STRUCTURAL BIOLOGY COMMUNICATIONS

Volume 75 (2019)

Supporting information for article:

Penetration of dyes into protein crystals

Alexander McPherson

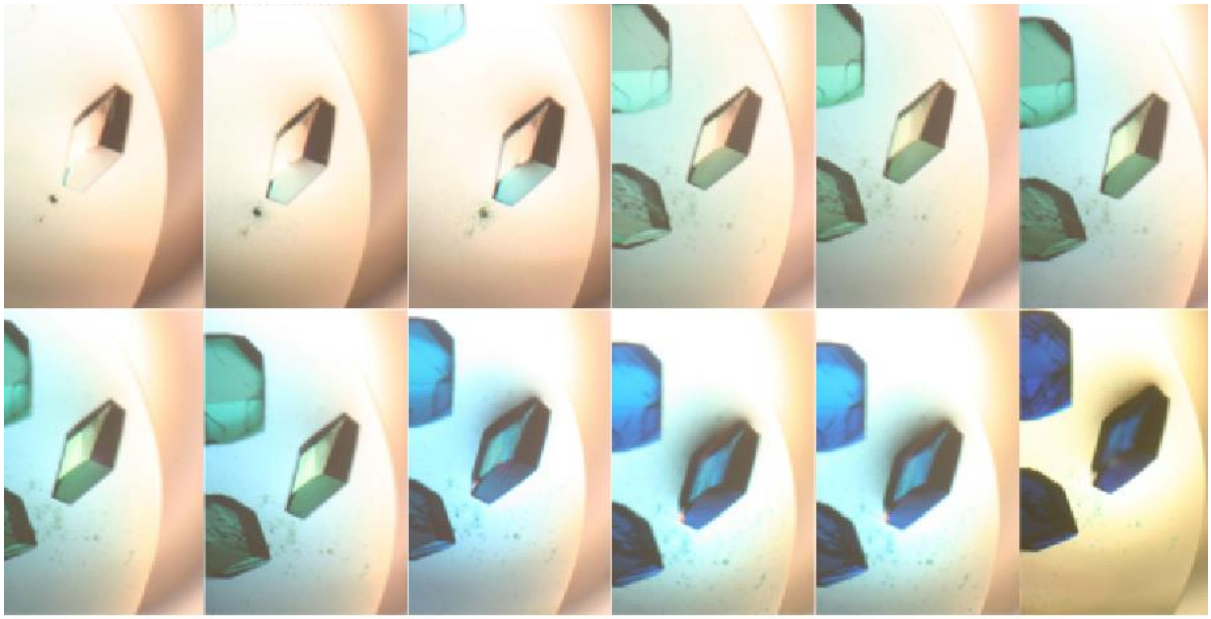

Figure S1 A crystal of tetragonal lysozyme (about 0.8 mm in largest dimension) is seen incorporating the dye bromophenol green over a period of about 18 hours.

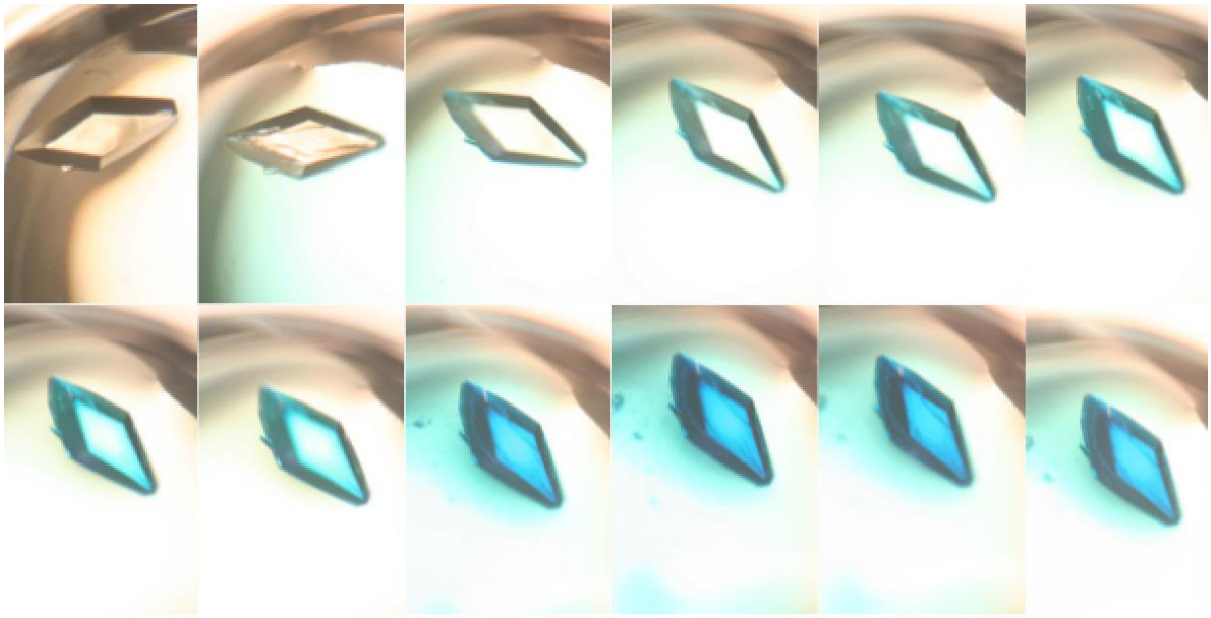

Figure S2 A crystal of tetragonal lysozyme (about 1 mm in length) is seen incorporating the dye methyl green over a period of about 18 hours.

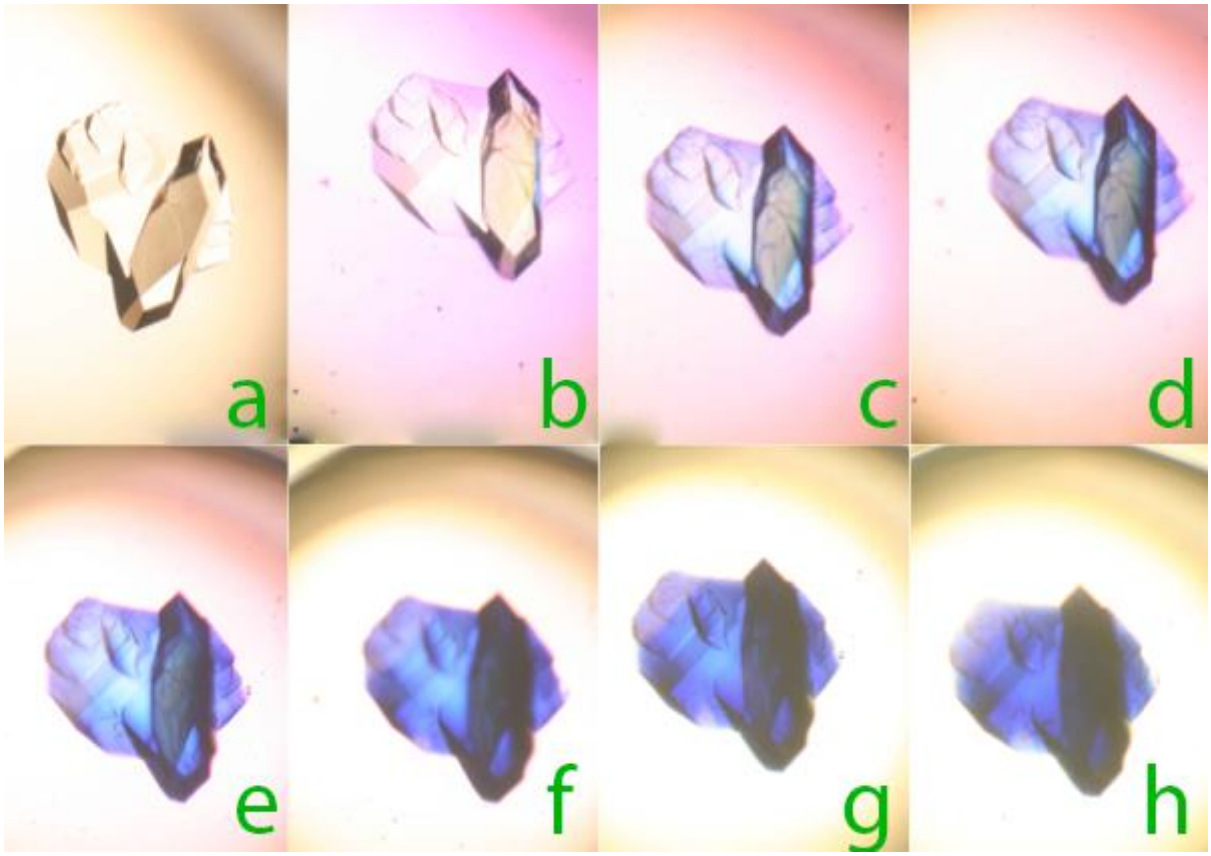

Figure S3 A cluster of tetragonal lysozyme crystals, the largest of which has a maximum dimension of about 1.2 mm, is seen incorporating the dye toluidine blue from its mother liquor. The total diffusion time to saturation was about 25 hours.

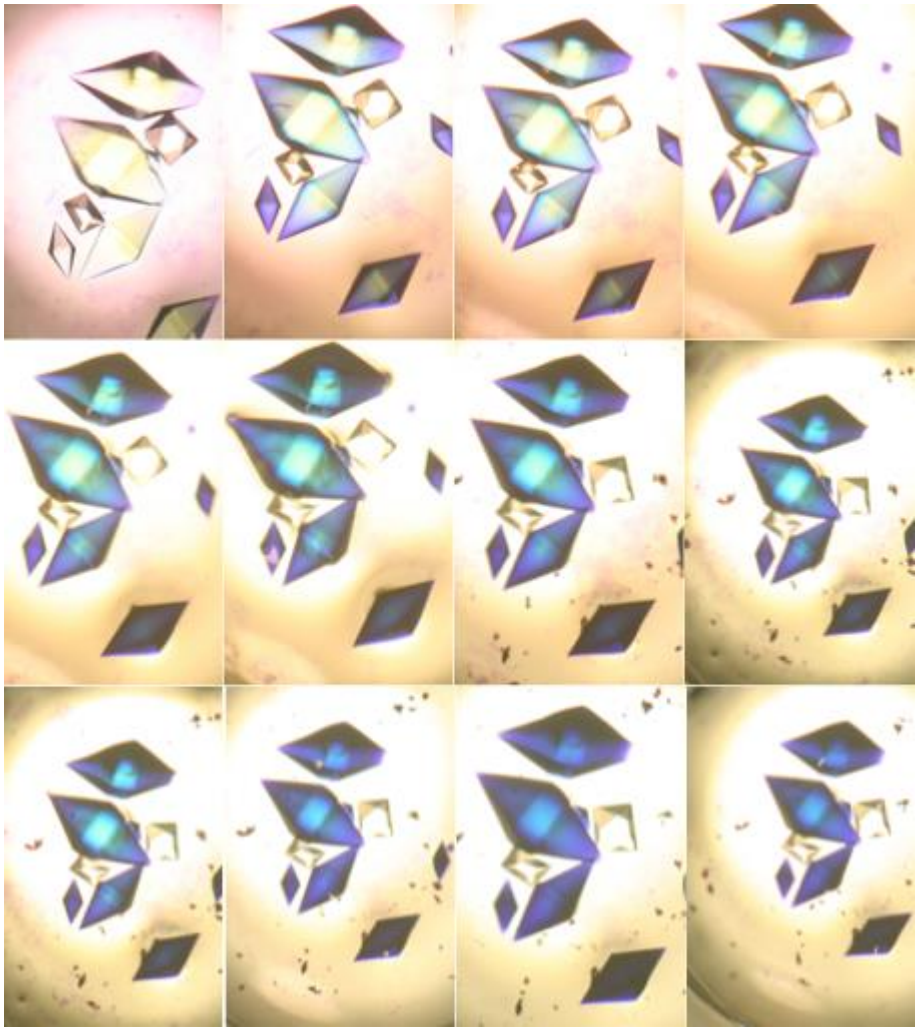

Figure S4 Crystals of tetragonal thaumatin having maximum dimensions of about 0.70 mm are seen incorporating the dye toluidine blue. The total diffusion time to saturation with the dye was about 22 hours.

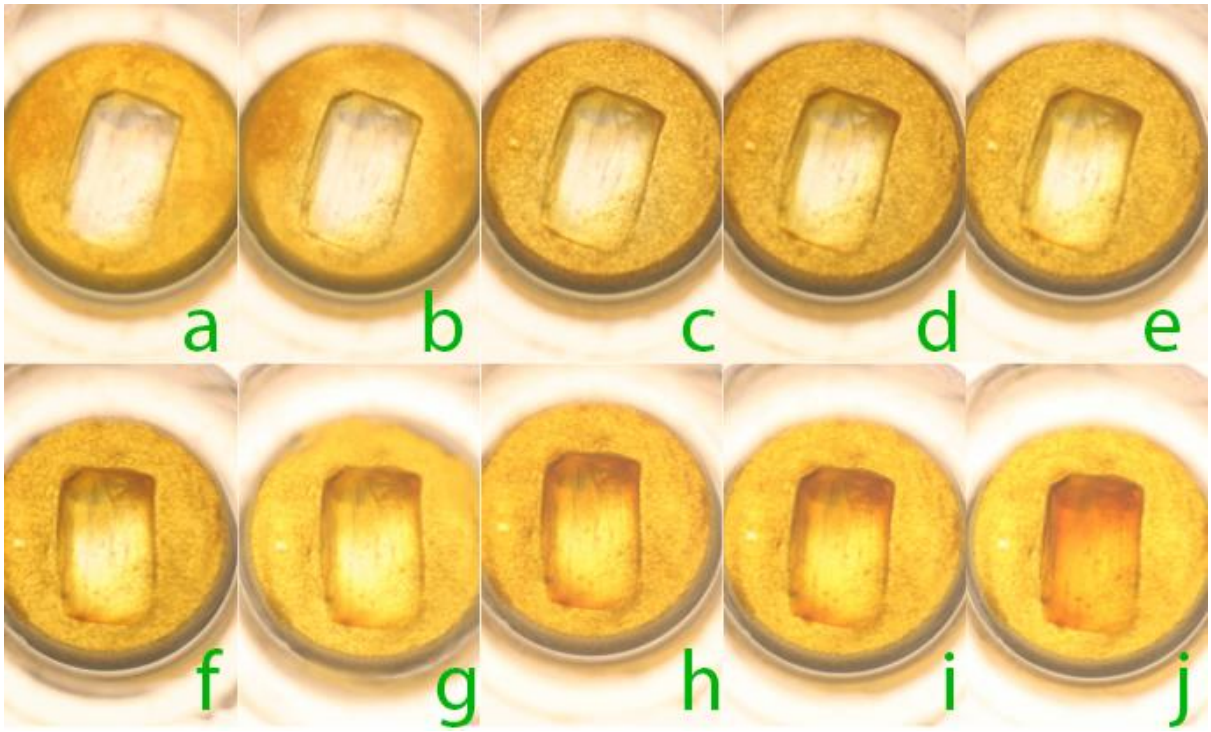

Figure S5 A single tetragonal lysozyme crystal of length approximately 6 mm and width 3 mm is seen staining with the dye thioflavin T, a strongly interacting dye. Even after 5 weeks (j) the crystal is still far from saturated with the dye.

Table S1 Dyes Investigated

DYE	Mwt.	pH or redox indicator
Bromophenol blue (4 bromine atoms)	670	pH
Rivanol (ethacridine lactate)	343	
Erythrosine B (4 iodine atoms)	880	
Xylene Cyanol	539	
Nitro Blue Tetrazolium Chloride	818	
Evans Blue (very low solubility)	960	
Chicago Sky Blue 6B (very low solubility)	993	
Brilliant Blue R (very low solubility)	826	
Basic Fuchsin	338	redox
Congo Red (Chlorophenol Red)	697	pH
Neutral Red	289	pH
Thioflavin T	319	
Methyl Orange	327	pH
Coomassie Brilliant Blue	826	
M – Cresol Purple	382	pH
Crystal Violet	408	redox
Methylene Blue	320	redox
Safranin Orange	351	
Azure Blue	306	
Methyl Green	653	redox
Bromothymol Blue (2 bromine atoms)	624	redox
Bromocresol purple (2 bromine atoms)	540	pH
Phenol Red	354	pH
Bromocresol Green (4 bromine atoms)	698	pH
Dimethyl Yellow (low solubility)	225	pH
Acriflavin	260	

Table S2 Crystallization Conditions

Orthorhombic crystals of canavalin: Canavalin was prepared from precanavalin by cleavage with purified bovine trypsin at 37 º as described in detail elsewhere (Smith *et al.*, 1982). It was crystallized at 22º C, dissolved with minimal NH_4OH and recrystallized by combining 30 to 40 mg/ml solutions of the cleaved protein with 2X Dulbecco's phosphate buffered saline (DPBS) at pH 6.0. Large crystals were obtained in Cryschem sitting drop vapor diffusion plates by redissolving the crystalline canavalin with minimal NH_4OH, and equilibrating 10 to 15 µl droplets against 0.6 ml reservoirs of 1X DPBS at pH 6.0.

Rhombohedral crystals of canavalin: Exactly the same procedure as above was followed except that the precanavalin was cleaved at 37º C, not by using purified bovine trypsin, but a crude aqueous extract of porcine pancreatin (Sigma – Aldrich, St. Louis, MO) made with DPBS and clarified by centrifugation. The cleavage may also be done with proteinase K or pronase.

Concanavalin A crystals: Concanavalin A (Sigma – Aldrich, St. Louis, MO) was dissolved in water at 25 to 30 mg/ml. Sitting drops of 10 to 12 µl volume consisting of 1: 1 mixtures of the protein with 2 M NH_4SO_4 were equilibrated at 22º C by vapor diffusion with 0.6 ml reservoirs of 2 M NH_4SO_4 with no buffer in either drop or reservoir.

Trypsin inhibited with benzamidine crystals: Purified bovine trypsin (Sigma- Aldrich) was dissolved in 0.1 M Tris – HCl buffer containing 4 mm $CaCl_2$ and 0.2 M benzamidine to a concentration of 40 mg/ ml. Large crystals were grown over a week's time at 22º C by vapor diffusion in sitting drop Cryschem plates. The sample drops were 12 to 16 µl in volume and consisted of 1: 1 mixtures of the protein solution with 1.35 to 1.75 M NH_4SO_4 in water. They could also be grown by substituting for the NH4SO4 sodium malonate in the range of 35% to 43% saturation at pH 7.

Beef Catalase crystals: Beef catalase in water plus trace thymol (Sigma – Aldrich) was concentrated to 25 to 30 mg/ml. Drops of 1 : 1 mixtures of the protein solution plus reservoir solution were equilibrated by vapor diffusion with 0.6 ml reservoirs at 32º C. The reservoirs were 25 mM to 50 mM Magnesium formate in water.

Tetragonal lysozyme crystals: Hen egg lysozyme (Sigma – Aldrich) was dissolved in H_2O to a concentration of 100 mg/ml. Drops of 10 to 14 µl of the protein solution with reservoir solution were equilibrated at 22 º C by sitting drop vapor diffusion against 0.6 ml reservoirs. The reservoirs were 6% to 8% w/v NaCl buffered with 0.1 M acetate at pH 4.8, or 0.1 M HEPES at pH 6.5 to 7.5. The largest crystals grew at room temperature over the period of a week from the higher pH samples.

Monoclinic lysozyme crystals: Using lysozyme dissolved in H_2O at 100 mg/ml concentration the same procedure was followed as for the tetragonal crystals above, except that the reservoir solution was 2.5% w/v NaNO3. The buffers were the same. It is essential not to let chloride ions creep into any solutions or the tetragonal crystal form will appear.

Thaumatin crystals: The sweet protein thaumatin (Sigma – Aldrich) was dissolved in 0.1 M HEPES at pH 6.5 to a concentration of 50 mg/ml. Drops 12 to 16 µ in volume of a 1 : 1 mixture of the protein solution with the reservoir solution were equilibrated at 22º C using sitting drop vapor diffusion with 0.6 ml reservoirs. The reservoir solution was varied between 0.5 M and 1.5 M unbuffered Na-K tartrate.

Beta-Lactoglobulin crystals: Beta-lactoglobulin from cow's milk (Sigma – Aldrich) was dissolved in 0.1 M citrate buffer at pH 3.0 to 3.5 to a concentration of 40 mg/ml/ 10 to 14 µl drops of 1 : 1 mixtures of the protein solution with reservoir solution were equilibrated at 22º C by sitting drop vapor diffusion with 0.6 ml reservoirs. The reservoirs were 3.5 M $(NH_4)SO_4$ in water. Upon combining the protein solution with the $(NH_4)_2SO_4$ solution a heavy precipitate form almost immediately, but the crystals grow in spite of the precipitate over several days to a week's time.

Glucose Isomerase crystals: Bacterial glucose isomerase (Hampton Research, Aliso Viejo, CA) as a crystalline suspension in 2 M $(NH_4)_2SO_4$ was centrifuged to pellet the protein microcrystals. These were dissolved in water to a concentration of about 30 mg/ml and dialyzed for 12 to 16 hours against deionized water to remove residual salt. Drops of 12 µl volume composed of 1 : 1 mixtures of the protein solution with reservoir solution were equilibrated at 22º C by sitting drop vapor diffusion with 0.6 ml reservoirs. The reservoirs were 18% PEG 3350 containing 30 mM MgCl2 buffered with 0.1 M Tris – HCl at pH 8.0. This procedure yielded large polyhedral crystals in 24 to 48 hours. If the pH of the reservoir solution is buffered at pH 5.5 to 6.5 then a second crystal form appears as large thin laths.

Concanavalin B crystals: The protein concanavalin B, which is an inactive form of chitinase, was prepared as previously described (Morrison *et al.*, 1984) from Jack Bean meal (Sigma – Aldrich). The protein is prepared as a microcrystalline suspension at very low ionic strength. It is necessary to dissolve the concanavalin B microcrystals in 5% w/v NaCl made 0.1 M in acetic acid, and this with gentle heating under a hot water tap. The protein solution was centrifuged at high speed and filtered through 0.22 um filters to minimize residual nuclei. The protein solution was mixed in a 1 : 1 ratio with reservoir solutions in the range of 42% to 48% saturated $(NH_4)_2SO_4$ in water. Drops of 1 to 14 µl of the protein – reservoir mixture were then equilibrated by sitting drop vapor diffusion in Cryschem plates against 0.6 ml reservoirs containing the $(NH_4)_2SO_4$ solutions. The plates were first incubated at 42º for two hours immediately after setting them up, and then they were transferred to 22º C for crystallization.

The same crystals of concanavalin B could also be grown using the same procedures but substituting 15% PEG 400 and some other polymers as the reservoir in place of $(NH_4)_2SO_4$, but this was not completely reproducible.

Satellite Tobacco Mosaic Virus (STMV) crystals: STMV was prepared from TMV – STMV co- infected tobacco leaves as described elsewhere (Koszelak *et al.*, 1989). The virus was dissolved to a concentration of 4 to 8 mg/ml in 0.1 M phosphate buffer at pH 6.0. 1 : 1 mixtures of the STMV solution with reservoir solutions in 8 to 12 µl drops were equilibrated at 22º C by vapor diffusion with 0.6 ml reservoirs containing $(NH_4)_2SO_4$ in the concentration range of 10% to 20% of saturation.

RNase S crystals: Three different crystal forms of RNase S (the subtilisin cleaved form of RNase A) were obtained by vapor diffusion in Cryschem plates. RNase S (Sigma – Aldrich) was dissolved in water to a concentration of 30 mg/ml and combined 1 : 1 with a reservoir solution to make the sample drops of 10 to 14 ul volume. The reservoir solutions and the crystals they yielded at 22º C were:

(a) monoclinic crystals of very thin flat plate habit with reservoirs of 25% PEG 1000.

(b) Thick monoclinic crystals of irregular habit with reservoirs of 2 M $(NH_4)_2SO_4$ containing also 5% Peg 400 buffered with 0.1 M sodium citrate at pH 4.0.

(c) Trigonal crystals of rhomboid habit from reservoirs of 25% PEG-MME.

Table S3 Crystal Properties

PROTEIN	**S.G.**	**UNIT CELL**	**SOLVENT**
Glucose Isomerase	I222	92.7 97.8 102.6	Vm = 2.64 56% solvent
	$P2_12_12$	98.5 129.6 78.3	Vm = 2.84 57% solvent
Concanavalin B	$P6_1$	81.0 81.0 101.0	Vm = 3.19 61% solvent
Thaumatin	$P4_12_12$	58.6 58.6 151.8	Vm = 2.93 58% solvent
Lysozyme	$P4_32_12$	78.7 78.7 38.6	Vm= 2.09 41% solvent
	$P2_1$	28.0 62.9 60.3 90.7°	Vm = 1.83 33% solvent
Concanavalin A	I222	63.1 87.0 89.2	Vm = 2.27 46% solvent
Trypsin + benzamidine	$P2_12_12_1$	54.7 58.3 67.6	Vm = 2.37 48% solvent
STMV	I222	174.3 191.8 202.5	Vm = 2.26 46% solvent
Canavalin	$C222_1$	137.0 150.0 133.0	Vm = 2.42 49% solvent
	R3	137.0 137.0 76.0	Vm = 2.42 49% solvent
Beef Catalase	$P2_12_12_1$	83.1 140.5 228.7	Vm = 2.78 56% solvent
Beta – Lactoglobulin	$P3_221$	54.0 54.0 112.7	Vm = 2.58 52% solvent
RNase S	C2	101.8 33.4 73.6 90.4°	Vm = 2.15 43% solvent
	$P3_221$	64.8 64.8 65.2	Vm = 2.73 55% solvent
	C2	74.5 48.9 96.4 91.5°	Vm = 2.14 42% solvent