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New morphology, symmetry, orientation and perfection of lysozyme crystals grown in a magnetic field when paramagnetic salts ($NiCl_2$, $CoCl_2$ and $MnCl_2$) are used as crystallizing agents

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Abstract

Chlorides with different paramagnetic cations such as Ni^{2+}, Co^{2+} and Mn^{2+} were used as crystallizing agents instead of NaCl to crystallize hen egg-white lysozyme. $NiCl_2$ was found to give two types of crystals with different morphologies: one (roof-like) is a new type of orthorhombic $P2_12_12_1$ crystal with lattice constants $a = 79.0$ Å, $b = 80.8$ Å, and $c = 37.5$ Å; the second is an ordinary tetragonal crystal of its characteristic shape with $a = b = 80$ Å and $c = 38$ Å. The appearance of the roof-like shape became dominant in the presence of a magnetic field. In the case of using $CoCl_2$ and $MnCl_2$, ordinary tetragonal crystals were formed. A striking fact was that the *a*-axis of the crystals oriented along the magnetic field when $CoCl_2$ was used, as opposed to the usual *c*-axis orientation. Large and optically perfect lysozyme crystals can be obtained in a magnetic field when $NiCl_2$ or $MnCl_2$ is used as a crystallizing agent. These profound effects of the paramagnetic cations may be caused by the coordination of two Ni^{2+} and Co^{2+} ions to a lysozyme molecule, which was found by X-ray crystallography.

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1. Introduction

In the past few years, effects of a magnetic field on protein crystal growth have been extensively studied ([1,2] and references therein). Various interesting phenomena were found, for example,

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magnetic orientation of crystals [3–5], a decrease both in the crystal growth [6,7] and dissolution [6,8] rates, improvement of crystal quality [9,10], an increase in viscosity of protein solutions [11], a decrease in diffusivity of lysozyme molecules in the solution [8], and damping of natural convection during crystal growth [12,13].

All these studies have used, as crystallizing agents, diamagnetic materials such as NaCl, $MgCl_2$, and PEG 3350. Weak magnetic repulsive force acts on diamagnetic materials. On the other hand, relatively strong attractive force acts on paramagnetic materials such as $NiCl_2$, $CoCl_2$, and $MnCl_2$. These paramagnetic salts have seldom been used so far as crystallizing agents of lysozyme, except for the lysozyme crystallization by $NiCl_2$ [14] and the solubility determination in the presence of $CoCl_2$ and $MnCl_2$ [15].

There has been no attempt to study the protein crystal growth in a strong magnetic field using paramagnetic materials as crystallizing agents. In this paper, we used three types of paramagnetic salts ($NiCl_2$, $CoCl_2$, and $MnCl_2$) as crystallizing agents to grow hen egg-white lysozyme crystals in a magnetic field. It is characteristic that the cations in these salts, Ni^{2+}, Co^{2+}, and Mn^{2+}, have magnetic moments, unlike Na^+, K^+, or Mg^{2+}. When these paramagnetic salts are used, aqueous protein solutions are paramagnetic. Especially in a magnetic field, this will make the growth environment different from the case when diamagnetic salts are used as crystallizing agents. We focus on the effects of a magnetic field on the crystal morphology, symmetry, orientation and optical perfection when these paramagnetic salts are used as crystallizing agents. The present study will show that the combined use of paramagnetic salts and magnetic fields may open a new method of designing and controlling protein crystal growth.

2. Experimental procedure

Hen egg-white lysozyme (Seikagaku Kogyo) was used without further purification. Three types of transition metal chlorides with different magnetic susceptibilities were used as crystallizing agents: $NiCl_2$ (magnetic susceptibility at room temperature $\chi_g = 27.7 \times 10^{-6}\,cm^3\,g^{-1}$), $CoCl_2$ ($\chi_g = 75.4 \times 10^{-6}\,cm^3\,g^{-1}$), and $MnCl_2$ ($\chi_g = 114 \times 10^{-6}\,cm^3\,g^{-1}$). A supersaturated solution was prepared by mixing an equal volume of the lysozyme solution and the paramagnetic chloride solution. Then, pH was controlled in the range of 4.31–5.52 by adding 1 N HCl. The pH was measured by a pH meter (PH8111-J, Yokogawa Inc.). The solution was then filtered through a filter with pore size 0.22 μm. Buffers were not used to avoid complexity arising from the coexistence of additional ions. The crystallization was carried out batchwise in a glass vessel with size ∅18 mm × 50 mm, and the solution height in the vessel was kept to be about 10 mm. The vessel was set inside a copper water jacket, and the temperature was controlled by flowing temperature-controlled water through the jacket. If not mentioned, the controlled temperature was kept at 21 °C. A set of vessel was placed at the center of a vertical superconducting magnet (JMTD-10T100 M, maximum field strength: 10 T; Japan Magnet Technology, Inc.), and another set outside the magnet. Fig. 1 demonstrates the experimental configurations. The space group and lattice constants of the

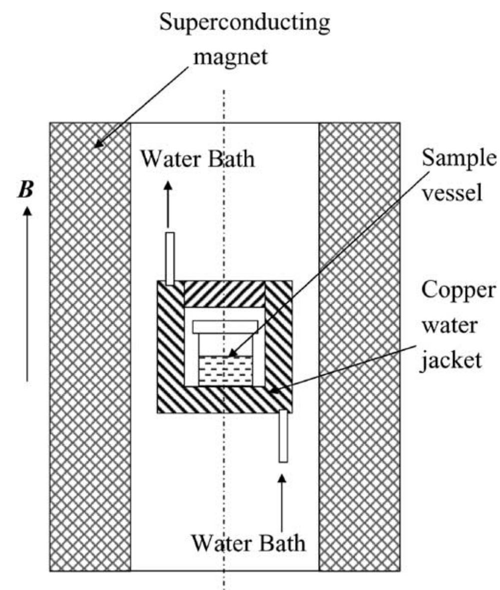


Fig. 1. Schematic illustration of the experimental configuration. *B* indicates the direction of magnetic flux density.

crystals were determined by X-ray diffraction method.

3. Experimental results and discussion

3.1. Lysozyme crystals with $NiCl_2$

The conditions for the preparation of the crystals with $NiCl_2$ were varied as follows: lysozyme = 40 mg/ml, $NiCl_2$ = 40–120 mg/ml and pH = 4.31–5.52. Crystal growth was most rapid when $NiCl_2$ concentration was around 80 mg/ml. This fact agrees with the data of lysozyme solubility in the literature, which shows a solubility minimum at around 80 mg/ml [14,15].

During the screening of the crystal growth conditions, we found that two types of crystals grew, depending on the $NiCl_2$ concentrations and pH. Fig. 2 shows the photographs of these two types of crystals formed in the absence (*a*, *b*) and presence (*a*′, *b*′) of a magnetic field of 10 T. The pictures were taken 2 days after supersaturating the solution with $NiCl_2$.

3.1.1. Distortion of the tetragonal lattice that gave a new type of orthorhombic crystals (roof-like crystals)

Figs. 2(a) and (a′) show the crystals grown in the absence and presence of a magnetic field of 10 T, respectively, when the conditions for the preparation are lysozyme = 40 mg/ml, $NiCl_2$ = 80 mg/ml and pH = 4.60. At 10 T (Fig. 2(a′)), all of them look like a roof with a ridge—the diagonal line is the ridge. They show the same faces toward our eyes, while at 0 T, the orientation is random.

X-ray analysis showed that this type of crystals belongs to orthorhombic symmetry $P2_12_12_1$ with $a = 79.0$ Å, $b = 80.8$ Å, and $c = 37.5$ Å. Clearly, these are different from the high-temperature-type orthorhombic crystals ($a = 56.44$ Å, $b = 73.73$ Å, and $c = 30.43$ Å) [16]. The lattice constants of this new type of orthorhombic crystals are nearly the same as the tetragonal crystals ($a = b = 79.20$ Å,

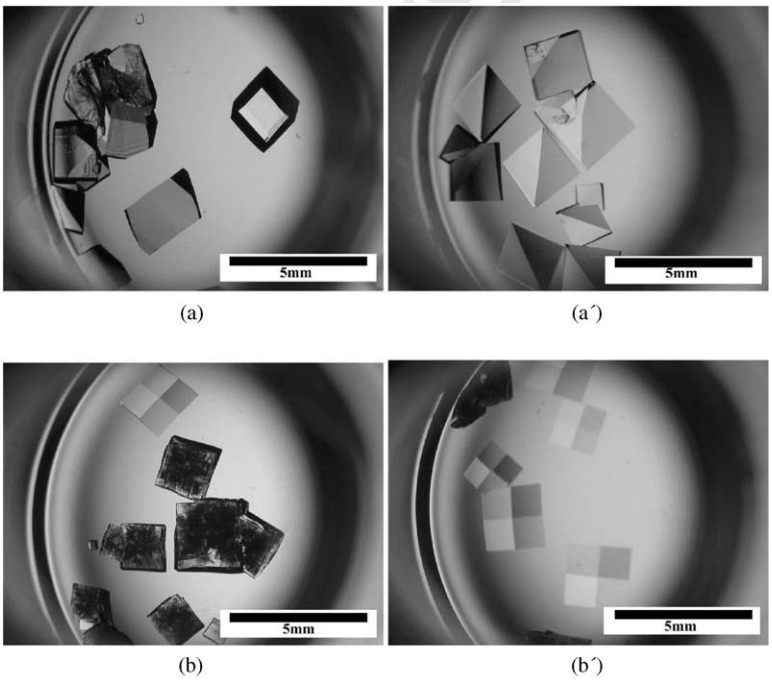
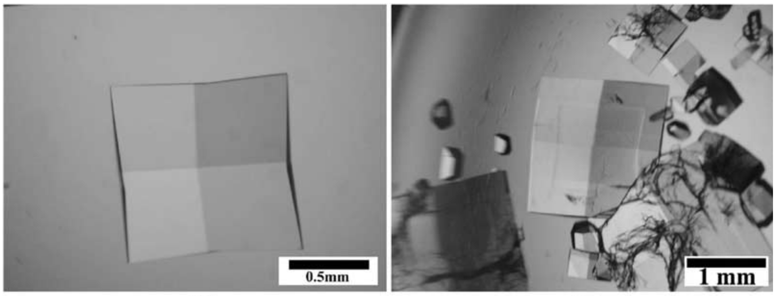


Fig. 2. Magnetic field effects on the morphology and orientation of lysozyme crystals crystallized with $NiCl_2$. Pictures were taken 2 days after supersaturating the solution with $NiCl_2$: (a) orthorhombic crystals under 0 T and (a′) orthorhombic (roof-like) crystals under 10 T; (b) ordinary tetragonal crystals under 0 T and (b′) ordinary tetragonal crystals under 10 T.



(a) (b)

Fig. 3. "Star-like" crystals. Preparation conditions: lysozyme = 40 mg/ml, $NiCl_2$ = 100 mg/ml, pH = 4.90. (a) A crystal grown 1 day under 10 T after the supersaturation and (b) a fully developed tetragonal crystal 1 week after taken out from the magnet.

$c = 37.94$ Å) [17], and different from all the known orthorhombic ones deposited in the Protein Data Bank. Therefore, we think that the roof-like crystals are new ones formed just like ordinary tetragonal crystals with some minor distortion of the molecular packing from the one in the tetragonal lattice. From the X-ray analysis, it was found that all the crystals in Fig. 2(a′) direct their *c*-axis parallel to the magnetic field. Furthermore, the X-ray analysis shows that two different Ni^{2+} ions are coordinated to Asp52 Oδ2 and Glu 35Oε1 atoms in a lysozyme molecule. The existence of the two Ni^{2+} ions is considered to cause the new packing. In addition, we noted that the appearance of the roof-like shape became dominant in the presence of a magnetic field.

3.1.2. Ordinary tetragonal crystals

Figs. 2(b) and (b′) are the pictures of crystals grown in the absence and presence of a magnetic field, respectively, when the conditions for crystal preparation are $NiCl_2$ = 100 mg/ml and pH = 4.90. These crystals show typical morphology of the well-known tetragonal crystals grown with NaCl. X-ray diffraction analysis confirmed that they belong to the tetragonal symmetry, with lattice constants of $a = b = 80$ Å and $c = 38$ Å. When the field was applied, the *c*-axis of these crystals was observed to be oriented along its direction.

3.1.3. Metastable, star-like growth shape of the ordinary tetragonal crystals

We found that a new crystal shape like a star appeared in the early stage of crystal growth. This was especially marked in the presence of the magnetic field. Fig. 3(a) shows such a crystal grown for 1 day at 10 T after supersaturation. The preparation conditions were the same as above for Figs. 2(b) and (b′). It was unstable and continued to grow to take eventually an ordinary tetragonal form. Fig. 3(b) gives a close look at one crystal when the solution was taken out from the magnet 1 day after the supersaturation and allowed to grow at room temperature for further 1 week. The crystal was developed to a typical tetragonal one but with some traces of its earlier shape. This indicates that the star-like shape is a metastable growth shape (not an equilibrium shape) of an ordinary tetragonal crystal. However, in the earlier stage of the growth, the {1 1 0} faces "split" into two vicinal faces, and this habit is quite different from that of normal tetragonal crystals [18]. The incorporation of Ni^{2+} into the crystal may cause the difference, but the mechanism is unknown at present.

3.2. Lysozyme crystals with $CoCl_2$

The conditions for preparation were screened as follows: lysozyme = 40 mg/ml, $CoCl_2$ = 60–140 mg/ml, and pH = 4.4–4.60. Crystal growth was most rapid when $CoCl_2$ concentration was around 80 mg/ml. This agrees with the solubility data of lysozyme in the literature, which shows a solubility minimum at around 80 mg/ml [15].

Fig. 4 shows the comparison of the crystals grown in the absence (a) and presence (b) of 10 T when the conditions for crystal preparation were

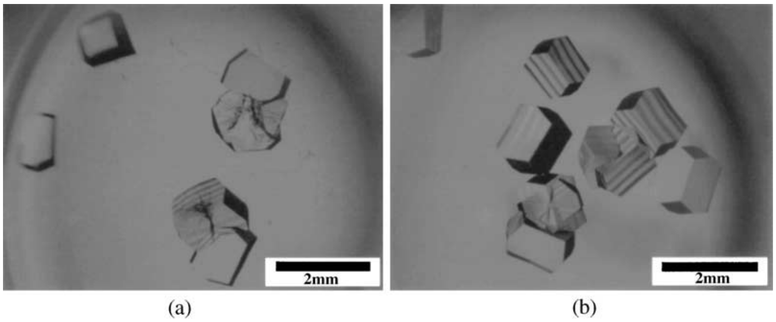


Fig. 4. Lysozyme crystals grown in the absence (a) and presence (b) of 10 T when $CoCl_2$ was used as a crystallizing agent. Pictures were taken 2 days after the supersaturation. Crystals preparation conditions: lysozyme = 40 mg/ml, $CoCl_2$ = 100 mg/ml, pH = 4.60.

lysozyme = 40 mg/ml, $CoCl_2$ = 100 mg/ml and pH = 4.60. Contrary to the case of $NiCl_2$, the morphology of the crystals seemed not to change drastically between the absence and presence of the magnetic fields; they looked like ordinary tetragonal crystals. X-ray diffraction analysis confirmed that they were the typical tetragonal crystals, with lattice constants $a = b = 79.5$ Å and $c = 37.6$ Å [19]. When crystals were grown in the absence of magnetic field, they were randomly oriented as shown in Fig. 4(a). However, it was surprising that, when grown at 10 T, they aligned their *a*-axis along the field direction as shown in Fig. 4(b). This fact is quite different from all the previous studies [3,4] in which the *c*-axis is aligned along the applied field. Furthermore, the X-ray analysis shows that two different Co^{2+} ions are coordinated to Asp52 Oδ2 and Glu 35Oε1 atoms in a lysozyme molecule, similar to the coordination of Ni^{2+} ions in a crystal shown in Fig. 2(a′). Clearly, the existence of the two Co^{2+} ions caused the crystals to respond to the magnetic field differently from the other lysozyme crystals. We suppose that the magnetic anisotropy coming from the two Co^{2+} ions is responsible for the new magnetic orientation.

3.3. Lysozyme crystals with $MnCl_2$

We tried crystallization from 40 mg/ml lysozyme at pH = 4.60 with 60–120 mg/ml $MnCl_2$. Crystal growth was slower than the former two salts. Usually visible crystals appeared 3–4 days after the supersaturation (at 20 °C). Crystal growth was most rapid when $MnCl_2$ concentration was around 80 mg/ml. It agrees with the data of lysozyme solubility in the literature [15], which shows a solubility minimum at around 80 mg/ml.

Fig. 5 shows the tetragonal lysozyme crystals 5 days after the supersaturation with $MnCl_2$. Crystal preparation conditions were: lysozyme = 40 mg/ml, $MnCl_2$ = 80 mg/ml, pH = 4.60. Fig. 5(a) shows the crystals grown outside the magnet, while Fig. 5(b) is the picture of the crystals grown initially inside the magnet for 2 days and then outside the magnet. Crystals appeared to be of typical tetragonal morphology. When crystals were grown initially for 2 days in the magnetic field, the crystals aligned their *c*-axis along the magnetic field. From these results we see that $MnCl_2$ seems not to show any strong effect on the behavior of the crystallization (or crystal morphology and orientation) in the magnetic field compared with $NiCl_2$ and $CoCl_2$.

3.4. Preliminary results of the effect of magnetic field on the perfection of crystals

Besides magnetic orientation, magnetic fields significantly affected the appearance of two types of crystals with $NiCl_2$ (Fig. 2). The crystals prepared in the absence of the magnetic field showed various irregular appearances, while in a magnetic field, they became more uniform. Such a phenomenon could be observed even when the field was as small as 1 T at which most crystals

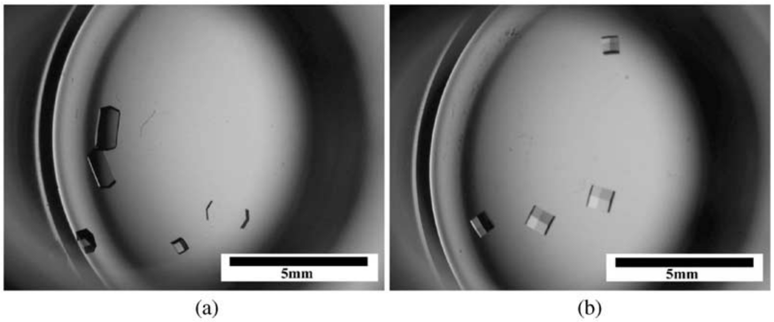


Fig. 5. Lysozyme crystals 5 days after the supersaturation with $MnCl_2$ crystals grown (a) outside the superconducting magnet and (b) initially inside the magnet for 2 days and then outside the magnet. Crystals preparation conditions: lysozyme = 40 mg/ml, $MnCl_2$ = 80 mg/ml, pH = 4.60.

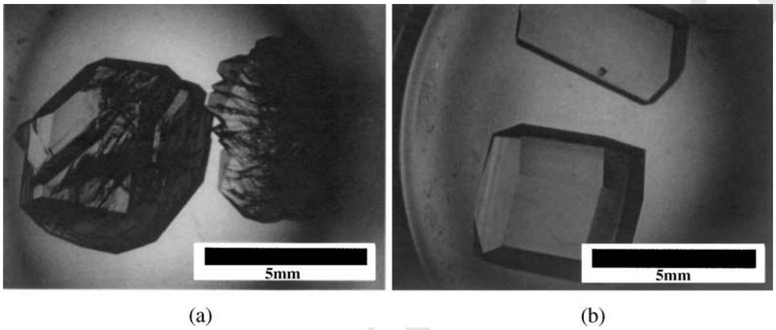


Fig. 6. Lysozyme crystals 4 months after supersaturating with $MnCl_2$ (a) grown outside the superconducting magnet and (b) grown initially inside the magnet for 4 days and then outside the magnet. Crystals preparation conditions: lysozyme = 40 mg/ml, $MnCl_2$ = 80 mg/ml, pH = 4.60.

were magnetically oriented. Furthermore, many crystals grown in the absence of the magnetic field seem to have a lot of micro-cracks so that the crystals look opaque (see Fig. 2(b)), while in the presence of the magnetic field (Fig. 2(b′)), they became much more transparent. From these phenomena, it seems that the magnetic field or magnetic orientation (or both) helped to grow better crystals. Even when the protein solution was taken out from the superconducting magnet 2 days after supersaturation and crystals were grown outside the magnet, crystals looked better than those grown in the absence of the field. Furthermore, crystals treated in the magnetic field can eventually reach a size more than 5 mm while maintaining the optical perfection.

A similar phenomenon was also observed when crystals grew in the presence of $MnCl_2$. Fig. 6(a) shows the crystals grown outside the magnet, while Fig. 6(b) shows the crystals which grew first inside the magnet for 3 days after the supersaturation and further grew for 4 months outside the magnet. The crystals initially grown inside the magnet finally looked much better than the control, although in the initial stage, the crystals grown both inside and outside of the magnet seemed to yield similar optically perfect crystals (see Fig. 5). The final size of crystals grown in the presence and absence of a magnetic field is about 5 mm, as shown in Fig. 6. Thus, large and optically perfect lysozyme crystals can be obtained when $NiCl_2$ or $MnCl_2$ is used as crystallizing agent and crystals grow in a magnetic field.

Structural imperfections in a protein seed crystal will induce lattice stress during crystal growth. A seed with the more defects will cause the larger stress and induce more cracks. This is a case similar to the observations in the Czochralski growth of inorganic materials [20]. The final appearance of lysozyme crystals initially prepared in a magnetic field suggests that the seed itself possesses better quality than its counterpart prepared in the absence of the magnetic field.

When $CoCl_2$ was used as a crystallizing agent, the crystals could grow to a size more than 3.5 mm. Apparent difference in optical perfection between the crystals grown in the presence and absence of a magnetic field was not clearly observed yet.

3.5. A role of paramagnetic cations as a crystallizing agent to design lysozyme crystal growth

The previous experiments carried out by other investigators indicated that different anions give lysozyme crystals of different symmetry and/or lattice constants [21–24]. On the other hand, different cations give crystals of the same symmetry and lattice constants [25]. Therefore, the role of the cations may be regarded as marginal, except for its profound effect on solubility [15].

In this paper, we have shown that the effect of cations can be more profound when used combinedly with a magnetic field. We have used salts like $NiCl_2$, $CoCl_2$ and $MnCl_2$ as crystallizing agents. They contain divalent, paramagnetic cations. All of them worked as crystallizing agents and showed a tendency to grow larger crystals than ordinary diamagnetic salts, and in the magnetic field, $NiCl_2$ and $MnCl_2$ could make large crystals that looked optically perfect. Furthermore, $NiCl_2$ was found to give crystals of a different symmetry and morphology. $CoCl_2$ could endow crystals with different magnetic orientations from ordinary ones. This is the first instance that the use of cations as the crystallizing agent can modulate crystal growth of lysozyme.

The effect of Ni^{2+}, Co^{2+}, and Mn^{2+} on lysozyme is an old problem. Ikeda and Hamaguchi [26] showed through circular dichroism measurements that the existence of these ions affected the enzymatic activity. McDonald and Phillips [27] indicated that the NMR spectrum of lysozyme changes in the presence of Co^{2+}. However, we consider that this is the first time that the crystals of hen egg-white lysozyme was grown in the sole presence of Ni^{2+}, Co^{2+}, or Mn^{2+} as crystallizing agents and the crystal structure was solved. As a result, it was found that two Ni^{2+} or Co^{2+} ions are incorporated into the crystal and the position was in the active cleft, in agreement with the effects of these ions on enzymatic activity [26,27]. These facts explain the profound influence of the paramagnetic cations on the properties of the lysozyme crystals that have been found here in a strong magnetic field.

4. Conclusions

In this paper, we studied lysozyme crystal growth with paramagnetic salts ($NiCl_2$, $CoCl_2$ and $MnCl_2$) as crystallizing agents in a magnetic field for the first time. Experimental results showed that the paramagnetic cations (Ni^{2+}, Co^{2+} and Mn^{2+}) exhibited profound effects on crystal growth as follows:

(1) $NiCl_2$ was found to give two types of crystals with different morphologies. One (roof-like) is orthorhombic $P2_12_12_1$ with $a = 79.0$ Å, $b = 80.8$ Å, and $c = 37.5$ Å and the second is ordinary tetragonal crystal of its characteristic shape with $a = b = 80$ Å and $c = 38$ Å.
(2) The above-mentioned roof-like shape as well as a metastable, star-like shape are considered as new morphologies of lysozyme crystals. Although they may come from the presence of Ni^{2+} ions, the occurrence was much frequent in a magnetic field.
(3) When $CoCl_2$ was used as a crystallizing agent, the tetragonal crystal aligned its *a*-axis along the magnetic field direction instead of its *c*-axis.
(4) When these paramagnetic salts are used as crystallization agents, large and optically perfect lysozyme crystals can be obtained if the crystals grow initially in a magnetic field.

As a reason for the profound effects of the paramagnetic cations, we may consider the co-ordination of two Co^{2+} and Ni^{2+} ions to a lysozyme molecule found by X-ray crystallography. The results of present study suggest that the combined use of paramagnetic salts and magnetic fields may open a new method of designing and controlling protein crystal growth.

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