Acta Cryst. (1996). D52, 806-812

Contaminant Effects on Protein Crystal Morphology in Different Growth Environments†

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(*Received 10 November 1995; accepted 7 February 1995*)

Abstract

Contaminant effects on the morphology of turkey egg-white lysozyme (TEWL) crystals grown in ungelled and in gelled growth media have been investigated. The latter may serve as a model system for future microgravity experiments. Hen egg-white lysozyme (HEWL) was added as the contaminant at levels ranging from 4 up to 70%(*w/w*) (total protein). Morphology measurements indicate a contaminant effect leading to a shortening along the *c* axis of the crystal. This shortening effect depends on the contaminant concentration. It is attenuated and varies more regularly in gelled than in ungelled growth media. The specificity of the HEWL contaminant effect was verified by addition of ribonuclease A, which did not influence crystal morphology. Contaminant inclusion into the growing TEWL crystals could be calculated directly from equilibrium protein concentration measurements. The level of HEWL inclusion is closely related to the concentration of HEWL in the growth solutions. The specificity of the observed effect as well as the differences between the two growth media are discussed.

1. Introduction

There is little need to stress the importance of obtaining large, well diffracting protein crystals for structure determination. Although crystallization success is supposed to depend critically on protein purity (*e.g.* Giegé, Lorber & Théobald-Dietrich, 1994), it is well known that protein solutions thought to be homogeneous still contain considerable levels of contaminants (*e.g.* Vekilov, Ataka & Katsura, 1995) and that polyacrylamide gel electrophoresis of dissolved crystals often shows multiple bands. The contaminants may stem from conformational heterogeneities, proteolytic fragments of the original protein, isoforms containing a few replacements in the amino-acid sequence, co-purified structurally unrelated proteins or contaminants other than proteins. Protein crystallization in the presence of a contaminant of similar structure has been studied here together with possible ways of minimizing the contaminant's influence on crystal growth.

† This is publication number 319 of the Institut be Biologie Structurale Jean-Pierre Ebel.

Table 1. *Replacements along the amino-acid sequences of turkey (TEWL) and hen egg-white lysozyme (HEWL) (data taken from Protein Data Bank entries 2lz2 and 6lyz)*

Residue No.	3	15	41	73	99	101	121
TEWL	Tyr	Leu	His	Lys	Ala	Gly	His
HEWL	Phe	His	Gln	Arg	Val	Asp	Gln

Crystallization behaviour can be influenced by rational addition of contaminants to a previously homogenous solution. The effect of these additives on crystallization are well established for small organic molecules (Weissbuch, Addadi, Lahav & Leiserowitz, 1991). Provided the additive is structurally related to the bulk material, the molecular recognition processes on the crystal surfaces are modified during growth (Clydesdale, Roberts & Docherty, 1994). Since each set of crystal faces displays specific binding energies, their interactions with the contaminant will also vary. This may lead to modifications of crystal morphologies and/or to reduction in symmetry (McBride, 1989; Lahav & Leiserowitz, 1993).

In the experiments presented here, turkey egg-white lysozyme (TEWL) served as the protein crystallization system, and hen egg-white lysozyme (HEWL) as the contaminant. These homologous lysozymes differ by only seven out of 129 amino acids (95% sequence identity, Table 1). Known X-ray crystal structures of these lysozymes (Protein Data Bank codes 2lz2 and 6lyz) indicate that these replacements are all located on the molecular surface, making HEWL sufficiently 'different' from TEWL to correspond to the model mentioned above, taken from small-molecule cases. TEWL/HEWL co-crystallization experiments have already shown that there exists a specific effect on the morphology of resulting TEWL crystals (Abergel, Nesa & Fontecilla-Camps, 1991). Except for a recently reported monoclinic form (Harata, 1993), TEWL is known to crystallize in only one other, hexagonal, space group. This makes TEWL a more suitable model system than HEWL, which crystallizes in several different polymorphs, sometimes under very similar conditions.

Preliminary observations have indicated that nucleation and growth effects observed in the TEWL/HEWL system are modified when using gelled growth media

ISSN 0907-4449 © 1996

instead of the standard liquid solution (Robert & Provost, 1993). We have investigated these phenomena further, verifying the HEWL contaminant effect both in ungelled and in gelled growth media. The gel environment reduces convective solutal flow around the growing crystal, presumably reducing solute transport to the crystal surface. Thus, it is often considered as a suitable model for crystal growth conditions in microgravity environments. The gel growth results obtained here will allow us to verify this hypothesis by comparing them with results from future contamination experiments in microgravity (these are under way).

Agarose gels were chosen for crystallization in gelled media because they solidify by cooling and do not produce side products during solidification as chemical gels do (*e.g.* silica gels). They also solidify quickly, so that good control over gelling conditions is ensured. Furthermore, they are compatible with the conventional hanging-drop technique (Provost & Robert, 1991), which makes results of ungelled and gelled crystallization conditions easier to compare.

The question of HEWL inclusion into the TEWL crystals has also been addressed here. Comparing the final to the initial TEWL concentration in crystallization drops allowed us to determine whether contaminant inclusion had occurred and to give an estimate of its extent.

2. Materials and methods

2.1. *Optimizing TEWL crystallizing conditions*

TEWL was crystallized in ungelled solution using conditions optimized previously (Hirschler, Charon & Fontecilla-Camps, 1995): vapor diffusion using flat sitting-drop insets (Soriano & Fontecilla-Camps, 1993) in Linbro plates with drops of 2 µl of commercial-grade TEWL (Sigma, crystallized, dialyzed and lyophilized) at 30 mg ml^{-1} and 2 µl precipitant solution {2.40 *M* NaCl, 0.1 *M* HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), Fluka BioChimica, 15 m*M* NaN_3} at pH 5.8 and a reservoir volume of 1 ml of precipitant solution at 293 K. These conditions typically yield 1 to 5 well shaped crystals of up to 1 mm in their longest dimension. The occasional appearance of polycrystalline material could not be suppressed either by filtering or dialysis of the protein solution. Crystal cell parameters determined on a Xentronics/Siemens area detector coupled to a Rigaku RU200 generator were $a = b = 73.2$, $c = 86.8$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0°$, space group $P6_122$, which agrees with data published previously (Protein Data Bank code 2lz2).

For TEWL crystallization in gel medium, low gelling temperature Seaplaque agarose (Fluka BioChimica) was used. Its low-temperature gelling point minimizes thermal stress on the protein. The gel crystallization conditions were separately optimized taking the conditions described above for ungelled media as a starting point. A 3.0%(*w*/*v*) aqueous solution of the agarose gel powder was boiled in a sealed Eppendorf tube for 1 h. After cooling to 323 K, one part of the gel solution was mixed with three parts of the precipitant solution described above and one part water, and maintained at 318 K. 2 µl of the protein solution were pipetted onto the sitting-drop inset as indicated above. 4 µl of the gel–precipitant–water solution were then pipetted onto the protein droplet. All these operations were performed at room temperature. The Linbro plates were stored at 293 K. These gel-grown crystals display an even more regular habit than crystals grown in ungelled media. The occasional growth of polycrystalline material encountered in ungelled growth conditions was also suppressed under these conditions.

2.2. *Crystallization of TEWL in the presence of contaminants*

HEWL dissolved in water (Sigma, three times crystallized, dialyzed and lyophilized) was centrifuged (Microsep 3K for two times 8 h at 6000*g*) and added to TEWL solutions up to a constant total protein concentration of 30 mg ml^{-1}. Highly purified pig pancreas ribonuclease A (Fontecill-Camps *et al.*, 1994) was a gift of Dr R. de Llorens of the Institut de Biologia Fonamental, Universitat Autonòma de Barcelona, Spain. Protein concentrations were determined by optical density (OD) measurements at 280 nm using calibration curves. These curves were obtained from OD measurements of weighted samples of commercial-grade protein. Crystals were grown in ungelled and gelled conditions using the optimized conditions. After 20 d the morphologies of resulting well developed crystals were measured. The crystals on their sitting-drop insets were removed from the crystallization plates and oriented on a spindle under a light microscope so that their lateral faces were perpendicular to the viewing direction. The length and width of these faces were then measured with the help of a microscopy graticule included in the eyepiece. In cases where the crystal could not be appropriately oriented, the edge between two adjacent lateral faces was brought into viewing position. The length and width were then measured, the latter being corrected for the 30° angle of the edge-on view. The morphological ratio (MR) was then calculated for each crystal independently,

$$\mathrm{MR} = \frac{\text{Length of the lateral faces (scale units)}}{\text{Width of the lateral faces (scale units)}}.$$

2.3. *Determining equilibrium drop conditions*

Samples of the crystal growth solution were taken from equilibrated drops and diluted for OD measurements at 280 nm. Care was taken to leave the crystals undisturbed. The OD measurements were corrected for the change of drop volume during equilibration (4.0 µl at initial conditions, 1.8 µl at equilibrium conditions). The volumes of the equilibrated drops were calculated from

their diameters and heights measured under a bisecting microscope, approximating their shape to truncated spheres in the calculation. HEWL contents of the resulting crystals were calculated using the measured decrease in HEWL concentration and the equilibrium TEWL concentrations in the drops (1.6 mg ml^{-1}, which were assumed to be constant in the presence of HEWL). Conductivity measurements were performed with a CDM92 conductometer using microprobes (Radiometer, Copenhagen) at 293 K.

2.4. *Determination of face indices*

Crystals were mounted in 0.7 mm glass capillaries. Zero-level precession photographs ($\mu = 8°$, Ni-filtered Cu $K\alpha$ radiation) were used to determine the indices of the lateral faces. Subsequently, the indices of the apical faces were deduced from the interfacial angles they formed with the lateral faces. Cell parameters determined in our laboratory (above) were used to calculate possible interfacial angles of low-index faces. These angles were compared to measured values. Microphotographs were taken from five appropriately oriented crystals originating from different pure and contaminated solutions. The interfacial angles were obtained from these photographs.

3. Results

TEWL crystallization conditions were optimized in order to produce small numbers of well developed crystals. Purification of commercial-grade TEWL did not further improve the crystallization results (not shown). Consequently, the protein was used directly from the bottle in the subsequent experiments. HEWL was filtered through 10 kDa cut-off filter membranes to remove small-molecule contaminants (*Materials and methods*). The total protein concentration (TEWL plus the contaminant HEWL) was kept constant at 30 mg ml^{-1} for all crystallization conditions.

Hexagonal crystals of TEWL grown in the presence of HEWL display a shortened morphology in the direction of their *c* axis when compared to crystals grown from contaminant-free TEWL solutions (Figs. 1*a*, 1*c* and 2*a*). Even at high HEWL concentrations, well developed crystals with the typical 6/*mmm* symmetry appeared (Fig. 1*c*). The shortening is expressed in terms of the length-to-width ratio of the lateral faces of the resulting crystals, here defined as the morphological ratio (MR). Precession photographs indicated that these lateral faces have indices of the type {110}. The measured interfacial angles formed with the apical faces were all within the same range of 125 ± 1.6° for crystals grown from both pure and contaminated solutions. This value agrees well with the calculated angle of 124.4° between the {110} and the {112} faces. Hence, the presence of HEWL did not change the indices of the expressed faces (Fig. 1*b*).

The standard deviations of the MR of crystals grown in ungelled media between 8 and 30% HEWL became unusually high (not shown). Closer examination of the data revealed the existence of two populations of crystals grown under identical conditions, presenting different degrees of shortening along the *c* axis. Replotting of these two types of crystals as distinct populations gives much more reasonable standard deviations (Fig. 2*a*). The reason for the presence of these two different crystal populations is not well understood, although the relative orientation of the crystals relative to the gravity vector may be the cause. Neither the induction time nor the final equilibrium conditions were different between the two populations (see below).

The number of crystals per drop did not change significantly in going from contaminant-free to contaminated ungelled solution (*Materials and methods*). Aggregates shaped as doubly capped mushrooms appeared occasionally at contaminant levels of 50% HEWL. They correspond to what has been described previously as crystals of transitional morphology (Abergel *et al.*, 1991).

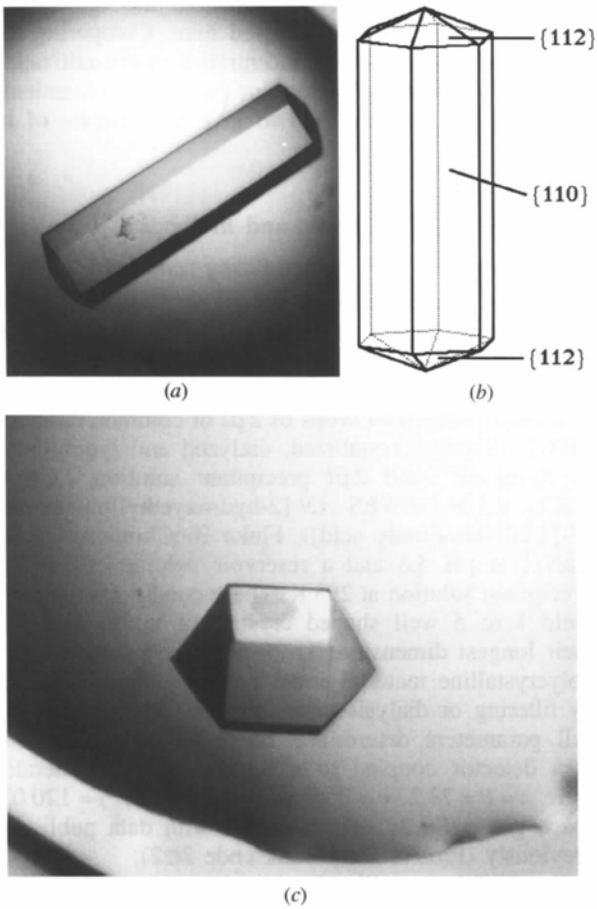
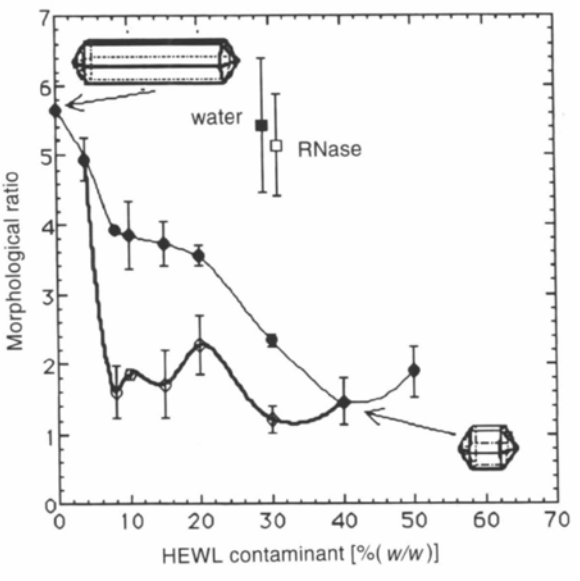
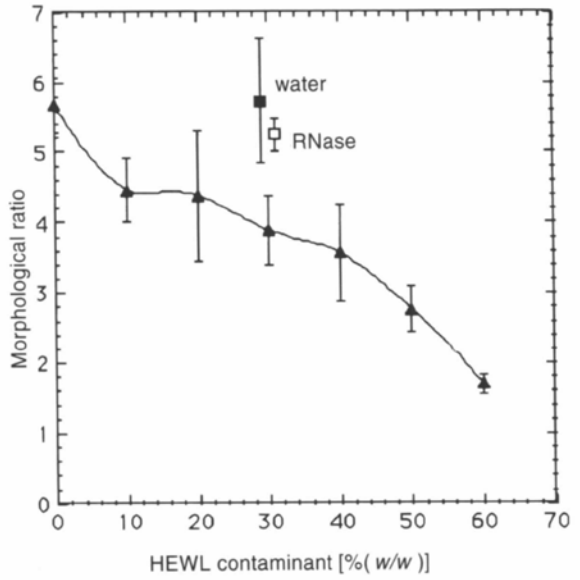


Fig. 1. (*a*) Photograph of a TEWL crystal grown from ungelled contaminant-free solution with a morphological ratio of 5.9 and (*b*) its schematic drawing with the indices of the faces. (*c*) Crystal obtained from ungelled TEWL solution in the presence of 40%(*w*/*w*) HEWL contaminant. The morphological ratio is reduced to 1.3.



(*a*)



(*b*)

Fig. 2. (*a*) Morphological ratio (MR) of TEWL crystals grown in the presence of HEWL in ungelled solution. Two populations of crystals with different MR's were obtained for HEWL contaminant levels between 8 and 30%(*w*/*w*). Both populations are shown here (open circles indicate the low-MR population). MR's of TEWL crystals obtained with water or ribonuclease A as control 'contaminants' are also shown (both at 30% level, slightly shifted for clarity). Insets illustrate crystals with MR's resulting from uncontaminated and from solution contaminated with 40%(*w*/*w*) HEWL (*b*) As (*a*), but the morphological ratios of crystals were obtained from agarose-gelled crystallization solutions. For a given contaminant concentration, the MR's of crystals from gelled media indicate less shortening along the *c* axis than that in crystals obtained from ungelled media. Hexagonal crystals are also obtained at higher contaminant content in gelled media than in ungelled media.

Crystals compatible with the symmetry of tetragonal HEWL appeared very rarely, even at a level of 60% HEWL, where the 'additive' actually becomes the major component.

Crystals grown from contaminant-free gelled solution display the same MR as those from contaminant-free ungelled solution. However, with increasing contaminant concentrations, the MR's of the former decrease much less rapidly than those of the crystal populations obtained in ungelled solutions (Fig. 2*b*). Thus, the extent of the shortening is not only dependent on the concentration of the added HEWL, but also on the medium used for crystallization. Furthermore the fall-off in MR is more regular for crystals grown in gel media. Well developed crystals displaying the usual sixfold symmetry are even obtained at contaminant levels of 60%. Beyond this level, crystals showed very pronounced roughening on their {112} faces (Fig. 3). The typical number of crystals per drop was slightly higher than in ungelled drops, which is in agreement with prior observations on crystallization in agarose gels (Provost & Robert, 1991). Just as in the case of ungelled droplets, this number did not change significantly upon addition of the contaminant. Preliminary experiments to determine diffraction limits indicate that uncontaminated TEWL crystals from gelled media seem to diffract to slightly higher resolution than those obtained from ungelled media (1.8 and 2.1 Å, respectively).

Control experiments at 30% contaminant level were performed in gelled and ungelled media using either a structurally unrelated protein or water as 'contaminants'. Ribonuclease A was chosen as the control protein contaminant. Its molecular mass (13.6 kDa) and p*I* are very similar to HEWL, but its structure is unrelated (5% amino-acid sequence identity only, Abergel *et al.*, 1991). The MR's of TEWL crystals grown in presence of 30% ribonuclease A correspond to MR's of crystals grown from contaminant-free solution (insets in Figs. 2*a* and

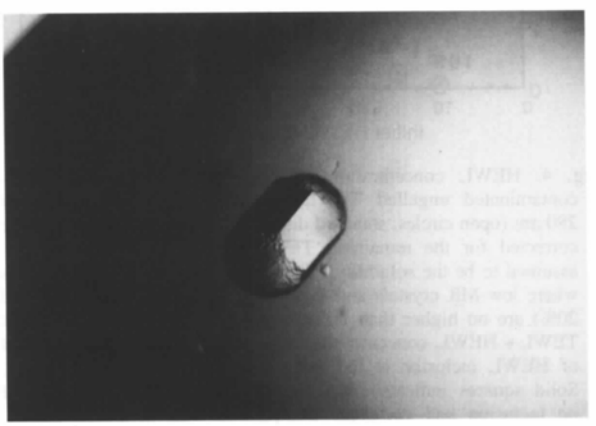


Fig. 3. Crystal obtained from gelled TEWL solution in the presence of 70%(*w*/*w*) HEWL contaminant. The lateral faces are well developed, whereas the apical faces show pronounced roughening.

2*b*). Thus, the observed shortening of the TEWL crystals is not a result of a non-specific protein contaminant effect. Neither does it result from a dilution effect, where different crystal morphologies may be caused by different supersaturation levels (Durbin & Feher, 1986; Forsythe & Pusey, 1994).

Subsequently, we tried to better understand the reasons for the co-existence of the two crystal populations grown from contaminated ungelled solution. One possible reason for this phenomenon could be that contaminants are included to different degrees into the two growing crystal populations, which would result in drops of different final HEWL concentration at equilibrium. To verify this, the final total protein concentrations of the different drops were measured. As can be seen in Fig. 4, the standard deviations of measurements in the range where the two crystal populations co-exist are comparable to those in the range where only one crystal population is present. The final total protein concentration simply depended on the initial HEWL concentration. Thus, different levels of contaminant inclusion are not likely to be responsible for the co-existence of the two crystal populations. An alternative explanation could be different inclusion levels of either precipitant or buffer, or both, into the growing crystals. We verified the final NaCl concentration by conductivity measurements. Again, no difference could be detected between the two kinds of drops (not shown). Using a different approach, we looked at the crystallization pathway. Close examination revealed that certain crystals in ungelled media first develop a standard morphology (Fig. 1*a*), but subsequently they started to develop a second growth layer onto their {110} surface (Fig. 5). This extra layer gives the crystals a 'thicker' appearance, changing their MR towards lower values.

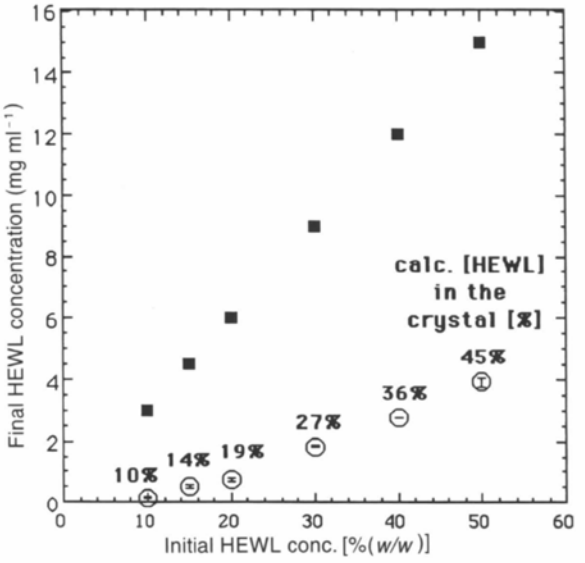


Fig. 4. HEWL concentration of equilibrated drops of HEWL-contaminated ungelled TEWL measured by its absorbance at 280 nm (open circles, standard deviations inset). Absorbances were corrected for the remaining TEWL concentration (1.6 mg ml^{-1}, assumed to be the solubility). Standard deviations at HEWL levels where low-MR crystals and high-MR crystals coexist (10, 15 and 20%) are no higher than those at higher HEWL levels (constant TEWL + HEWL concentration of 30 mg ml^{-1}). Thus, the amount of HEWL inclusion is the same for the two types of crystals. Solid squares indicate calculated final HEWL concentrations if no inclusion had occurred. Comparison with measured HEWL concentration shows a decrease of HEWL concentration over the entire contaminant range, indicating HEWL contaminant inclusion into the TEWL crystals. Inset are calculated percentages of crystalline HEWL resulting from this inclusion.

The measurements of equilibrated crystallization drops described above showed that the measured total protein concentrations in all the experiments were well below the calculated final HEWL concentration expected if all the contaminant molecules remained in solution. This loss demonstrates that inclusion of HEWL into the growing TEWL crystals does occur. The possibility of a reduction of HEWL concentration by formation of significant amounts of precipitate is excluded, since all drops stayed optically transparent. The calculated HEWL content in these crystals are shown in Fig. 4. Overall, they correspond to the HEWL concentration of the solution the crystals grew in. This agreement is high for HEWL concentrations of 10, 15 and 20%, and is slightly lower for higher HEWL concentrations. Although in the following the crystals will be referred to as TEWL crystals, they actually are mixed crystals of TEWL and of HEWL.

4. Discussion

The shortening effect of HEWL on the morphology of TEWL crystals along their *c* axis is specific to this structurally related additive. This was shown by addition of ribonuclease A, a protein similar to avian lysozymes in size and p*I* but displaying only 5% amino-acid sequence identity with them. Neither is it due

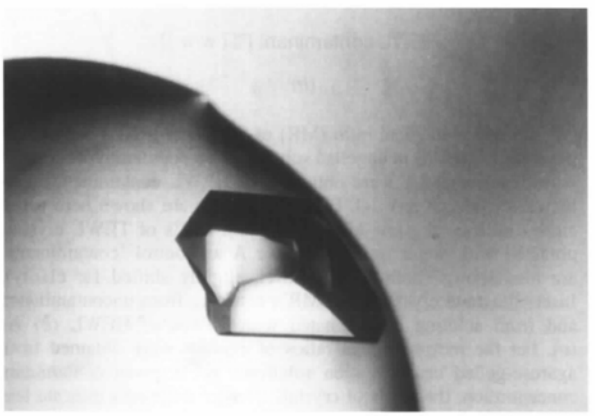


Fig. 5. Crystal obtained from ungelled TEWL solution contaminated by 10%(*w*/*w*) HEWL, developing a second layer around its periphery.

to different supersaturation levels, as could be shown by addition of water. Both the quantitative and the qualitative influences of the HEWL contaminant are functions of the growth environment. Comparing it to ungelled media, gelled media reduces the shortening effect of HEWL and leads to only one crystal population, with an almost linear decrease of MR with increasing HEWL concentration.

The contaminant effect of HEWL observed here can be rationalized by analogy with observations of additive effects on crystals of small organic molecules (Weissbuch *et al.*, 1991 and references therein). By virtue of its similar structure HEWL may interact with the various faces of the growing TEWL crystal. The apical {112} faces display more affinity for the contaminant than the lateral {110} faces. This has been shown by roughening of the {112} faces observed at maximum contaminant concentration. Growth kinetics of the {112} faces will then be more affected than growth kinetics of the lateral {110} faces (Markman, Elias, Addadi, Cohen & Berkovitch-Yellin, 1992), leading to the observed shortened morphology. Simulations of the recognition process between crystal surfaces and contaminant should allow us to verify the hypothesis presented here in a qualitative manner (work in progress).

The different effect of the contaminant in gelled and ungelled media may be explained in terms of reduced convective flow in the latter. The growth process with its incorporation of protein molecules into the crystal leads to a depletion of molecules in the crystal vicinity (Pusey, Snyder & Naumann, 1986; Broom, Witherow, Snyder & Carter, 1988). In ungelled media convective flow continuously moves fresh solution towards the growing crystal surfaces (Pusey & Naumann, 1986; Vekilov *et al.*, 1995), compensating for any significant concentration changes in the crystal vicinity. This is different in gelled media, where convection is minimized. This may lead to secondary contaminant effects resulting from contaminant inclusion and/or accumulation at the interface. A possible explanation for this is given below.

We have shown that (1) the interactions of the additive with the two types of crystal faces are different and (2) the contaminant is included into the growing crystal. Hence, it is very likely that the degree of contaminant inclusion will also vary for the two types of faces, leading to a different degree of accumulation of contaminant molecules on the crystal faces. The lower the affinity of the interfaces for the contaminant, the higher this accumulation will be. Under a convective regime, these contaminant build-ups may be 'washed off' the crystal/solution interface. Thus, the solution composition at this interface is similar to that of the bulk. This is not so in gelled media, where the contaminant, besides being incorporated into the apical faces, will accumulate at the crystal/solution interface over the lateral faces. Under this diffusional regime, the depletion zone will become increasingly rich in contaminant, hindering the growth of the lateral faces as a result of depletion of TEWL. Thus, the kinetics of both types of faces are slowed down on the apical faces, due to attachment and incorporation of the contaminant, and on the lateral faces because of its accumulation at the crystal interface. In this case the observed shortening along the c axis induced by the contaminant is compensated for by its effects on the lateral faces resulting in MR values which are similar to those of crystals from uncontaminated solutions. This hypothesis is being verified by comparing crystal face growth rates in the presence of contaminants in gelled and ungelled media (work in progress).

The shortening effect in gelled media can be explained using only one crystal population. This is different from the behavior of TEWL in ungelled crystal growth where the results can be best fitted with two crystal populations. The existence of two populations cannot be accounted for by different inclusion levels of HEWL or of precipitant agent into growing TEWL crystals, as we have shown by measurements of equilibrated ungelled crystallization drops. One possible cause is the development of a secondary layer on their {110} faces. An alternative cause may be related to the observed difference in contaminant-induced shortening effects in gelled and ungelled media. Since it appears to originate from the difference between diffusional and convective flow, the two crystal populations observed in ungelled media may as well be caused by different orientations of the crystals in the drop and subsequent different effects of convective flow on the crystal faces. This possibility is also being investigated.

The effect of a contaminant over its concentration range is different for the two media. In the gelled environment the MR decreases almost linearly with increasing contaminant concentration. In ungelled media the initially steep decrease levels off at an MR of approximately 1.5. According to the model for contaminant interactions used above, the growth rates of apical faces are slowed down in the presence of contaminants, as reflected by the final crystal morphology. Hence, Figs. 2(*a*) and 2(*b*) can be interpreted to represent the relative growth ratios of the crystal faces for different contaminant concentrations. Using the model of Kubota & Mullin (1995), the almost linear fall-off of the curve obtained in gelled media (Fig. 2*b*) represents the case of high contaminant effectiveness, eventually leading to a complete cessation of growth (impurity effectiveness $\alpha > 1$). The two curves obtained in ungelled media correspond to smaller impurity effectiveness, as they level off until they reach a non-zero value ($1 > \alpha > 0$).

5. Conclusions

Contaminants like HEWL, with a three-dimensional structure similar to the principal compound, modify its crystal growth process in a specific manner. The impact

of the effect depends on the crystal growth medium. Gelled growth media attenuated the contaminant effect compared with ungelled growth media, and rendered it more regular over the range of contaminant concentrations. The specificity of the contaminant effect observed here for HEWL, but not for ribonuclease A, shows the importance of defining more precisely the structural relationship between the 'contaminant' and the main compound in protein crystallization studies.

The contaminant effect on the crystallization process may or may not be desirable. As an example, small-molecule crystal engineering routinely makes use of additives to influence crystal morphology. Ungelled growth solutions are clearly preferable to maximize the additive's impact. Protein crystallization, on the other hand, suffers greatly from the presence of undesired contaminants even after thorough purification. If our results are applicable to other proteins, then protein crystal growth in gelled media is an easy-to-implement method to minimize the impact of contaminants and to improve crystallization results. Future experiments in microgravity will show how much the contaminant effect is attenuated in this environment. This will allow us to elucidate further the role of gels in protein crystal growth as a ground-based model system for crystallization under microgravity.

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