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The effects of acetate buffer concentration on lysozyme solubility

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The effects of acetate buffer concentration on lysozyme solubility

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Abstract

The micro-solubility column technique was employed to systematically investigate the effects of buffer concentration on tetragonal lysozyme solubility. While keeping the NaCl concentrations constant at 2%, 3%, 4%, 5% and 7%, and the pH at 4.0, we have studied the solubility of tetragonal lysozyme over an acetate buffer concentration range of 0.01M to 0.5M as a function of temperature. The lysozyme solubility decreased with increasing acetate concentration from 0.01M to 0.1M. This decrease may simply be due to the net increase in solvent ionic strength. Increasing the acetate concentration beyond 0.1M resulted in an increase in the lysozyme solubility, which reached a peak at ~ 0.3M acetate concentration. This increase was believed to be due to the increased binding of acetate to the anionic binding sites of lysozyme, preventing their occupation by chloride. In keeping with the previously observed reversal of the Hoffmeister series for effectiveness of anions in crystallizing lysozyme, acetate would be a less effective precipitant than chloride. Further increasing the acetate concentration beyond 0.3M resulted in a subsequent gradual decrease in the lysozyme solubility at all NaCl concentrations.

1. Introduction

The tetragonal form of chicken egg white lysozyme has become the de facto standard protein for crystal growth studies. A large body of data on the crystal growth rate, nucleation rate and solubility (for example, see Refs. [1–5], meeting proceedings) exists because of the ease and reproducibility of working with this protein. Consequently, the crystal nucleation and growth behavior of this protein is better characterized than that of any other. For example, the growth rate at a given supersaturation varies with pH, temperature and salt concentration [6–8], which is believed to be due to the complex behavior of this protein in solution [6,9].

A knowledge of the phase behavior is fundamental to studies of the crystal growth process. The saturation concentration of a solute is the equilibrium concentration that is attained in the presence of its solid phase, and is dependent upon the nature of that phase. Proteins are typically crystallized or precipitated by the addition of a precipitating molecular species, which may for example be a neutral salt, a high molecular weight polymer such as PEG, or small organic compounds such as methylpentanediol [10]. Lysozyme crystallizations are typically from neutral salts. Hofmeister [11] empirically determined that different ions were more or less effective in desolubilizing proteins, giving rise to what is now called the Hofmeister or lyotropic series. Lysozyme

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solubility has been shown to be predominately a function of the anion species, whose order of precedence is

$$SO_4^{2-} > HPO_4^{2-} > CH_3CO_2^- > HCO_3^- > Cl^- > Br^- > NO_3^- > I^- > SCN^- .$$

The arrangement of the ions in this series is from less to more chaotropic, left to right. Hofmeister determined that more chaotropic ions tended to solubilize proteins, and that those to the left in the above series were better precipitants than those to the right. However, it has been shown that for lysozyme the solubility is the reverse order of this series [12–14]. The anionic species not only determines the solubility of the protein at any given condition, but also the crystal form obtained. Thus, more chaotropic anions (NO_3^- to SCN^-) give monoclinic crystals [12,13], while ions to the left of NO_3^- give tetragonal crystals at room temperature. Demonstrably, then, lysozyme solubility and phase behavior are also a function of their surrounding environmental composition.

Most lysozyme crystal growth and solubility studies have been in sodium acetate buffer, between pH 4.0 to 5.4. One major impediment of the direct comparison to other data has been the variety of conditions studied. Extensive solubility data is now available as a function of both pH and temperature for tetragonal lysozyme in 0.1M acetate buffer [15,16]. However, many researchers have worked at other buffer concentrations or not used buffers at all. One heretofore overlooked variable has been the effects of the buffer concentration on the crystal growth process. Perhaps this is because typical buffer concentrations are relatively low compared to the precipitant concentration. However, acetate is also known to be a weak precipitant of lysozyme [12]. This work was initiated in an effort to understand the effects of buffer concentration on the solubility of lysozyme. Additionally, it also gives some insight into how lysozyme is driven from solution by precipitating anions.

2. Materials and methods

Chicken egg white lysozyme (Sigma) was prepared for solubility column packing essentially as previously described [14,17,18]. The protein was dialyzed against pH 4.0 sodium acetate (NaAc) buffer at the appropriate buffer molar concentration (see below), then against pH 4.0 acetate buffer plus 2% (w/v) sodium chloride, at room temperature. The crystals and some of the supernatant from this procedure were set aside, and the remaining supernatant divided among four dialysis bags. At this point, crystalline suspensions were prepared by dialysis against 3.0%, 4.0%, 5.0% and 7.0% salt solutions at the appropriate buffer concentration.

The crystals from the above dialysis procedures provided material to pack the solubility columns. Reservoir solutions were prepared from the supernatant and dialyzate solutions. The tetragonal habit of the crystals was verified by simple visual inspection. After the solubility columns were packed, several milliliters of the appropriate reservoir solution were passed through them to ensure equilibration.

All non-protein materials were reagent grade or better. Acetate buffer solutions were prepared by adding the appropriate amount of acetic acid to ~ 950 mL of dH_2O, then adding the proper amount of sodium chloride, followed by adjusting the pH to 4.0 with saturated NaOH. The volumes were adjusted to 1.0 L after the pH titration procedure. All pH measurements were determined at room temperature. Lysozyme concentrations, after dilution into dH_2O, were determined using $A(1\%, 281.5\ nm) = 26.4$ [19].

The solubility apparatus was slightly modified from that previously reported [14]. In this case, the thermoelectric cooling plate was replaced by a stainless steel tube loop, with cooling provided by a refrigerated cooling circulator. This eliminated problems encountered due to failure of the previously used thermoelectric devices.

3. Results and discussion

Tetragonal lysozyme solubilities were collected at 0.01M, 0.05M, 0.15M, 0.2M, 0.3M, 0.4M and 0.5M sodium acetate buffer concentrations, and at 2.0%, 3.0%, 4.0%, 5.0% and 7.0% NaCl for each buffer concentration. Most solubility data were collected over the range of 4–25°C, with several passes re-

peated over this range in ~0.5 to 2.0 degree steps. As with previous solubility data from this laboratory, polynomial coefficients for best fits to each data set are given in Table 1. Some data sets, indicated by a *, were only collected from 9–25°C due to problems with the cooling system, and the coefficients given are only good over that range. The coefficients given for the 0.1M buffer concentration are from Cacioppo and Pusey [15]. Typically, ≥ 50 data points were used to determine the coefficients for each set of conditions.

For any given buffer concentration, the overall shape of the solubility versus temperature curves were similar to those previously obtained at 0.1M

Table 1
Coefficients from solubility data fit to a third-order polynomial

Molarity	% NaCl	A	$B \times 10^2$	$C \times 10^3$	$D \times 10^5$	Average deviation %
0.01 *	2.0	22.33	−248.3	160.1	78.95	8.0
0.01 *	3.0	−1.274	74.25	−31.63	131.4	6.3
0.01 *	4.0	2.810	−37.17	46.8	−96.44	7.7
0.01 *	5.0	−4.304	104.5	−56.35	118.0	9.6
0.01 *	7.0	−0.6891	28.78	−17.14	41.66	4.0
0.05 *	2.0	−35.06	871.2	−511.5	1274	5.7
0.05 *	3.0	−0.9917	75.87	−39.22	151.5	7.6
0.05 *	4.0	0.6162	30.64	−20.24	79.11	7.2
0.05 *	5.0	−2.385	67.66	−36.16	77.84	4.4
0.05 *	7.0	−0.2344	14.63	−4.663	8.806	7.5
0.10	2.0	−2.385	200.2	−169.4	684.2	4.7
0.10	3.0	−0.931	65.57	−54.52	208.9	5.7
0.10	4.0	−0.2784	21.10	−13.85	50.91	5.7
0.10	5.0	0.05134	7.642	−5.852	30.75	3.9
0.10	7.0	0.1444	0.0291	1.127	3.229	4.2
0.15 *	2.0	0.6501	215.3	−177.8	835.4	10.0
0.15 *	3.0	−1.957	108.4	−64.93	207.5	8.7
0.15 *	4.1	3.057	−26.59	19.59	−11.92	8.3
0.15	5.0	0.6938	6.345	−0.9337	16.09	11.7
0.15	7.0	0.5419	2.094	−1.069	9.346	10.2
0.20 *	2.0	−20.10	590.1	−374.8	983.0	9.9
0.20 *	3.0	19.43	−292.6	171.2	−234.0	10.1
0.20	4.0	1.258	13.88	−4.697	37.88	11.4
0.20	5.0	1.159	7.772	−7.160	35.77	12.3
0.20	7.0	1.952	−2.182	0.9851	6.858	8.8
0.30	2.0	0.6074	166.2	−113.3	515.0	8.5
0.30 *	3.0	5.966	−64.25	43.82	14.14	8.3
0.30 *	4.0	2.969	−34.37	28.42	−33.60	9.8
0.30	5.0	1.110	−4.964	6.573	−2.684	8.8
0.30	7.0	1.462	−4.880	5.914	−7.468	7.9
0.40 *	2.0	16.16	−246.1	198.4	−245.1	8.7
0.40 *	3.0	3.391	−42.12	42.32	−33.37	9.4
0.40	4.0	0.6952	9.327	−4.618	36.21	7.6
0.40	5.0	1.05	−5.643	5.839	2.21	9.1
0.40	7.0	0.9537	4.024	−0.3597	7.198	10.0
0.50	2.0	6.280	−6.032	14.28	115.0	8.6
0.50	3.0	0.7474	26.93	−16.29	81.97	7.3
0.50	4.0	0.4695	11.27	−5.348	31.92	6.7
0.50	5.0	0.6207	7.219	−3.906	23.76	6.9
0.50	7.0	0.5780	3.818	−1.571	8.982	9.7

Coefficients indicated by an * are only valid over the range from 10 to 25°C, the rest from 4 to 25°C.
Using the following equation, $A + BX + CX^2 + DX^3$, where $X = °C$, one can calculate the solubility. For example, at 0.1M NaAc, 3% NaCl, 20°C the solubility is 7.14 mg/ml.

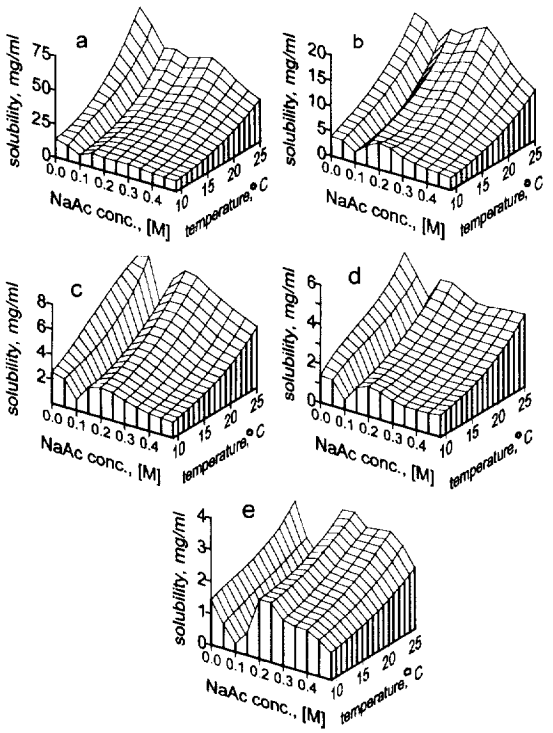

Fig. 1. Surface plots of lysozyme solubility as a function of the buffer concentration and temperature at fixed NaCl concentrations. Note that the Z axes are all on different scales. (a) 2% NaCl, (b) 3% NaCl, (c) 4% NaCl, (d) 5% NaCl, and (e) 7% NaCl.

acetate concentration. The major differences were observed when the solubility was plotted as a function of the buffer concentration and temperature. These plots, for each NaCl concentration, are shown in Fig. 1. The most obvious features, common to all of these plots, were (1) a pronounced minimum in solubility at ~ 0.1M buffer concentration, (2) steeply decreasing solubilities with increasing buffer concentration over the 0.01–0.1M concentration range (3) increasing solubilities with buffer concentration above 0.1M, and (4) a subsequent decrease in the solubilities at buffer concentrations greater than ~ 0.3M.

While the data in Fig. 1 indicates how the absolute solubilities respond to changing buffer concentrations, they do not clearly show where the system is most sensitive to these changes. This is shown in Fig. 2, where for each salt concentration the solubilities at 10°C and 20°C are normalized to those at 0.1M NaAc. These plots show that while the absolute changes in solubilities may be slight at higher NaCl concentrations, the percentage changes are considerably larger than at lower NaCl concentrations.

We had previously suggested that the large solubility differences noted between data collected by this lab and that collected by other groups at a lower buffer concentration was more likely due to differences in technique, or quality of the protein used [15], than to any buffer effects. From the results in Fig. 2, this assessment was obviously not correct. Depending upon the temperature and salt concentration, solubilities at 0.05M NaAc ranged from ~ 1.5–5 times greater than those at 0.1M NaAc. However, these rates of change were greater than those found for lysozyme at low NaCl concentrations. Over the 4–25°C range (0.1M NaAc, pH 4.0), lysozyme solubilities at 0.34M NaCl (2%) go from ~ 2.1 × (4°C) to 2.77 × (25°C) those at 0.43M NaCl (2.5%) (unpublished data). From Fig. 2, we see that a 0.05M change in the buffer concentration, whether decreasing from 0.1M to 0.05M or increasing from 0.1M to 0.15M, results in 1.5 to ~ 6.5 fold changes in the solubility. On a per mole basis, at low

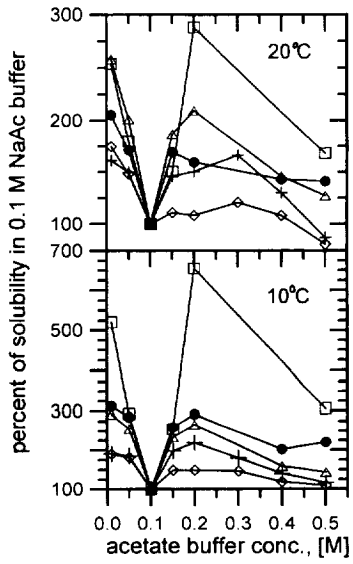

Fig. 2. The relative effects of the buffer concentration on solubility, as a percentage of the solubility at 0.1M NaAc. Legend: 2% NaCl (◇), 3% NaCl (+), 4% NaCl (△), 5% NaCl (●), and 7% NaCl (□).

buffer concentrations, lysozyme is more sensitive to the NaAc concentration than to NaCl. More interesting from a crystal growth standpoint is that this relative sensitivity increases with increasing NaCl concentrations. Slight changes in the buffer concentration can affect the solubility (and thus the supersaturation level), and these effects are more dramatic the higher the salt concentration.

From its position on the lyotropic series and based upon previous solubility data [12], acetate is a less effective precipitant for lysozyme than chloride. Because of this, it is unlikely that the effects at concentrations ≤ 0.1M are due to the direct desolubilization of lysozyme by the acetate ion. Ries-Kautt and Ducruix [12] have found that lysozyme solubility in ammonium acetate is ~ 10–12 times greater than in sodium chloride. However, their data also shows an ~ 3 fold increase in solubility between sodium and ammonium chlorides. We may reasonably assume then that solubilities in sodium acetate should be ~ 3–4 times those for sodium chloride. Ion binding studies in solution and in the crystal (work in progress) suggest that there are a minimum number of sites on the protein surface which have to be occupied by counter ions for full solubility to be reached. Occupation of sites beyond this level then results in the progressive decrease in protein–solvent affinity, and a concomitant increase in protein–protein interactions. While acetate is a less effective precipitant, it may still bind equally or more tightly to and preferentially occupy these minimally required "salting in" sites over chloride ion. The displaced chlorides would then be free to bind to a reduced number of remaining sites, thereby reducing the solubility. This argument does not adequately explain the proportionately greater sensitivity of the solubility to the acetate concentration at lower protein concentrations, where one would expect that the higher chloride concentrations would result in a reduction in the numbers of bound acetate.

The above argument makes the assumptions that the chloride and acetate binding sites are all the same and that selected sites are more important to others in determining the proteins solubility. This remains to be shown. Initially, our assumption had been that chloride was bound primarily to the basic amino acid side chains (lysine and arginine). Crystallographic data have since shown that this is not the case (work in progress). We can speculate that acetate ions may also (weakly) interact with hydrophobic pockets by means of the methyl group. Such an interaction would both cover a hydrophobic region and replace it with a charged, more hydrophilic group enhancing the protein solution interactions (i.e., the acetate would act as a weak detergent).

This speculation does not account for the rapid decrease in solubility found when the acetate concentration is increased from 0.001 to 0.1M. Also, it does not explain the (apparently) hyper-sensitivity to acetate over the 0.01M to 0.15M concentration range at higher chloride concentrations. This high sensitivity can translate into very large supersaturation swings on the crystal face in the presence of any buffer concentration gradients, and may be a heretofore "hidden" cause of variations in growth rate data and growth cessation. Studies of the solubility of lysozyme in acetate buffer (without other anions present) and of acetate binding to lysozyme are now under way and should help clarify this problem.

The increase in solubility above 0.1M acetate suggests that there is competitive displacement of chloride from sites on the protein by the buffer anions. Again, from Fig. 2, it would appear that this effect is proportionately greater the lower the protein concentration (i.e., the higher the chloride concentration). The increases in solubility continue to ~ 0.2–0.3M acetate, after which the solubility again decreases. This final decrease is most likely due to the concentrations of acetate becoming sufficiently high to drive protein precipitation.

The interpretation, that the mechanism by which acetate and chloride ions affect lysozyme solubility through direct binding to the protein surface, runs counter to the preferential interaction studies of Timasheff [20]. Their data indicated that at high concentrations (0.5–1.0M) both anions are strongly excluded from, and that there is little binding to, the protein. Extensive binding to the protein surface was characteristic of destabilizing or salting-in effects. However, kinetic and equilibrium binding data ([21], and work in progress) show that the formation of protein–protein bonds, as would be occurring at high precipitant concentrations, occurs by the breaking of extensive protein–anion interactions, releasing those anions back to the solution.

On the basis of the evidence at hand, we can only

speculate about the interplay between chloride, acetate and lysozyme which leads to the results presented above. Explanations for much of this behavior are not evident. For example, observations not introduced into the above is the apparent shift in the solubility minimum at 2% NaCl, from 0.1M to 0.2M acetate, between 10 and 25°C, or why the solubility minimum should generally be so firmly "rooted" at 0.1M acetate. While determining the origins of the buffer effects on solubility remains to be resolved, one practical result is immediately apparent. Accurate and reliable crystal growth data can only come from a strict control over all relevant parameters, which can only be accomplished by the careful preparation of the material to be used. On the basis of the above it is obvious that the buffer concentration is a major factor in determining the data obtained, and should be controlled accordingly.

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