A comparison of salts for the crystallization of macromolecules

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Abstract

Thirty-one proteins and viruses that we knew from our own experience could be crystallized, or had been reported to have been crystallized by others, were investigated. In this experiment, each protein or virus was subjected to a crystallization screen of 12 different salts, each titrated to pH 7.2 beforehand, at concentrations ranging from 20% saturation to 90% saturation. Eight macromolecules failed to crystallize at all from any salt and were omitted from consideration. From the remaining 23 proteins, each salt was scored according to how many proteins and viruses it successfully crystallized. Among several results, one was particularly striking. Sodium malonate clearly was much more successful than any other salt, resulting in the crystallization of 19 of the 23 macromolecules, almost twice as effective as the next most successful salt, which was a draw between sodium acetate, sodium tartrate, sodium formate, and ammonium sulfate (11 of 22). The high success rate of sodium malonate in producing crystals was even more impressive when an overall unique success rate with individual macromolecules was considered.

Crystallization of biological macromolecules, including proteins, nucleic acids, viruses, and other macromolecular assemblies, has assumed an important role in molecular biology because it is a prerequisite for their structure determination by x-ray diffraction. Crystallization of macromolecules remains problematic, however, and still is dependent on empirical approaches (McPherson 1998; Bergfors 1999). Confining the most probable sets of crystallization conditions and reagents to a manageable array is, therefore, highly desirable.

We attempted in this experiment to identify which salts, a major class of precipitants used in macromolecule crystallization, are most likely to yield success, and which of them offer low probability of promoting nucleation and growth. Proteins used for evaluating the efficiency of the salts were those that could be crystallized by at least some means. Most had been crystallized previously in this labo-

ratory. Often, however, the proteins had not been crystallized previously from salt, but from polyethylene glycol (PEG), organic solvents, or by adjustment of pH.

All salts were treated the same to prevent bias, that is, all experimental trials were carried out at the same pH 7.2, at the same temperature, 17°C, and in identically the same apparatus.

Results

The results of the experiment are presented in histogram form in Figure 1 and as a matrix in Table 1. When the successes for each protein are weighted as described in Materials and Methods, then the histogram in Figure 2 is obtained. The highest success rate was achieved with sodium malonate, which was significantly greater than the next most successful salts. No salt failed entirely, though LiCl produced crystals of only a single protein and was least effective. Several proteins were uniquely crystallized by only a single salt; they were xylanase (MgSO₄), myoglobin (malonate), bovine trypsin (MgSO₄) and RNase A (formate). Most of the proteins that failed to crystallize at all (β -lactalbumin, for example) were those we had crystal-

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Fig. 1. Histogram of the number of proteins successfully crystallized for each of the 12 precipitant salts investigated.

lized previously from PEG or propanol or at a pH distant from 7.2.

Discussion

An unexpected result that emerged was the significantly higher success rate in crystallizing macromolecules shown by sodium malonate, even with respect to ammonium sulfate, which traditionally has been the salt of choice. The results here, assuming they are representative of most macromolecules, suggest that malonate should be one of the first precipitants included in crystallization attempts, and one to be emphasized in crystallization trial matrices. If only a very small amount of biological material is available, and critical selection of precipitants is necessary, then sodium malonate should be considered strongly. With only four exceptions, malonate produced crystals for every protein for which crystals were obtained at all, and in one case yielded crystals when all others failed. One of the exceptions was the protein thaumatin, which requires tartrate ions to construct the tetragonal lattice of its most easily grown crystal form (Ko et al. 1994). There, individual tartrate molecules link together three thaumatin molecules through hydrogen bonds. Two other exceptions were crystallized only by MgSO_4 , and no other salt, suggesting that in those cases the cation may have played an important role. Though it might of course be said

for any of the salts here, it is nonetheless possible that were pH explored, and not held fixed, malonates' success rate might have been even higher.

Malonate, whose structure is seen in Figure 3, is an organic acid of relatively high charge density, which carries two negative charges at neutral pH. It has a very high solubility in water and is likely to be a "cosmotrop" in terms of its interaction with water (Collins et al. 1985, Collins 1995). Therefore, it is likely to stabilize protein structure as well as conform with local water structure.

From Table 2 it can be seen that sodium acetate and lithium chloride have solubilities in water that are even higher than sodium malonate. The ions of these salts, however, carry a single negative charge per ion at neutral pH. This is important because ionic strength, which drives the "salting out" phenomenon, is a function of the square of the ion valence (Cohn and Ferry 1943). Thus, at comparable concentrations, malonate produces four times the ionic strength of acetate or chloride. As a consequence, a common observation was that whenever a protein crystallized from multiple salts, it did so from malonate at 10% to 40% lower levels of saturation.

Saturated sodium malonate at pH 7.2 is ~4 M, but as the pH decreases, it approaches 15 M in its acid form. Sodium acetate and formate, even at neutral pH, are soluble to nearly 15 M. Thus, there are only about 3 to 12 molecules

Table 1. *Proteins and viruses investigated*

Protein	Organism/organ source	Reference
α -lactalbumin	Cow milk	Sigma Biochemical Co.
Elastase	Pig pancreas	Sigma Biochemical Co.
Trypsin plus benzamidine	Pig pancreas	Sigma Biochemical Co.
Antibody IDEC CE 9.1	Human-simian chimera-antigen CD4	IDEC Pharmaceuticals, Inc.
Hemoglobin	Goat blood	Sigma Biochemical Co.
Antibody IDEC-151	Human-simian chimera antigen CD4	IDEC Pharmaceuticals, Inc.
Lipase	Bacterial	NOVO Pharmaceuticals, Inc.
Cucumber mosaic virus (CMV)	Infected tobacco leaves	This laboratory
Satellite tobacco mosaic virus (STMV)	Infected tobacco leaves	This laboratory
Myoglobin	Horse muscle	Sigma Biochemical Co.
Glucose Isomerase	Bacteria	Hampton Research
Antibody 61.1.3	Mouse IgG1-antigen phenobarbital	QED Pharmaceuticals, Inc.
Trypsin plus benzamidine	Cow pancreas	Sigma Biochemical Co.
Thaumatin	Serendipity berry	Sigma Biochemical Co.
Ribonuclease A	Cow pancreas	Sigma Biochemical Co.
Satellite panicum mosaic virus (SPMV)	Infected millet leaves	This laboratory
Catalase	Cow liver	Sigma Biochemical Co.
Edestin	Hemp seed	Sigma Biochemical Co.
Bromegrass mosaic virus T = 1 particles	Barley leaves	This laboratory
Pepsin	Cow stomach	Sigma Biochemical Co.
Concanavalin B	Jack Bean	This laboratory
Xylanase	Bacteria	Hampton Research
Lysozyme	Hen egg	Sigma Biochemical Co.

of water for every organic ion. Because malonate, acetate, and formate have seven, four, and three nonhydrogen atoms, respectively, both in terms of mass and occupied volume, the ions are comparable to, or in excess of, water by as much as twofold in some cases.

The manner by which these ions interact with a protein's surface begins to assume considerable significance at these concentrations. This is true particularly when the concentration of protein also is high, as in crystallization mother liquors, where the center-to-center distances between mac-

Fig. 2. Histogram of the weighted score for each salt, where the weight applied for each protein was the reciprocal of the total number of salts which successfully crystallized that specific protein. This gives a measure of the uniqueness of the successes.

Fig. 3. The structures of the ions utilized in the investigation.

romolecules are relatively small. For example, in a 20-mg/mL lysozyme solution, the macromolecules are separated on average by only two to three molecular diameters (Fredericks et al. 1994).

The small organic acids, citrate, tartrate, malonate, acetate, and formate, as an ensemble, make a strong showing. Each of the organic acids is competitive with ammonium sulfate, the differences being barely significant. This suggests that the organic acids are, as a class, valuable precipitants for crystal growth. Neither of the chlorides, lithium or sodium, appear to offer any advantage, nor for that matter does phosphate.

As noted above, thaumatin crystallizes under many conditions, and from a variety of salts, so long as some tartrate

Table 2. *Precipitant salts investigated*

Salts	Mol. wt.	Solubility in H ₂ O
Ammonium sulfate	132.14	5.80 M
Sodium malonate	148.03	14.80 M
Lithium sulfate	109.95	3.50 M
Ammonium phosphate	132.07	4.45 M
Sodium phosphate	163.94	1.74 M
Sodium citrate	258.07	2.98 M
Sodium acetate	82.04	15.2 M
Sodium tartrate	210.15	1.95 M
Magnesium sulfate	120.36	10.4 M
Sodium chloride	58.44	6.11 M
Ammonium formate	63.06	11.30 M
Lithium chloride	42.39	18.1 M

is included as well (unpubl. data). There also are other cases where organic ions are similarly obligatory components. These examples suggest that an even more potent crystallization precipitants might be devised based on high concentrations of malonate, but also containing other organic acids. The set could be expanded to include other small organic ions such as succinate or isocitrate. Cocktails of small organic ions carried in high concentrations of malonate may, therefore, prove to be a profitable trail to follow.

The organic acids offer at least one additional advantage over sulfate, phosphate, and chlorides. Crystals grown from

Table 3. *Matrix of success of each salt with each macromolecule*

Protein virus	Ammonium sulfate	Sodium malonate	Lithium sulfate	Ammonium phosphate	Sodium phosphate	Sodium citrate	Sodium acetate	Sodium tartrate	Magnesium sulfate	Sodium chloride	Ammonium formate	Lithium chloride
α-Lactalbumin	X	X	X									
Elastase	X	X										
Porcine trypsin + benzamidine	X	X	X	X	X	X	X	X	X	X	X	
Antibody IDEC CE9.1		X				X	X	X				
Hemoglobin	X	X		X								
Antibody IDEC-151		X					X					
Lipase		X						X				
CMV	X	X					X	X			X	
STMV	X	X	X	X	X	X	X	X	X		X	
Myoglobin		X										
Glucose isomerase	X	X	X	X		X	X	X	X		X	
Antibody 61.1.3	X	X	X	X	X	X	X	X	X	X	X	
Bovine trypsin + benzamidine									X			
Thaumatococcus								X		X	X	
Ribonuclease A											X	
SPMV		X		X			X				X	
BMV T = 1 particles		X		X		X						
Pepsin		X				X						
Concanavalin B	X	X		X		X		X				
Xylanase									X			
Lysozyme		X					X			X	X	X
Catalase		X	X	X	X	X	X	X				
Edestin	X	X				X					X	

these latter salts are difficult problems for cryocrystallography (Garman and Schneider 1997), which appears most successful on crystals grown from PEG or 2-methyl-2,4-pentanediol (MPD). High concentrations (>50% saturated) of tartrate and malonate, however, freeze as a glass and inherently serve as useful cryoprotectants. Thus, crystals grown from malonate may need the concentration of the salt in the mother liquor increased to >50% if necessary, but otherwise may require no additional cryoprotectant. Crystals grown from salts such as ammonium sulfate may, in at least some cases, gradually be exchanged into malonate or tartrate mother liquors.

Materials and methods

The proteins and viruses investigated, as well as their sources, are shown in Table 3, the salts in Table 2. Saturated solutions of each salt were made and titrated to pH 7.2 with an appropriate acid or base. Considerable caution must be exercised in preparing the saturated solutions of the organic acids. In the case of malonate, it is made by neutralizing malonic acid with concentrated sodium hydroxide and titrating it with the base to the desired pH. This can be a violent reaction and must be carried out slowly, in a vented hood, wearing safety gloves and eyewear and otherwise observing proper safety procedures. This pH was chosen on the basis of the pH distribution of successful crystallizations obtained from database searches (Gilliland 1988, Hampton Research Catalog 1995). Solutions of 20%, 30%, 40%, 50%, 70%, and 90% saturation then were made by dilution with water of the saturated solutions and these were used in the crystallization trials.

All experiments were carried out in Cryschem sitting-drop plates sealed with clear plastic tape. Reservoirs were 0.6 mL of the salt solution and drops were composed of 5 μ L of the stock protein solution and 5 μ L of the reservoir. Each protein was screened against the six concentrations (20% through 90%) of the 12 salts, thereby utilizing three Cryschem plates. The plates were placed in an incubator at 17°C and examined periodically under microscopes over 6 weeks.

In scoring a salt for success, the only consideration was whether any macromolecule crystals were observed for any concentration of that salt. That is, whether crystals appeared at several concentrations of the salt, or at only a single concentration, was not taken as relevant to the final score.

Different macromolecules produced different overall success rates. Some, such as satellite tobacco mosaic virus (STMV), crystallized from virtually every salt, and others (trypsin, myoglobin) crystallized from only a single salt. To take this into account, a success rate for each salt also was computed based on the uniqueness of the successes. In this scoring, each protein success was given a value dependent on the inverse of the number of salts, which gave crystals of that protein, i.e. $\frac{1}{12}, \frac{2}{12}, \dots, \frac{12}{12}$.

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