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The Use of Two Novel Methods to Grow Protein Crystals by Microdialysis and Vapor Diffusion in an Agarose Gel

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Abstract

The crystals of most proteins are poorly ordered and diffract to lower resolutions than other crystals of simple and inorganic compounds. The use of two novel methods for gel protein crystal growth, utilizing liquid diffusion and vapor diffusion, are described for the growth of lysozyme and canavalin. Crystallization using gels has been demonstrated to improve crystal quality by reducing convective flow, sedimentation, nucleation and twinning. Preliminary X-ray diffraction data are also presented.

Introduction

The growth of high-quality three-dimensional protein crystals has been described as the bottleneck in protein crystallography (DeLucas & Bugg, 1987). Efforts to produce higher quality crystals have led to the search for conditions which may benefit crystal growth. Crystal growth in microgravity has been utilized as a tool to attempt to grow higher quality crystals (Littke & John, 1984; DeLucas *et al.*, 1989; DeLucas, Smith, Smith *et al.*, 1991; DeLucas, Smith, Carter *et al.*, 1991; Stoddard, Strong, Farber, Arnott & Petsko, 1991). The use of gels to aid the growth of higher quality protein crystals has also been utilized (Robert & Lefaucheux, 1988; Provost & Robert, 1991; Miller, He & Carter, 1992; Narayana Kalkura & Devanarayanan, 1991; Robert, Provost & Lefaucheux, 1992).

The use of gels may benefit crystal growth in several ways. By reducing or eliminating density-driven and Marangoni convective flow patterns, a more controlled environment may be generated around crystal growth surfaces. The sedimentation of nucleated crystals is minimized with the gel matrix. This property allows the determination of nucleation sites as well as allowing an isotropic environment around the crystal. The nucleation of crystals may be reduced in gels. In the case of crystals that readily twin, twinning may be reduced in gels.

The use of gels was not initially conceived for use with protein crystals. Inorganic compounds have been crystallized in gels for almost a century (Liesegang, 1906; Hatschek, 1911; Henisch, 1970, 1988). The growth of protein crystals in gels, up to this point, seems to be somewhat focused on the use of capillary tubes or test tubes (Robert & Lefaucheux, 1988; Miller *et al.*, 1992; Narayana Kalkura & Devanarayanan, 1991; Robert *et al.*, 1992). In this approach, the gel acts as an intermediary between the protein solution and salt solution. The difficulty with this approach is that since the diffusion coefficient is normally greater for the protein than the precipitant, crystals will normally form not only in the gel, but also in the protein solution, thereby wasting valuable protein and partially defeating the purpose of using a gel.

Protein crystals grown in gels have exhibited lower percentages of growth defects by X-ray topography analysis (Robert & Lefaucheux, 1988; Provost & Robert, 1991). A comparison between human serum albumin crystals grown on Earth, in gels and in microgravity has been made (Miller *et al.*, 1992); however, this comparison was limited to the use of capillary tube gel growth. The results may be significantly different with the use of an alternative hardware.

In this paper, the use of microdialysis-gel liquid diffusion hardware and sitting drop-gel vapor diffusion hardware is described. Hen egg-white lysozyme will be used for these experiments due to its availability and relative wealth of literature from experiments conducted on Earth and in microgravity (Pusey, 1991, and references therein). Canavalin will also be used for these experiments due to the interest that it has been given in microgravity experiments (DeLucas *et al.*, 1989; DeLucas, Smith, Smith *et al.*, 1991; McPherson, Greenwood & Day, 1991; Day & McPherson, 1992). Preliminary X-ray data for both proteins and both sets of hardware will also be presented.

Materials

The gels used were physical hydrogels of agarose (SeaKhem, FMC Bioproducts) at concentrations between 0.2 and 0.6%. Hen egg-white lysozyme

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(grade 1, Sigma) solutions were at concentrations between 10 and 70 mg ml^{-1} in buffer. Canavalin,* supplied in a microcrystalline suspension in DPBS† buffer, was elevated to pH 9 with ammonium hydroxide for crystallization. In the case of lysozyme, buffers consisted of sodium acetate (pH 4.4, 4.5 and 4.7) and Tris–HCl‡ (pH 8.5); salts consisted of sodium chloride and sodium citrate; precipitants consisted of PEG 400§ and ammonium sulfate; and temperatures were 277, 295 and 300.5 K. In the case of canavalin, buffers include DPBS (pH 6.8) and dH_2O, and temperatures were 277 and 300.5 K.

Hardware

In these experiments, microdialysis-gel and sitting drop-gel hardware was used. The microdialysis-gel hardware employs a dialysis membrane thereby making it a liquid-diffusion technique, whereas the sitting drop-gel hardware is a vapor-diffusion technique.

The microdialysis-gel hardware (Smith & Thiessen, 1994) uses microdialysis buttons (Cambridge Repetition Engineers) with a dialysis membrane. Heated agarose gel is pipetted into the button well and is then allowed to cool. A sheath is then placed around the button to provide a second, larger well. Protein solution is pipetted into this well, and the protein is allowed to diffuse into the gel overnight. The protein solution and sheath are then removed. Dialysis membrane is applied to the button and fastened with a rubber o-ring. The button is then placed in a reservoir solution for crystal growth. The membrane acts to restrict the protein to within the gel; however, it lets the reservoir solution diffuse with no restriction. The apparatus may be housed in a 24-well Linbro tissue-culture plate (ICN Flow Laboratories).

The sitting drop-gel hardware uses Cryschem plates (Charlie Supper Co.) or other apparatus for sitting-drop experiments. Heated agarose gel is pipetted into the cup or well and is allowed to cool. A sheath is placed around the finger to provide a second, larger well.¶ Protein solution is pipetted into this larger well and allowed to diffuse into the gel overnight. The protein solution and sheath are removed. A reservoir solution is pipetted into the reservoir, and the system is closed. Because this is vapor-diffusion hardware, the protein never has an opportunity to diffuse into the reservoir.

* Canavalin was a kind gift from Dr Alexander McPherson, University of California, Riverside, USA.

† DPBS, Dulbecco's phosphate-buffered saline.

‡ Tris–HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

§ PEG 400, polyethylene glycol 400, M_r = 380–420.

¶ This sheath is necessary in the case of Cryschem plates. It may not be necessary in other apparatus.

Results

In experiments where crystalline structures were present, photomicrographs were taken on an Olympus CHT microscope with an OM-2 camera at regular time intervals to both record and monitor growth. After photomicrography, some crystals were mounted for X-ray diffraction.

Both lysozyme and canavalin crystals grew in both the microdialysis-gel and sitting drop-gel hardware. The lysozyme crystals tended to have greater success in the microdialysis-gel hardware at 300.5 K with 0.3–0.4% agarose concentration. Crystals of lysozyme formed with all additives, but the quality of the crystals varied. The lysozyme crystals generally had the highest success with sodium chloride/sodium acetate, pH 4.7; however, there were many quality crystals grown with the organic solvent (PEG 400). On average, lysozyme crystals grew to 1 mm, with the largest at 1.7 mm. X-ray diffraction shots were taken of lysozyme crystals which had strong data to 1.8 Å resolution.*

Quantitative analysis of the lysozyme crystals revealed an interesting property of the gels (Fig. 1). As a general trend, it is apparent that the number of crystals is directly proportional to the agarose concentration up to 0.4%. Above 0.4% concentration, the general trend is that the number of crystals is inversely proportional to the agarose concentration.

Canavalin crystals tended to grow better in the sitting drop-gel hardware at 277 K with 0.4% agarose concentration.† High-quality canavalin crystals formed with both buffers. Rhombohedral canva-

* Data collected on a Siemens multiwire area detector for 100 s frame^{-1} and −0.25° frame^{-1} at the Center for Macromolecular Crystallography at the Univeristy of Alabama at Birmingham by C. Smith.

† Canavalin was only grown in 0.4% agarose.

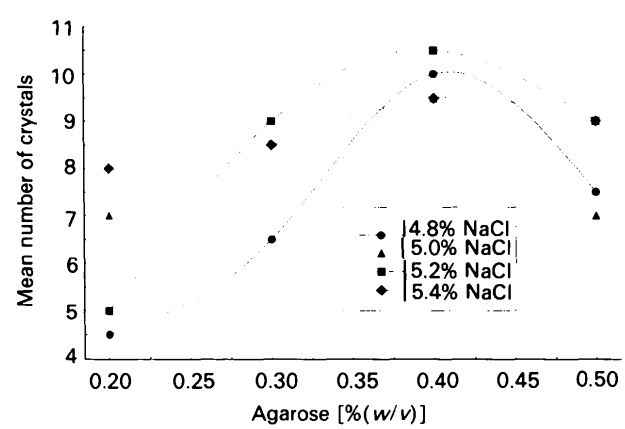

Fig. 1. A representative graph of the mean number of lysozyme crystals as a function of agarose concentration. The conditions in these experiments were: 50 mg ml^{-1} lysozyme; 0.1 *M* sodium acetate, pH 4.7; and 300.5 K.

lin crystals were grown up to 500 μm; however, hexagonal crystals were not grown to a size large enough for diffraction. X-ray diffraction shots were taken of rhombohedral canavalin crystals which had data to 2.5 Å resolution, with a full data set possible around 2.7–2.8 Å resolution.* Unfortunately, the crystals were over eight months old at the time of their diffraction. Normally, hexagonal canavalin crystals formed on Earth display cusps at the growing end of the crystal. This phenomenon is explained by the fact that a protein deficit must occur at the center of the crystal as the exterior edges may have enough protein to grow past the center. Hexagonal canavalin crystals grown in microgravity exhibit hexagonal vacuoles in place of the cusps (Day & McPherson, 1992). Similarly, the hexagonal canavalin crystals grown in gel exhibit these hexagonal vacuoles at the growing end.

Discussion

To evaluate the use of agarose gels in the growth of high-quality protein crystals, it is necessary to analyze more than simply the resolution of the formed crystals. It is necessary to realize that the gel presents an entirely new set of conditions; therefore, it is unrealistic to believe that the best crystallization conditions for a protein growth without a gel would be the same as those with a gel. It is necessary, furthermore, to look at the general trends in the crystal growth with a gel compared to without a gel, not simply which method can provide immediate higher resolution.

The determination of the best concentration for gel is a delicate balance between maintaining the stability of the gel matrix but without restricting the crystal growth. In Fig. 1, it is observed that the greatest number of crystals formed at 0.4% agarose. One might assume that this is the best balance of factors; however, it would be necessary to test a greater concentration range to give a more definitive answer.

It has been observed that the crystals grown in gels appear to have well defined facets. This may be attributed to a lessening in convective flow around the crysal during growth. This may also be attributed to the gel matrix preventing density-driven movement of the nuclei. The crystals are distributed throughout the gel. The crystals were grown to good size in the gel. This size may be attributed partially to reduced nucleation; hence, the fewer crystals to deplete the protein supply, the larger the crystals may form. Lysozyme and canavalin crystals have a lessened tendency to twin in the gel than out of the gel.

The physical properties of the gel must be taken into account. Some lysozyme crystals were observed to grow together. I would suggest that the crystals actually nucleated independently and were forced together, by the gel matrix, as they grew larger. It is also necessary to study the effect of gel on crystal packing patterns and the rate of protein diffusion.

The microdialysis-gel method may easily be adapted to use buttons with well volumes from 5 to 100 ml. The sitting drop-gel method would be limited in volume only by the size of the cup.

A difficulty with the use of gels is the retrieval of the crystal from the gel without damaging it. The gel does not readily give up its crystals. A sufficient method for the removal of crystals has been described (Robert *et al.*, 1992); however, it is difficult to do so without damaging the crystal. It has been observed that crystals are extremely sensitive to even minute changes in their environment (DeLucas *et al.*, 1986). It may be advantageous to leave the crystal in the gel when mounting it, thereby keeping it in its 'mother liquor.'

It has been observed that the crystals are exceptionally stable in the gel. Canavalin crystals have been observed to maintain structural integrity for over eight months, and lysozyme crystals for over two years. It was observed that when the crystals are brought out of the gel for mounting (especially in the case of canavalin), their degradation is immediate and visually apparent. In the case of canavalin, almost all crystals disintegrated and cracked upon removal from the gel. All canavalin crystals were visually cloudy immediately after removal from the gel.

Applications

It has been suggested that gel protein crystal experiments may serve as an alternative method to microgravity experiments (Robert & Lefauxcheux, 1988). Gels may provide an environment where similar effects may be achieved; however, it is not possible to mimic all positive properties of microgravity. I would suggest that gel experiments should not be envisaged as a substitute for microgravity experiments; however, protein crystal growth in gels should be an additional step before utilizing microgravity. Because of the limited space on microgravity carriers, the cost of that limited space and the relative infrequency of microgravity missions (DeLucas *et al.*, 1989), it is absolutely necessary to make every attempt to determine the structures of proteins without wasting this valuable resource. If gels may be used to produce high enough quality crystals for structure resolution, then they are the obvious choice

* Data collected on a San Diego multiwire area detector with a Rigaku RU-200 X-ray generator set at 50 kV, 80 mA, with helium shield, and a 0.3 mm collimator for 20 s frame^{-1} at the Vanderbilt Medical Center with W. Anderson.

for their repeatability, cost and ease. However, for those proteins where gels do not increase their quality enough for high-resolution studies, then the resource of microgravity should be utilized.

Gels may also prove to be valuable in testing flight hardware and for the simulation of microgravity experiments here on Earth. Because of the limited time of microgravity missions (usually less than 14 d at present), many crystals that take weeks or months to grow on the ground may simply not have enough time to grow: gels provide a possible alternative to this problem. I would also suggest that the incorporation of gels with the use of microgravity may produce the best possible circumstances for protein crystal growth and recovery. G-jitter, or the vibrations caused by the crew and the orbiter itself, has been cited as a possible damaging effect to protein crystal growth using the Shuttle as well as the reentry g levels (possibly in excess of $1.5g$) (DeLucas & Bugg, 1990). In addition, on parachute-based recovery systems (*i.e.* Russian Photon), g levels may exceed $10g$, where the addition of gels may be the difference between having crystals and not having crystals upon touchdown. The gel could act as an elastic wall to help absorb the vibrations instead of making the crystal absorb the vibrations as in conventional systems, and the addition of a gel should not require major redesign in many existing microgravity experiment modules.

Concluding remarks

By considering the properties of gels, it has been demonstrated that the use of gels gives access to conditions otherwise unattainable in conventional solution crystal growth. The advantages and disadvantages of gel techniques have been demonstrated in the case of lysozyme and canavalin using microdialysis-gel and sitting drop-gel hardwares. These techniques should give greater versatility to protein crystal growth experiments. The crystals in this study demonstrated that high-quality three-dimensional crystals could be grown with the use of gels. The crystals grown in gels had orderly morphologies, grew to a good size, had no tendency to sediment, were fewer in number than found in their conventional counterparts, and did not appear to twin. In the case of canavalin, a unique effect, seen previously only in microgravity, was observed with hexagonal crystals grown in a gel. Further research must be performed to understand the physical properties of gels and their interactions with proteins, test the use of a wide variety of gels to a wide variety of proteins, and to determine the effect of gels used in combination with microgravity. A precedent has been set to see more successful gel protein crystal growth.

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