Published in final edited form as:
J Struct Biol. 2014 November ; 188(2): 102–106. doi:10.1016/j.jsb.2014.09.011.

Efficient Cryoprotection of Macromolecular Crystals using Vapor Diffusion of Volatile Alcohols

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Abstract

Macromolecular X-ray crystallography, usually done at cryogenic temperature to limit radiation damage, often requires liquid cryoprotective soaking that can be labor intensive and damaging to crystals. Here we describe a method for cryoprotection that uses vapor diffusion of volatile cryoprotective agents into loop-mounted crystals. The crystal is mounted into a vial containing a small volume of an alcohol-based cryosolution. After a short incubation with the looped crystal sitting in the cryosolution vapor, the crystal is transferred directly from the vial into the cooling medium. Effective for several different protein crystals, the approach obviates the need for liquid soaking and opens up a heretofore underutilized class of cryoprotective agents for macromolecular crystallography.

Keywords

X-ray crystallography; cryoprotection; vapor diffusion

1. Introduction

Data collection at cryogenic temperature has become the normal approach for structure determination via X-ray diffraction. The low temperature (typically 100 K) slows radiation damage and is especially useful at high intensity synchrotron radiation sources (Kmetko et al., 2006; Owen et al., 2006). However, cryogenic cooling itself can damage the crystal and compromise diffraction quality, often due to ice formation (Haas and Rossmann, 1970; Juers and Matthews, 2001; Juers and Matthews, 2004; Kriminski et al., 2002; Low et al., 1966). Cooling-induced damage is typically reduced by cooling faster and/or adding cryoprotective agents such that the system cools through the freezing point of water to the glass transition before ice can form (Chinte et al., 2005; Shah et al., 2011; Warkentin et al., 2013). The use

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of pressure to prevent the formation of ice I during cooling has also been successfully applied to several systems (Burkhardt et al., 2012; Kim et al., 2005; Thomanek et al., 1973).

Many different cryoprotective agents have been identified (Bujacz et al., 2010; Gulick et al., 2002; Holyoak et al., 2003; Hope, 1988; Marshall et al., 2012; Mueller-Dieckmann et al., 2011; Pemberton et al., 2012; Rubinson et al., 2000; Vera and Stura, 2014), including sugars, linear and branched polyols, salts, organic solvents, amino acids, methylamine osmolytes and viscous hydrocarbons. In some cases, an adequate cryoprotective agent is already present in the crystallization buffer and the crystal can be cryocooled directly from the growth drop. However, very often an additional cryoprotection step is performed by soaking the crystal in a cryosolution, which can be laborious and damaging to crystals due to handling and osmotic stresses. Approaches to cryoprotection that limit such treatments would be advantageous.

Volatile alcohols are known to be efficient cryoprotective agents and have been useful for cryopreservation of microorganisms (Hubalek, 2003) and for low temperature crystallography in the liquid state (Douzou et al., 1975). Recent experiments showed that both methanol and ethanol require lower concentrations (w/v) than traditional cryoprotective agents (e.g. glycerol and ethylene glycol) to prevent ice formation in small volumes of plunge-cooled solution (Warkentin et al., 2013). Despite their effectiveness, volatile alcohols have seen little use for cryoprotection in macromolecular crystallography, due in part to the difficulty of working with their high vapor pressures. Of the ~100,000 structures in the protein data bank, just 0.2% have methanol or ethanol present in the model, while 14% include either glycerol or ethylene glycol (Berman et al., 2000). A recently described vial mounting methods offers the possibility of turning the high vapor pressure into an advantage to deliver the volatile alcohol to a loop-mounted crystal (Farley and Juers, 2014). Here we show the approach is rapid and effective for several different protein crystals. Subsequent cryocooling yields high quality diffraction without ice formation. The approach does not require liquid soaking and opens up a new class of cryoprotective agents for macromolecular crystallography.

2. Materials and Methods

2.1 Crystals

Chemicals were from Hampton Research (Aliso Viejo, California, USA; glucose isomerase #HR7–100) or Sigma-Aldrich (St. Louis, Missouri, USA; all other chemicals). Orthorhombic glucose isomerase crystals were used as provided by the supplier. All other crystals were grown using hanging drop vapor diffusion with 24 well plates (Hampton Research, Aliso Viejo, CA) at 294–298 K (277 K for hexagonal thaumatin) and used within a few months of growth. Tetragonal lysozyme (#L6876) well: 20 mM NaOAc 4.5, 3–5% w/v NaCl; protein: 80–100 mg/mL in 20 mM NaOAc 4.5(Forsythe et al., 1999). Orthorhombic and trigonal trypsin (#T8003) well: 100 mM Tris 8.0, 25% w/v PEG 8000, 0.2 M $AmSO_4$, 0.1 M benzamidine HCl; protein: 50 mg/mL in water (Leiros et al., 2001); Tetragonal thaumatin (#T7638) well: 0.2 M – 0.9 M Na/K tartrate; protein: 35–70 mg/mL in 100mM HEPES 7.3 (Ko et al., 1994). Hexagonal thaumatin (#T7638) well: 0.1 M NaOAc 4.5, 0.175 M $AmSO_4$, 0.1 M $LiSO_4$, 0.1 M $MgCl_2$, 15% (v/v) glycerol, 2% (w/v) PEG 400;

protein: 35 mg/mL in 100 mM HEPES 7.3(Charron et al., 2004); Thermolysin (#P1512) well: 30% sat'd $AmSO_4$; protein: 150 mg/mL in 45% v/v DMSO (hexagonal); 100 mg/mL in 45% v/v DMSO, 0.5 M $ZnCl_2$ (tetragonal) (Hausrath and Matthews, 2002). Tetragonal proteinase K (#P6556) well: .3–.4 M Na/K tartrate or 12–15% w/v PEG 8K; protein: 30–50 mg/mL in water. Cubic insulin (#I5523) well: 345–525 mM NaPhosphate dibasic, 10 mM EDTA 9.2; protein: 15 mg/mL in 18 mM NaPhosphate dibasic, 10mM EDTA 10.5(Gursky et al., 1992). In all cases, drop sizes were 6–9 μL and were ½ well/½ protein, except for thermolysin, which used just the protein solution given set up over the well. Prior to cryocooling, some thermolysin crystals were serial diluted (2–3 minutes) into DMSO-free protein solution (i.e. water for hexagonal crystals and 0.5 M $ZnCl_2$ for tetragonal crystals) to ensure the absence of the natural cryoprotective effects of DMSO. Similarly, some glucose isomerase crystals were serial diluted over 2–3 minutes from their 0.9 M $AmSO_4$ solution into 0.25 M $AmSO_4$.

2.2 Cryosolutions

Cryosolutions were based on four volatile alcohols – methanol, ethanol, isopropanol, and tert-butanol. Binary cryosolutions (alcohol/water) were prepared gravimetrically, while well-based cryosolutions were prepared volumetrically using 2X well solution, water and the alcohol. Because the low surface tension can make vial mounting difficult (see below), we also tested a cryosolution of 7.5 % agar, 40% methanol and 52.5 % water (by weight). The agar was dissolved in hot water and pipetted into a cryovial. Then the methanol was added and the solution was mixed, covered with a crystal-cap and O-ring and allowed to cool.

2.3 Vial Mounting and Cryoprotection

Vial mounting proceeded as previously reported (Farley and Juers, 2014). Briefly, a cryovial (Hampton Research, Aliso Viejo, California, USA) was prepared by plugging the liquid nitrogen escape holes with clay and fitting an O-ring (amazon.com, nitrile rubber, 50A durometer hardness; 3/8" ID×1/16" thick) on the crystal cap (SPINE, Hampton Research). Crystals were mounted by placing the crystal growth coverslip in a humid flow of 85–98% RH, looping the crystal using cryoloops of 20 μm diameter nylon with microtubes snapped at the 18 mm notch (Hampton Research) and inserting into a vial containing 500 μL of cryosolution. Crystals were mounted directly from drops without adding extra solution. (Sometime crystals were pushed into the drop.) The vial was allowed to sit for some time period (a few seconds up to 16 hours). Our default condition was 2 minute equilibration against 40% w/w methanol. After equilibrating, the crystal was directly mounted on the diffractometer from the vial. It is recommended that the vial undergo minimal handling and that the crystal cap be manipulated with a thermally insulated wand in order to uniformly maintain the cap-vial system at ambient temperature. The vial mounting technique should be practiced to achieve the smooth motions required to prevent crystals from being dislodged from the loop. The goniometer should be positioned such that the vial is at least horizontal and ideally angled downward as it is removed from the crystal cap, keeping the low surface tension cryosolution towards the bottom of the vial. The cryosolution can also be prepared as an agar gel to limit its movement during mounting (see above).

2.4 X-ray Data Collection

X-ray data were collected using an Agilent Xcalibur X-ray diffractometer with a Nova X-ray source and Onyx detector (Agilent Technologies, Santa Clara, California, USA) using the following parameters: 50 kV, 0.8 mA, crystal to detector distance = 65.000 mm, theta (the detector angle) = 3.5°, oscillation width = 0.25°, number of frames: 2×6, separated by 90 degrees. The detector edge was set to 1.8 Å for all crystals, regardless of their diffraction power. Exposure times were 15 or 30 seconds, the latter if the shorter exposure did not yield 2.0 Å data. Data were processed with CrysalisPro (Agilent) in Pre-experiment mode, which outputs cell parameters, an estimate of the diffraction limit and the mosaicity.

3. Results and Discussion

Fig. 1 compares diffraction images from crystals incubated in-vial over crystal growth well solution vs an alcohol-based cryosolution. The crystals equilibrated over alcohols show high quality diffraction to at least 2.0 Å resolution, comparable to crystals cryoprotected by soaking in traditional cryoprotectants (i.e. ethylene glycol, glucose, MPD) while the negative controls show ice and reduced diffraction power. Many of the crystals diffracted to much higher resolution than 2.0 Å, and data sets were collected to 0.95, 1.3, 1.5, 1.5, 1.5, 1.8, and 1.9 Å resolution for trypsin (orthorhombic), proteinase K, lysozyme, glucose isomerase, thaumatin (tetragonal), thermolysin (tetragonal) and insulin respectively. The approach was effective for eliminating ice from well-diffracting crystals as well as the complete cryoprotection of crystals for which the negative control destroyed the crystal lattice. Initially, in-vial equilibration times of tens of minutes were used, since we found previously that small unit cell changes occur on that time scale for vial mounts of thaumatin crystals (Farley and Juers, 2014). Subsequently, for most of the proteins we tested shorter equilibrations (except for trigonal trypsin for which we only had two crystals) finding 10 seconds – 3 minutes produced high quality diffraction.

The method was successful with all ten crystals tested Eight crystals could be cryoprotected using the vapor of a simple binary solution of water and alcohol and two crystals required supplementing the crystal growth solution with alcohol. For tetragonal thermolysin, methanol/water produced high mosaicity and an apparent change in space group but tert-butanol/water yielded diffraction nearly equal to a positive control (diffraction to 2.0 Å vs 1.9 Å for a soak in 50% w/w glucose). Hexagonal thaumatin dissolved upon exposure to methanol/water vapor, but using the well solution supplemented with 5% v/v methanol yielded high quality diffraction beyond 2.0 Å. For PEG grown proteinase K, alcohol only solutions usually yielded high mosaicity, as did well solution supplemented with methanol. But well solution supplemented with 60% ethanol or 50% isopropanol yielded high quality diffraction with Bragg spots beyond 1.2 Å. Tartrate grown Proteinase K could be cryoprotected with 40% methanol, but with somewhat higher mosaicity (~0.7° vs ~0.5°) than the ethanol or isopropanol protected PEG grown crystals. Additionally, lysozyme crystals (grown from 5% NaCl) were tested with ethanol (90% w/w), isopropanol (85% w/w), tert-butanol (75% w/w) and methanol/water/agar (see methods), yielding diffraction similar to the 40% w/w methanol/water cryoprotected crystal.

The mechanism of cryoprotection of the volatile alcohols can be understood in the framework of critical droplet theory, in which all standard cryoprotective agents (e.g. methanol, ethanol, glycerol, glucose) function simply by sterically hindering ice nucleation (Warkentin et al., 2013). The presence of these solutes decreases the probability of finding a region of pure water of sufficient size to crystallize, which increases the free energy of ice nucleation. Vapor diffusion apparently delivers the alcohol to high enough concentration to prevent ice nucleation both external and internal to the crystal. Initial examination of electron density maps for lysozyme, thaumatin, proteinase K, glucose isomerase and trypsin indicates the presence of some bound alcohol molecules, consistent with rapid delivery to the crystal and subsequent diffusion into the crystals. Further work is underway to understand the extent of diffusion along the solvent channels and binding to the protein during the short equilibration.

As described, the method includes humid flow for manipulating crystals, an O-ring to help seal the crystal capvial junction, and crystals directly mounted on the cryostream from the vial. These enhancements have clear benefits, including improved reproducibility of cell parameters, the possibility of long in-vial incubations, more time for crystal handling, and more reliable removal of external solution (Farley and Juers, 2014). Simpler approaches were also tested with lysozyme, glucose isomerase, thaumatin and tetragonal thermolysin. Using a vial at ambient humidity without an O-ring yielded high quality diffraction data without ice (Fig. 2). Using vial equilibrated crystals cooled by rapidly removing them from the vials and plunging into liquid nitrogen also yielded data of similar quality.

The main advantage of the method is its ease of use. The large vapor pressure (Table 1) facilitates transport of the cryoprotective agent to the crystal, obviating the need for liquid soaking, which can be laborious if serial soaks are required and damaging from handling and osmotic stresses. Another benefit of organic solvents is that they tend to reduce protein solubility by decreasing the dielectric constant of the medium (McPherson, 1999), unlike glycerol and ethylene glycol, which solubilize proteins (Auton et al., 2011).

The required conditions will depend on particulars of each crystal/solvent system. Minimum concentrations for cryoprotection were 20% – 40% w/w for the binary cryosolutions and usually somewhat lower for the well-based cryosolutions (Table 2). Well-based cryosolutions were effective for all crystals tested, so a conservative approach would be to start with them. In some cases, the diffraction quality depended on the alcohol concentration, so a range of concentrations should be tested. Minimum equilibration times also varied, but were relatively short – ranging from 30 seconds for lysozyme crystals to 3 minutes for very large – 800^3 μm^3 – insulin crystals). Longer equilibrations tended to reduce cell parameters (i.e. for insulin from 77.9 Å vs 77.6 Å for 45 second and 30 min incubations respectively). It should be noted that crystal packing changes associated with dehydration can occur slowly (Sanchez-Weatherby et al., 2009) (Farley and Juers, 2014). Therefore, in the event that short equilibrations are unsuccessful, we suggest that overnight incubations be considered, using an appropriately sealed vial/crystal cap.

Compared to crystals cryoprotected via liquid soaking with traditional cryoprotectants the unit cell volumes of the volatile alcohol cryoprotected crystals were smaller by 0–2%, which

could be due to dehydration or greater thermal contraction of the alcohol solutions (Alcorn and Juers, 2010). Dehydration can increase the extent of crystal contacts and improve diffraction (Kiefersauer et al., 2000) (Russi et al., 2011), but can also be detrimental (Bernal and Crowfoot, 1934). The smaller cell volume may have been part of the reason tetragonal thermolysin required tert-butanol instead of methanol, and follow-up studies are being conducted to further understand this result. Because the alcohols tested and traditional cryoprotective agents sample different ranges of thermal contraction, the approach described may be viewed as complementary to liquid soaking with traditional cryoprotective agents.

4. Conclusions

We have shown that the vial mounting method in concert with a volatile alcohol/water cryosolution combine to yield a new, effective approach for cryoprotecting macromolecular crystals. The approach is rapid, uses simply prepared cryosolutions, limits crystal handling and does not require liquid soaking.

Acknowledgments

This work was supported in part by a grant from the National Institutes of Health (GM090248).

Abbreviations

DMSO	dimethyl sulfoxide
AmSO4	ammonium sulfate
NaOAc	sodium acetate
PEG	polyethylene glycol
Tris	tris(hydroxymethyl)aminomethane
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MPD	2-methyl-2,4-pentanediol
MeOH	methanol
EtOH	ethanol
iPrOH	isopropanol
tBuOH	t-butanol

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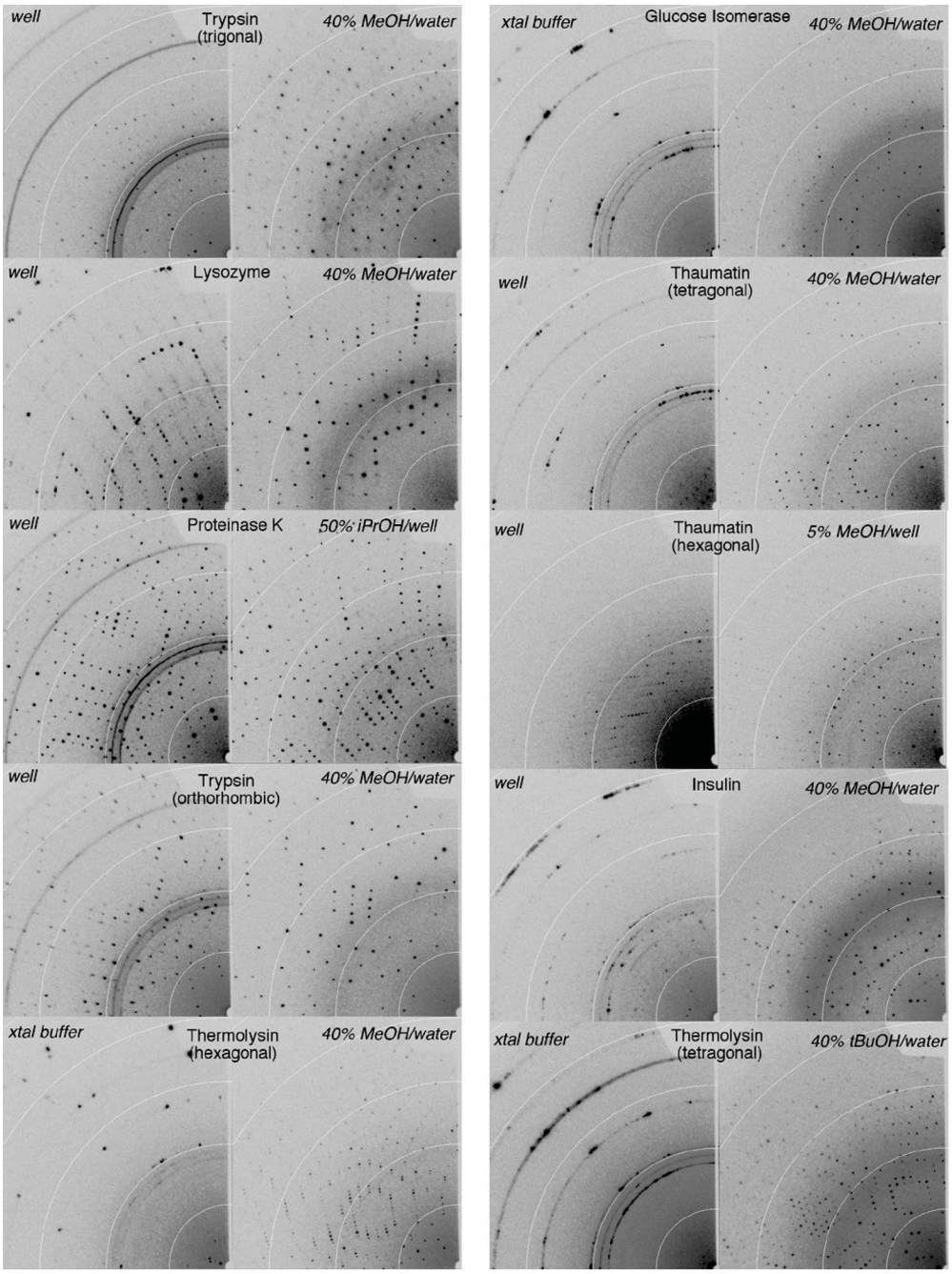

Figure 1.

Diffraction images from crystals equilibrated in-vial over crystal buffer (left hand image each pair) or an alcohol solution (right). Incubation times for the well solution measurements are a few minutes. Incubation times for the alcohol incubations are 10–120 seconds, except for trigonal trypsin, which was 15 minutes. The detector is set so the resolution at the edge is 1.8 Å and the four inner resolution rings are at 6.6, 3.5, 2.5 and 2.0 Å. All of the alcohol cryoprotected crystals diffract to at least 2.0 Å resolution. (a) trigonal trypsin, lysozyme, proteinase K, orthorhombic trypsin, hexagonal thermolysin (b) glucose

isomerase, tetragonal thaumatin, hexagonal thaumatin, insulin, tetragonal thermolysin. While glucose isomerase, and the two thermolysin crystal forms were soaked in xtal buffer (see methods) to reduce the natural cryoprotective effects of their crystallization buffers, they can also be mounted directly from the drop over alcohol solutions with high quality diffraction.

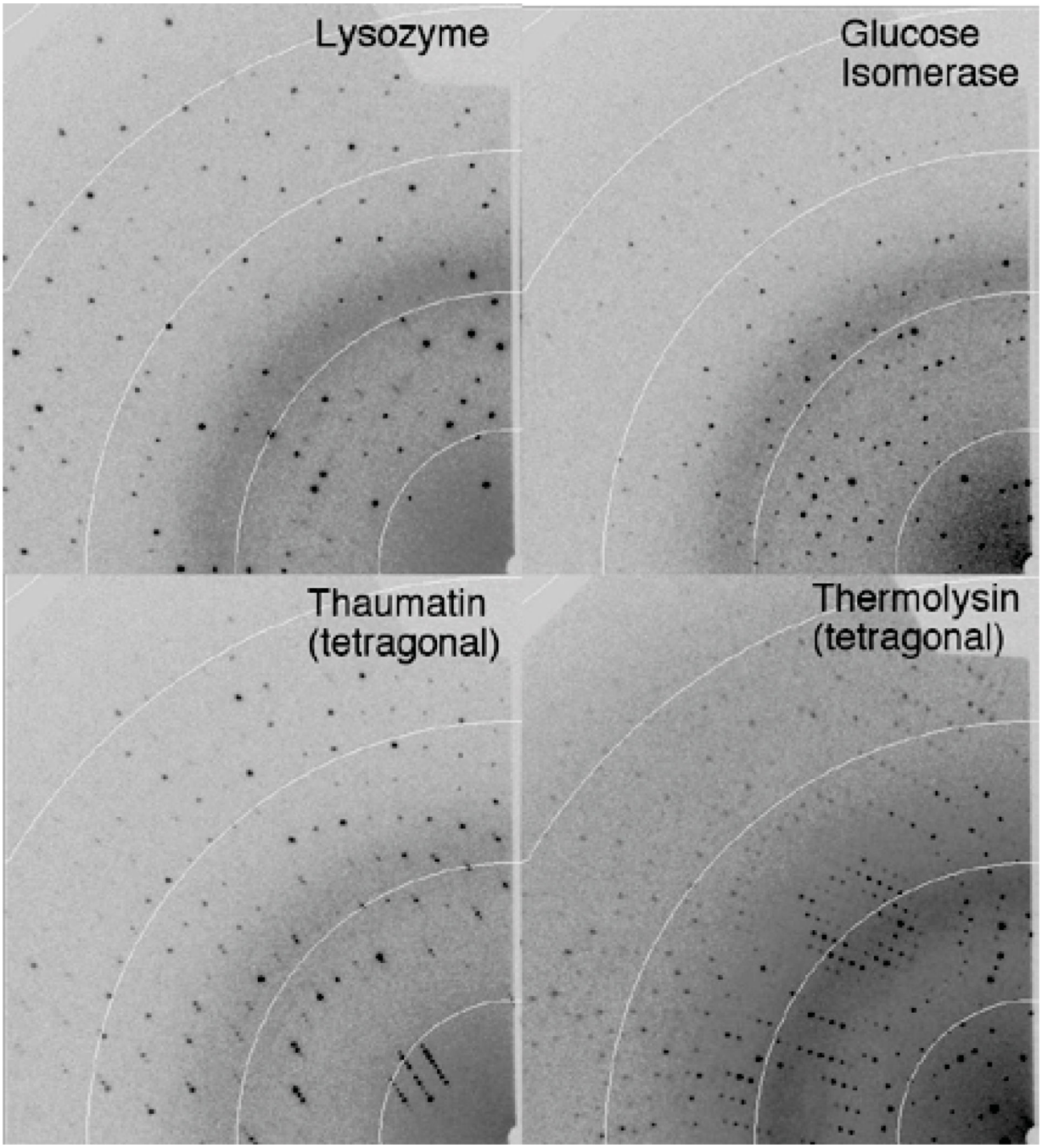

Figure 2.

Diffraction images from crystals transferred to vials without the use of humid flow or an O-ring on the vial. Liquid nitrogen escape holes were plugged with clay. Crystals were mounted directly from the vial onto the cryostream. Lysozyme: 40% MeOH, mosaicity 0.55°, <I/σ> = 8.0. Glucose isomerase: 40% MeOH, mosaicity 0.56°, <I/σ> = 2.6. Thaumatin: 40% MeOH, mosaicity 0.59°, <I/σ> = 4.9. Thermolysin: 40% tBuOH, mosaicity 0.61°, <I/σ> = 2.9. <I/σ> is given for the 2.0 Å resolution bin.

Table 1

Vapor pressures of some cryoprotective agents, in mm Hg at 298 K.

Molecule	Vapor Pressure (Yaws, 1999)
Methanol	125
Ethanol	59
Isopropanol	45
Tert-butanol	42
Water	24
DMF	4
DMSO	0.6
Ethylene glycol	0.1

Table 2

Some characteristics of the crystals and diffraction conditions.

Crystal	Solvent Content[a]	Primary component of crystal buffer prior to cryoprotection.	Binary Cryo (w/w) [b]	Xtal buffer Cryo(v/v)[b]	Mosaicity[c]	I/sigma (2.0 Å)[d]
Trypsin (trigonal)	40 %	25% P8K, 0.2 M $AmSO_4$	-	-	0.59	6.4
Lysozyme	41	0.5 M NaCl	40% MeOH	30% MeOH	0.57	12.8
Proteinase K	44	12% PEG 8K	55% EtOH	50% EtOH	0.56	15.1
Trypsin (orthorhombic)	47	25% P8K, 0.2 M $AmSO_4$	20% MeOH	5% MeOH	0.45	10.1
Thermolysin (hexagonal)	50	water	30% MeOH	30% MeOH	0.57	4.0
Glucose isomerase	57	0.25 M $AmSO_4$	40% MeOH	40% MeOH	0.57	3.0
Thaumatin (tetragonal)	58	0.2 M Na/K tartrate	40% MeOH	40% MeOH	0.49	2.8
Thaumatin (hexagonal)	62	0.2 M $AmSO_4$, 15% glycerol	Xtal dissolved	5% MeOH	0.57	3.4
Insulin	65	0.25 M NaPhosphate	40% MeOH	40% MeOH	0.61	2.6
Thermolysin (tetragonal)	66	0.5 M $ZnCl_2$	30% BuOH	30% BuOH	0.58	2.8

[a] Solvent content is calculated based the cell volume and SEQRES data in the pdb file (Matthews, 1968) (Kantardjieff and Rupp, 2003).

[b] Minimal concentration of volatile alcohol required for cryoprotection using 2 minute in-vial equilibrations. Diffraction characteristics for the images shown in Fig. 1 (right).

[c] The average of e_1, e_2 and e_2 output by CrysalisPRO.

[d] <I/σ> for the highest resolution bin (2.0 Å). Exposure times were 15 seconds, except for tetragonal thermolysin, tetragonal thaumatin, and glucose isomerase, which were 30 seconds. Crystal sizes were ~100–500 μm.