Agarose as crystallisation media for proteins II: trapping of gel fibres into the crystals

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The crystallisation pressure exerted by protein crystals growing in agarose gel does not disrupt the gel network. However, protein crystals trap agarose fibres when they grow in agarose gels. The fibres of agarose are distributed randomly in the crystals explaining why they do not appreciably affect the diffraction quality of the crystal.

Keywords: **protein, crystallisation, agarose, gels**

1. Introduction

The use of gels for small and large macromolecules is a technique currently used in crystallisation experiments in order to avoid sedimentation and convective fluid motion (Henisch, 1988; Robert & Lefaucheux, 1988; García-Ruiz, 1991; Robert *et al.*, 1999). Obviously, it involves the use of a chemical additive (the gel precursor), which might interact chemically with macromolecules, eventually affecting the quality of the diffraction behavior of the crystals. Among typical gel precursors used for protein crystallisation such as agarose, silica, acrylamide or sephadex, agarose gels are the most employed hydrogels in protein crystallisation. They are easy to prepare, are stable upon a wide range of pH (3.0-9.0) and show a high mechanical resistance as well as elasticity at low agarose concentration (< 6% w/v). In addition, they are familiar for most biochemists and molecular biologists as they are commonly used in electrophoresis and cell culture media.

Recently, two different studies have demonstrated that, at least for lysozyme, the interactions between the molecules in solution and in solutions gelled with agarose are identical (Finet *et al.*, 1998; Vidal *et al.*, 1998). In the first part of this series of papers (Garcia-Ruiz *et al.*, 2001) we presented a study focusing on the minimum agarose concentration able to reduce sedimentation and convective flow. Interferometric observations linked to rheological measurements yielded the result those agarose concentrations as low as 0.04% (w/v) were able to overcome buoyancy and crystal sedimentation. Here we focus on the mechanical interaction between the crystal and the gel and on the incorporation of agarose fibres into the crystal.

2. Materials and methods

All the proteins were purchased from Sigma. Thaumatin (*thaumatococcus danielli*), code T-7638 (Lot.108F299) and lysozyme L-6876 (Lot. 65H7025) were used without further purification. The proteins, supplied as a dry protein power, were dissolved in the corresponding crystallisation buffer and passed through a 0.45 µm pore size filter (Millipore, Millex-HV_{13}). The concentration was checked by absorbance measurement at 280 nm using 2.66 $ml \cdot mg^{-1}cm^{-1}$ for lysozyme and 1.22 $ml \cdot mg^{-1}cm^{-1}$ for thaumatin as extinction coefficients. Ferritin was obtained as concentrated solution (102 mg/ml) also from Sigma, code F-4503 (Lot.12H70402), and diluted in the crystallisation buffer to the desire concentration or as a monomer solution purified from the same source (Thomas *et al*, 1998). Other chemicals were purchased from Sigma. The crystallisation conditions are summarized in Table I. The agarose used in this study was supplied by Hispanagar with a melting point of 363 K and gelling point 310 K.

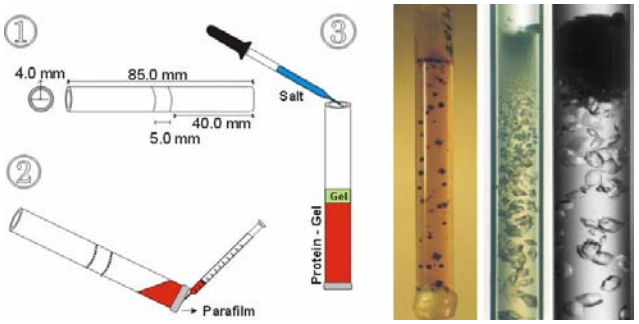

Figure 1

Sequences of the experimental set-up. On the right, three examples of protein crystals grown in agarose gels are shown: from left to right, ferritin, thaumatin and lysozyme crystals are shown.

As shown in Figure 1, the experiments were performed with the counter-diffusion geometry in a three chamber configuration method (Garcia-Ruiz, 1991). An 85-90 mm long glass tube with a 4.0 mm inner diameter was used as crystallisation device. A 1.0% (*w/v*) agarose sol was obtained by adding agarose powder to the buffer solution in a reflux flask under continuous stirring. The flask is then heated in a water bath to reach a temperature five degrees above the agarose melting point until a clear solution is obtained. The sol is then cooled down to five degrees above the agarose gelling point and kept at this temperature with continuous stirring. The protein solution is pre-warmed in the same water bath and mixed with the agarose-sol at equal volumes to obtain a 0.5% (*w/v*) final agarose concentration. The solution was immediately loaded across one of the ends of the tube previously sealed with parafilm, using a one ml syringe up to fill the 40 mm long protein chamber. After gelling of the protein-agarose sol, a 5 mm layer of 1.0% *(w/v)* agarose sol is poured on top of the gel-protein layer and allowed to cool. This layer acts as a physical buffer to reduce the high initial supersaturation values. Finally, the precipitant solution is added on top of the agarose layer up to fill the 40 mm long precipitant chamber. The experiments are then stored at room temperature.

The cylindrical agarose gel containing the crystals was extracted from the tube by increasing the pressure at the precipitant end using a syringe. For the crystal dissolution experiments individual crystal were harvested and separated from the gel using a thin paintbrush and crystal manipulation tools from Hampton Research. The selected crystal was gently transferred under an Olympus binocular microscope equipped with a camera. The crystal was then soaked in deionised water. A blue-dye was added to the solution to increase the contrast between the gel matrix and the solution.

High resolution electron micrographs were obtained with a Hitachi S-2300 electron scanning microscope working at 4 to 7 Kv. The sample preparation procedure detailed next was developed by combining and modifying the protocols described by Durbin & Feher (1990) to dry protein crystals and by Medin (1995) to dry agarose gel without disrupting their structure. The 0.5% *(w/v)* cylindrical agarose gel containing lysozyme crystals was cut into one centimeter slices, placed over a 6 cm diameter aluminum disk

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sample holder and immediately immersed in liquid nitrogen. The samples were then transferred to a vacuum chamber pre-cooled at 193.5 K and equilibrate in an argon atmosphere. In the last step the pressure was fixed to 10^{-6} atmospheres and then the temperature was increased to room temperature over 48 hours. The one centimeter dry samples were then cut in small pieces using a scalpel under a binocular microscope. Individual crystals were fractured to display the inner core. Each specimen was then placed on a two-faced stick carbon disk already glued on an aluminium mushroom-shape holder and coated with gold. The samples must be always maintained under a dry environment otherwise the gold deposition procedure may fail.

3. Results and discussion

A crystal growing inside a gel has to overcome the resistance offered by the gel network. The situation is similar to the crystallisation of a soluble salt inside a porous rock or when a crystal grows upwards with a weight on it. Because of the existence of supersaturation and the accretion process, the crystal exerts a crystallisation pressure that was calculated by Correns (1949) on the basis of the Riecke's principle to be:

$$P_c = \left(\frac{RT}{\Omega}\right) \ln \frac{C}{C_e}$$

R being the gas constant (0.082 lt atm mol^{-1} K^{-1}), and T the temperature in ºK. Basically, this expression shows that the crystallisation pressure increases with the supersaturation and with the molar volume Ω of the crystallising compound. Winkler and Singer (1972) show values of crystallisation pressure in the order of tens of bars for several salts. Figure 2 shows the values of the crystallisation pressure at T=300ºK as a function of supersaturation for three different substances, two proteins (lysozyme with $\Omega = 3 \times 10^{-20}$ cm^3 and ferritin with $\Omega = 10^{-18}$ cm^3) and one inorganic small molecule (potassium dihydrogen phosphate with $\Omega = 10^{-22}$ cm^3).

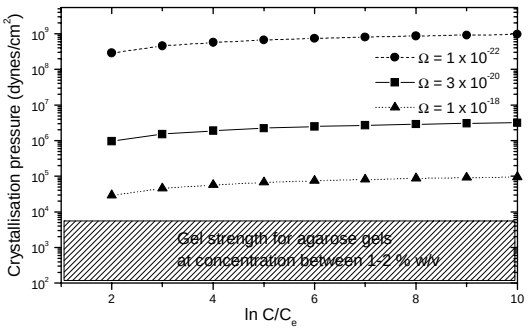

Figure 2

Crystallisation pressure as a function of supersaturation for three different molecules of molar volume $3 \cdot 10^{-22}$ cm^3 (potassium hydrogen phosphate), 10^{-20} cm^3 (lysozyme) and 10^{-18} cm^3 (ferritin).

On the other hand, the pressure that must be applied to a gel to fracture it is known as gel strength, and are reported by the suppliers in gr/cm^2. Gel strength varies with the type of gel and with the gel concentration. For the concentration range between 1 and 2% (*w/v*) of agarose, typical values vary between 400 and 5000 gr/cm^2 and depends directly on concentration. As shown in Figure 2, the values of the crystallisation pressure for all these three compounds are higher than the agarose gel strength. Therefore it should be expected that crystals will fracture the gel as a result of the very growth process. In fact, it is well known that crystals grown in different types of gel, either physical or chemical gels, provokes the breaking of the gel in the vicinity of the crystal, forming gel-void cavities that, because of the shape, are called cusp-cavities (Henisch, 1988). As shown in Figure 3, these cusp-like cavities mainly form near the corners of the crystal because of the higher supersaturation at these locations, which implies higher crystallisation pressure according to the Corren's equation. Surprisingly, this behaviour frequently reported during the crystallisation of small molecules has never been observed for protein crystals, either for lysozyme, thaumatin and ferritin crystals grown in this study or for other proteins grown in gels in the Laboratorio de Estudios Cristalográficos in the last years. Even no subtle disturbances of the gel surrounding the crystals were ever observed during our studies with Mach-Zhender interferometry.

To obtain a realistic picture of the crystal-to-gel contacts a large number of high resolution electron microscopy techniques and sample preparations were tested. Only the freeze-drying preparation described above consistently gives undamaged samples. Figure 4 shows micrographs of lysozyme crystals grown in 0.5% *(w/v)* agarose gel. The micrograph in Figure 4a shows that the gel has neither been split nor separated from the growing crystal faces. This is also observed in a typical region for cups-cavities formation shown in Figure 4b, where it can be noted that the gel fibres cover the crystal corner with no visible crack formation.

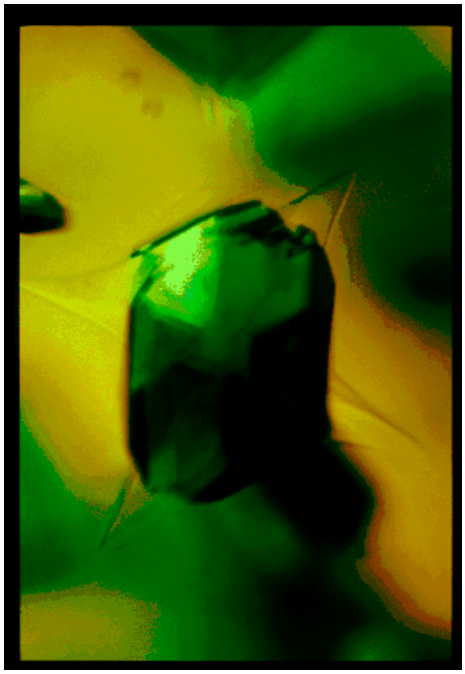

Figure 3

Potassium tartrate single crystal grown in gel showing the disruption of the gel structure due to the crystallisation pressure. Longest dimension of the crystal is 6 mm.

However, like many inorganic crystals, protein crystals trap the gel as they grow. Dissolution experiments of protein crystals were performed to determine if, as in the case of protein crystals grown in silica gel (Garcia-Ruiz *et al*., 1998), the agarose gel is incorporated during the crystal growth process. Single protein crystals were extracted from the gel as described above and dissolved in undersaturated solutions. As shown in Figure 5, the shape and size of the piece of gel observed after complete dissolution of the protein matches with the size and shape of the original crystal. In a separated experiment, one centimeter piece of agarose gel containing grown

lysozyme crystals was sunken in water until complete dissolution of the protein. The remaining crystal-free gel was examined under the microscope showing a homogeneous and continuous matrix with no traces of either the dissolved protein crystal or cracks of the agarose gel network.

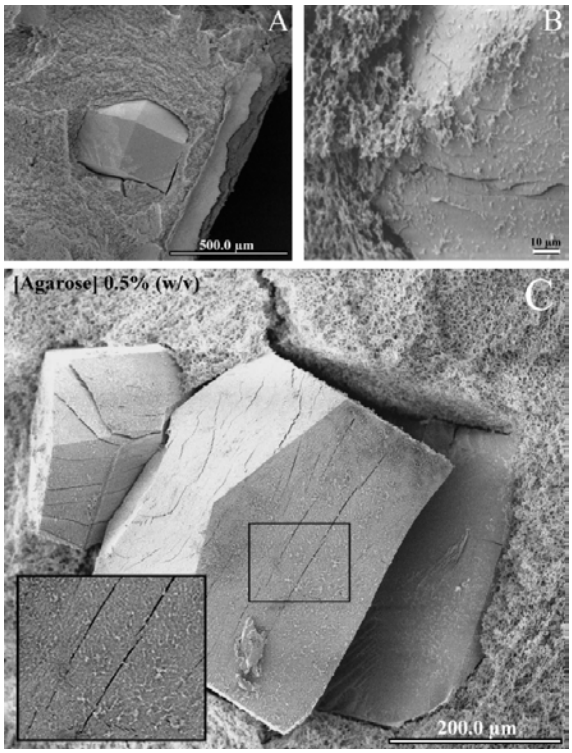

Figure 4

Scanning electron microscopy view of lysozyme crystals grown in gels obtained by the freezing technique. (a) {101} faces of a lysozyme crystal inside an agarose gel at a concentration of 0.5% (w/v). Note that the crystal is tightly surrounded by the agarose gel; (b, and c) Note the gel fibres outside, on the crystal surface and inside the crystal.

Figure 4c shows a tetragonal lysozyme crystal which was fractured to observe its core. Note that the gel fibres appear onto the crystal surfaces but also in the inner of the crystal. This random gel-like location of the agarose fibres in the core of the crystals agrees with our X-ray diffraction data where the existence of an ordered network of gel (for instance into the channel of the protein structure) has never been detected. It also explains that the X-ray diffraction quality of the crystals is not significantly affected by the trapping of the gel (Garcia-Ruiz *et al*, 1998; Lorber e*t al*., 1999a; 1999b; Gavira 2000; Biertümpfel *et al*., 2002; Garcia-Ruiz *et al*., 2002; Mañas *et al*., 2002), although more detailed study of this problem must be performed in the future.

4. Conclusions

Calculations show that the crystallisation pressure exerted by growing protein crystals is higher than the strength of the agarose gel. However, unlike for small molecules inorganic crystals, the crystallisation pressure does not disrupt mechanically the agarose gel structure. Dissolution experiments and scanning electron microscopy of freeze-drying crystals grown in agarose gels definitely show that, as reported previously for the case of silica gels, macromolecular crystals trap the fibres of agarose during the growth process. The agarose fibres do not occupy specific locations in the crystal but they are randomly distributed.

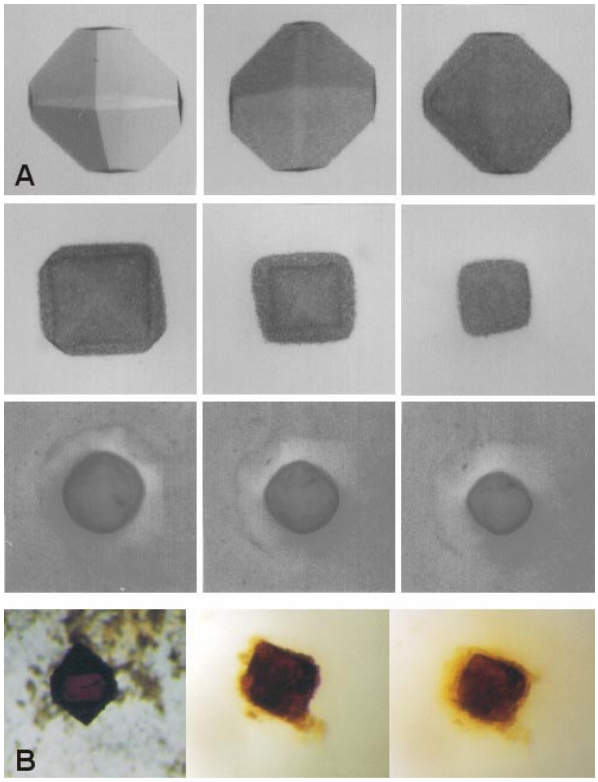

Figure 5

Time sequences corresponding to two different dissolution experiments. (A) shows a nine frames sequence of the dissolution of a lysozyme crystal grown in agarose gels at a concentration of 0.5% w/v. The last three pictures were obtained after adding a dye to increase the contrast. Note that after the protein is dissolved, a gel phantom remains showing the same shape as the crystal. The three frames in (B) correspond to the dissolution of a ferritin crystal.

Table 1 Crystallisation conditions

Protein	Concentration	Precipitant	Buffer
Lysozyme	100-200 mg/ml	20 % w/v NaCL	0.05 M AcNa pH 4.50
Ferritin	10-20 mg/ml	6.2% w/v $CdSO_4$	0.2 M AcNa pH 5.03
Thaumatin	200-100 mg/ml	20.0% w/v NaK-Tartrate	0.1 M Na Phosphate pH 6.00

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