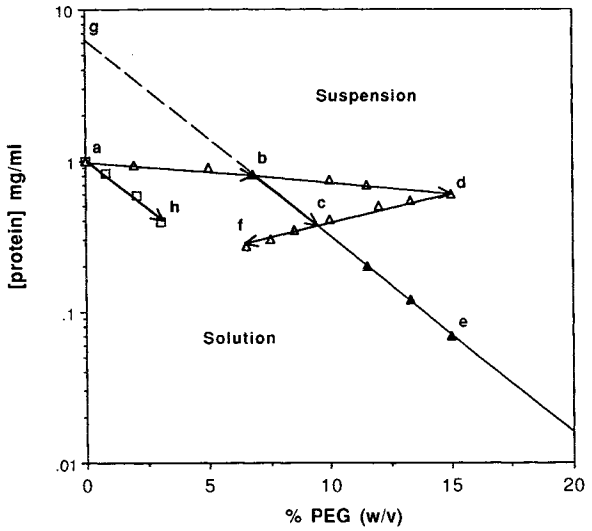

Fig. 5. Example of phase solubility diagram used in estimating protein solubility by volume exclusion methods.

Effect of rbSt Concentration

RbSt apparent solubility was seen to increase slightly with increasing initial protein concentration, independent of the method (Table 1). Other investigators observed that the fraction of protein found in the supernatant was independent of the initial protein concentration over a 50-fold range for albumin and a 10-fold range for β-lactoglobulin, deoxyhemoglobin, BSA and oxyhemoglobin (8,10,14). In the case of rbSt, a two-fold difference in estimated solubility was noted with a 50-fold change in initial rbSt concentration (Table 1).

Reversibility

Apparent solubility was obtained from precipitation curves as well as from resolubilization curves, showing good correlation between the two volume-exclusion methods and other independent data (Table 1). This method demonstrates the versatility of volume-exclusion with non-ionic polymers and substantiates the findings that precipitation by this method does not denature the protein in an irreversible fashion (2,8,10). The potential for reversible denaturation followed by reversible precipitation of a partially unfolded intermediate was not specifically evaluated. However, given that PEG's can specifically interact with partially unfolded intermediates (19) and that these intermediates have been observed with rbSt (20,21), such possibilities can not be ruled out. Reversible precipitation allows for curves to be obtained with far less material and ensures that the precipitation range may be double-checked with no additional use of material.

Effect of PEG

Volume-exclusion was shown to become more effective with increasing molecular weight of PEG, where less PEG was needed to precipitate rbSt with increasing PEG molecular weight. This trend agreed with that found by Ingham, Polson and others (2,5,8,9,16). It was also noted that the effect of PEG molecular weight was more pronounced at lower molecular weights. For example, the change in slope between PEG 300 and PEG 900 was much more dramatic than PEG 3350 to PEG 8000. Honig and Kula utilized this concept and determined that for selective protein precipitation, smaller molecular weight PEG's, with relatively shallower slopes, were more effective at precipitating a specific protein component cleanly (22). Conversely, for solubility determination of a pure protein, the choice of a larger molecular weight PEG may minimize the distance from the y-intercept and the subsequent error in extrapolated solubilities. However, this must be balanced against the greater chance for error given a steeper precipitation slope. From an experimental perspective there appears to be little value in using MW $>$ 3350 or MW $<$ 900 for solubility estimates.

Effect of Ionic Strength

Ionic strength was shown to affect rbSt solubility curves in several ways. At pH 7.4, increasing ionic strength increased the solubility of rbSt in the presence and absence of PEG (Figure 3). This salting-in effect has also seen for albumin, chymotrypsin and chymotrypsinogen by other researchers (3,8,16). The extrapolated solubilities from PEG studies ([rbSt] = 0.1 mg/ml) indicated an ionic strength effect similar to that determined in the absence of PEG, but underestimated the values by almost ten fold (Figure 3). This was consistent with the underestimated values previously observed when initial rbSt concentrations approaching 0.1 mg/ml were used (Table 1).

Effect of pH

Solubility of rbSt as a function of pH was shown to increase with increasing distance from the pI, indicating that protein solubility was at a minimum when at a net neutral charge (17). Other workers have also noted similar pH effects (3,8,9,22). The solubility-pH profiles of rbSt were obtained in varying dilute concentrations of PEG by conventional addition of excess protein to the solutions at various pH measurements. The concentration of PEG was shown to shift the entire curve to a lower solubility without altering the minima or overall shape of the pH profile. This indicated that the entire pH-solubility profile of rbSt, needed for preformulation, may be obtained with minimal material. However, some difficulties may be encountered if increasing concentrations of PEG significantly alter the protein's pKa's. Consequently, it may be best to limit the PEG concentration to less than 5% when attempting to determine the effect of pH on solubility.

CONCLUSIONS

Some practical factors to be considered when using the method of PEG-induced protein precipitation can be summarized and best understood in accordance with Figure 5: 1) the slope of the phase solubility line, **g** to **e**, should be maximized by using higher molecular weight PEG's, especially when the molecular weight of the protein is below 20 kD, 2) the dilution-protein concentration slope, **a** to **d**, should be minimized by using a high % PEG stock solution for aliquot addition (i.e. >30% PEG), thus avoiding a situation such as described for **a** to **h**, where addition of 5% PEG solution would not result in precipitation, 3) the point of saturation solubility should be reached with as low a concentration of PEG as possible, through use of as high a protein concentration as available, minimizing extrapolation distance, 4) the potential for changes in ionization equilibria of both protein, amino acids and buffers should not be ignored with higher PEG concentrations and 5) the potential for conformational alterations of proteins by PEG is possible if macromolecules such as PEG are used at higher temperatures or if partially unfolded intermediates are present (13,19).

Finally, while volume-exclusion principles may be utilized to estimate protein solubilities with less than 200 μg of protein, some precautions are necessary. For situations where protein-protein interactions are minimal, extrapolated solubilities are consistent with those obtained by conventional methods. Proteins which are highly soluble or have high protein-protein interaction terms may give intercepts which include an activity related term, and therefore do not depict practical solubility limits (9). Consequently, care should be taken in use of data for proteins with unknown properties and these estimates should be verified with conventional measurements when sufficient material is available.

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