Korean J. Cryst., Vol. 5, No. 1, pp 33~38, 1994.

증기확산법에 의한 단백질 결정화에 미치는 Reservior 용액의 영향

이정희, 정용제
충북대학교 생화학과

The Effects of Solvents in Reservior Solution on Protein Crystallization Using Vapor Diffusion Method

Junghee Lee and Yong Je Chung
Department of Biochemistry, Chungbuk National University

요 약

Hen egg white lysozyme과 equine serum albumin을 모델 단백질로 하여 'heterogeneous' vapor diffusion실험을 수행하였다. 즉, droplet과 reservoir용액에 각각 다른 침전제를 사용하여 vapor diffusion에 의하여 평형에 이르도록 하였다. 실험 결과는 NaCl 혹은 ammonium sulfate 대신 polyethylene glycol이 reservoir 용액에 포함되므로써 droplet과 reservoir 용액 사이의 평형 속도가 감소되는 것을 보여 주었다. Heterogeneous vapor diffusion 법을 통하여 reservoir용액에 포함된 이온성 염과 비이온성 침전제의 양을 적절히 조절하여 평형속도를 조절할 수 있음을 보였다.

Abstract

'Heterogeneous' vapor diffusion experiments were carried out using hen egg white lysozyme and equine serum albumin as model proteins : droplets were equilibrated against reservoir solutions containing an alternative precipitant which is different from that in the droplets. Experimental results showed that the use of polyethylene glycol as an alternative precipitant instead of NaCl or ammonium sulfate reduces equilibration rate between droplet and reservoir solution. By using the heterogeneous vapor diffusion technique it is possible to control the equilibration rate by adjusting the relative amounts of ionic salts and nonionic precipitants in reservoir solutions.

1. Introduction

The crystallographic study of proteins is often limited by the difficulty of growing large crystal suitble for X-ray analysis. Many laboratories have made efforts leading to a more rational approach to protein crystallization. However, the protein crystallization is still considered to be an empirical

and time-consuming procedure.

Vapor diffusion method[1,2] is currently one of the most popular techniques used for screening crystallization conditions and for growing diffraction quality protein crystals. To optimize the quality of the crystals formed we need a better understanding of the factors affecting the crystallization of proteins. In this connection, many studies have been performed to characterize the effects of the factors, including equilibration rate[3], protein impurity[4], gravity[5], additives[6,7]. These studies were carried out under the conditions in which both droplet and reservoir solution in the vapor diffusion setting contain the same kind of precipitant(s). The setting is referred to as 'homogeneous' vapor diffusion setting in this papper. In vapor diffusion method, equilibration between droplet and reservioir solution is accomplished by evaporation of volatile constituents such as water and equilibration rate is governed by the nature of precipitant[3]. Therefore, we may be able to control the rate by using a 'heterogeneous' vapor diffusion setting with the droplet containing different precipitant(s) from that in reservior solution.

In this papper, we report the results from the heterogeneous vapor diffusion experiments using a model system, the crystal growth of hen egg white lysozyme (HEWL) and equine serum albumin (ESA) against the reservoir solutions containing polyethylene glycol (PEG), 2-methyl-2, 4-pentanediol (MPD), and ammonium sulfate (AS)/NaCl as an alternative precipitant(s).

2. Materials and Methods

2.1. Materials

ESA, HEWL, PEG 400, PEG 4000, PEG 8000, NaCl, ammonium sulfate, acetic acid, and sodium acetate were purchased from Sigma. MPD was from Aldrich.

2.2. Determination of corresponding concentration of target precipitants

Sitting drop method was used for the experiment. Droplets of the mother liquor containing increasing concentrations of the precipitant (AS for ESA and NaCl for HEW lysozyme) were placed on Corning three-depression glass plates. The droplets of 100 μ1 on the plates are sealed in transparent plastic boxes (50 X 130 X 30 cm) containing 30ml of reservoir solution with a given concentration of precipitant. Sitting droplets and reservoir solutions were allowed to equilibrate for 5 days. Volume of droplets was measured by using Hamilton microsyringe. This procedure was adapted from Schreuder et al.[8].

2.3. Crystallization setup

Hanging drop method was used to crystallize model proteins under conditions being investigated. Droplets on cover slips were prepared by mixing 2 μ1 of protein solution and 2 μ1 of original reservoir solutions (the initial concentration of a precipitant in a droplet is half of that in corresponding reservoir solution). Here, 'original' reservoir solution represents the solution containing precipitants used to crystallize the model proteins (AS for ESA and NaCl for HEWL). The droplets were then equilibrated against 700 1 of reservoir solutions in the wells of Linbro tissue culture plate (6 wells in a row and 4 rows in a plate) at room temperature. Each well in the 1st row contained reservoir solutions with increasing concentrations of original precipitant (homogenous vapor diffusion setting.) On the other hand, for each well in the 2nd, the 3rd, and the 4th rows, hanging drop setting was performed by equilibrating the droplets against alternative reservoir solutions containing one of target precipitants (PEG, MPD, AS, or NaCl) instead

of original precipitant (heterogeneous vapor diffusion setting), represented in Table 1. In order to compensate possible errors in corresponding concentration, a wide range of concentration for the alternative precipitant was investigated.

TABLE 1 Vapor diffusion setting using 24-well Linbro tissue culture plate.

	①	②	③	④	⑤	⑥
ⓐ	(1)(a)	(2)(a)	(3)(a)	(4)(a)	(5)(a)	(6)(a)
ⓑ	(1)(b)	(2)(b)	(3)(b)	(4)(b)	(5)(b)	(6)(b)
ⓒ	(1)(c)	(2)(c)	(3)(c)	(4)(c)	(5)(c)	(6)(c)
ⓓ	(1)(d)	(2)(d)	(3)(d)	(4)(d)	(5)(d)	(6)(d)

①-⑥ : Column number of crystallization plate.
ⓐ-ⓓ : Row number of crystallization plate.
(ⓐ-homogeneous setting, ⓑ,ⓒ,ⓓ-heterogeneous setting)
(1)-(6) : Droplets containing original mother liquor. The concentration of precipitant indroplets grdually increases from ① to ⑥.
(a) : Reservoir solutions containing the same presipitant as droplets.
(b)-(d) : Reservoir solutions containing different precipitant from droplets. The concentration of precipitant increasesfrom ⓑ to ⓓ.
Detailed description is given in the text.

Tetragonal from crystals of HEWL : Tetragonal crystals of lysozyme were grown according to the procedure of Alderton and Fevold[9]. Lysozyme (10mg /ml) was dissolved in 50 mM sodium acetate buffer solution (pH 4.5). Original reservoir solutions contained 50 mM sodium acetate buffer (pH 4.5) with increasing concentrations of NaCl (3.5 to 6.0% with 0.5 increment). PEG 400, PEG 4000, PEG 8000, AS, and MPD as alternative precipitants were investigated.

ESA crystals : ESA was crystallized according to the procedure of McClure and Craven10). 50mM concentration of sodium acetate buffer (pH 5.0) was used to dissolve ESA (30 mg/ml) and to prepare reservoir solutions. Original reservoir solutions contained saturated ammonium sulfate (SAS) ranging 35 to 47.5 % by increments of 2.5 %. PEG 400, PEG 4000, PEG 8000, NaCl, and MPD as alternative precipitants were investigated.

2.4. Data Analysis

The growing crystals were investigated visually through a stereo microscope. For each box, crystals grown against alternative precipitants were compared with the best crystals in the 1st row (i.e. crystals from original condition) in terms of crystals size. the number of crystals in a well, the level of twinning, and the nucleation rate.

3. Results and Discussion

3.1. Determination of corresponding concentrations of target precipitants

Equilibration studies showed that 5 % NaCl (precipitant for tetragonal crystals of lysozyme) solution is approximately equivalent to 30 % PEGs, 30 % SAS and 20 % MPD , and 40 % SAS (precipitant for tetragonal crystals of lysozyme) solution to 40 % PEG, 2.0 M NaCl and 50 % MPD. We expected that errors could be significant due to the small size of droplets. Therefore, subsequent experiments using the results were performed over the extented range of concentration from the experimental values.

3.2. The effects of solvent in reservoir solution on protein crystallization

Tetragonal crystals of lysozyme : The use of PEGs (PEG 400, 4000, 8000) as an alternative precipitant in reservoir solution seemed to be generally positive. The heterogeneous vapor diffusion setting with PEG showed comparable quality crystals in size and the degree of twinning with

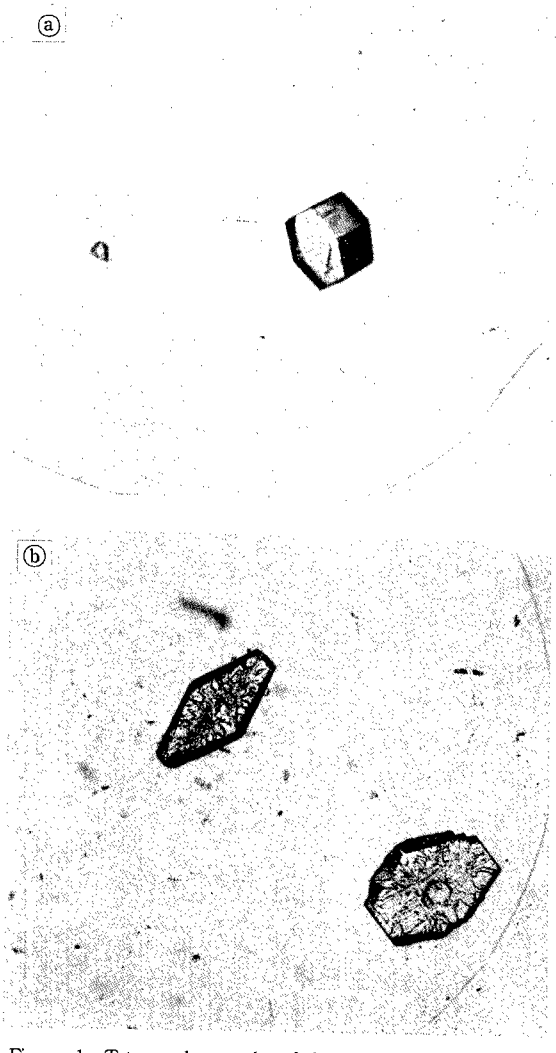

Figure 1. Tetregonal crystals of hen egg white lysozyme grown by heterogeneous vapor diffusion method.
ⓐ PEG 8000 as an alternative precipitant
ⓑ Ammonium sulfate as an alternative precipitant

those from the homogenous setting, as shown in Figure 1.ⓐ. Good quality of HEWL crystals were obtained over the wide range of PEG concentrations (35 to 45 %, 10 mg/ml protein concentration). On the other hand, NaCl in homogeneous setting is limited to relatively narrow range of concentrations (3 to 6 %). This could be a result of slower equilibration rate of PEG solutions than that of NaCl solution. The use of AS did notimproved crystal

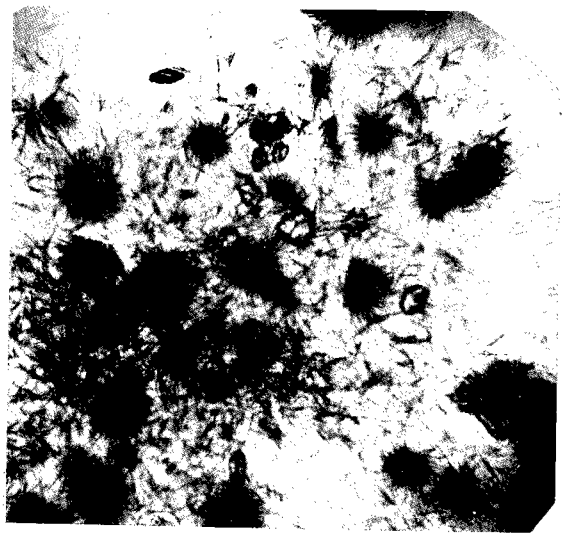

Figure 2. Needle-like crystals of hen egg white lysozyme grown by heterogeneous vapor diffusion method using 45 % saturated ammonium sulfate as analternative precipitant.

quality in terms of parameters observed. At initial stage, it seemed that crystal growth with AS looked the same as that from original reservoir solution. However, cracks on the crystals from the AS reservoir solution were increased upon proceeding of the crystal growth (Figure 1.ⓑ). It appeared that the cracks might be induced from the difference in evaporation rates of water on NaCl and AS solutions. An interesting result from the experiment was that needle-shaped crystals as well as tetragonal crystals were grown from reservoir solutions containing 45 % SAS, as shown in Figure 2. The 45 % is much higher than corresponding concentration (approximately 30 % SAS against 5 % NaCl). The crystals were too small to be characterized by X-ray diffraction method.To grow large enough crystals, seeding experiments are under way. In the case of MPD, no crystals were grown.

ESA crystals : It appeared that the use of NaCl as an alternative precipitant for reservoir solution resulted in the same effects on ESA crystallization

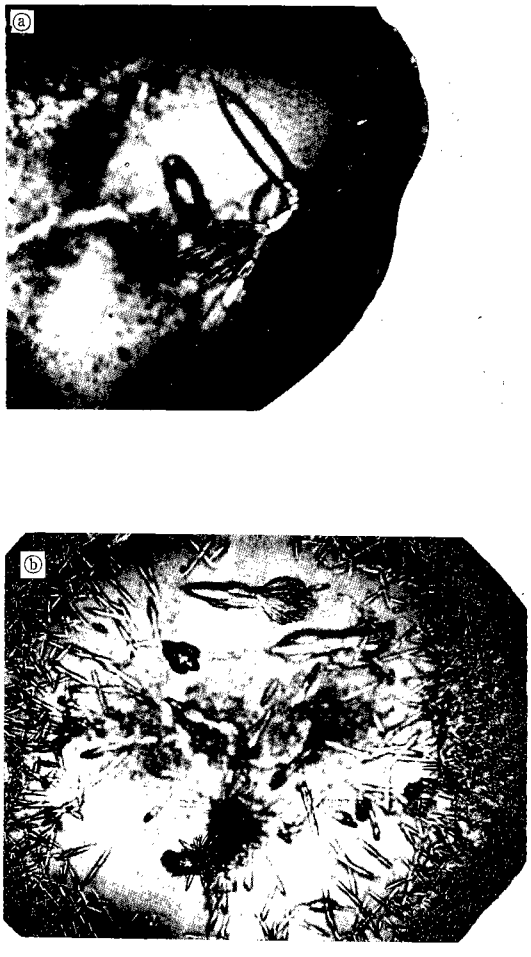

Figure 3. Crystals of equine serum albumin grown by heterogeneous vapor diffusion method.
ⓐ PEG 400 as an alternative precipitant
ⓑ 40 % PEG 4000 and 5 % saturated ammonium sulfate as alternative precipitant

as original precipitant (AS). On the other hand, the heterogeneous vapor diffusion setting using MPD, PEG 4000 and PEG 8000 showed no crystals under the conditions with the concentration ranging from 10 % to 50 % by 5 % increment. Turbid precipitates were always formed in every well. Such kind of precipitates were often observed from the homogeneous vapor diffusion setting with lower concentration of AS (below 30 % SAS). It might imply that slower equilibration rate under the conditions could result in lower supersaturation rate : i.e. supersaturation required for nucleation must be reached fast enough to overcome the rate of formation of amorphous precipitates. As a result, to increase equilibration rate a small amount of AS was added to reservoir solutions, containing PEGs or MPD. Indeed, by addition of 5 % SAS to the reservoir solutions, hexagonal pencil-like crystals were grown from the heterogeneous vapor diffusion settings with PEG 4000 or PEG 8000 but not with MPD, shown in Figure 3. From this result, it was believed that an appropriate equilibration rate was achieved by the addition of AS. PEG 400 showed different properties from other PEGs. Good quality of crystals were obtained from reservoir solutions containing 30 % of PEG 400. It was interpreted that the evaporation rate of water in PEG 400 solution could be faster than that in PEG 4000 and PEG 8000 solutions Generally, the quality of crystals was not improved from heterogeneous setting.

Slower equilibration rate is generally benefit to protein crystallization, producing larger crystals. For example, in addition to our results of lysozyme crystallization discussed above, it was reported that the crystal size of lysozyme was significantly increased by reducing equilibration rate[11]. Growth of hexagonal crystals of ESA, however, requires somewhat faster rate. It is hard to say that this is due to the nature of protein itself or other factors. By using MPD as an alternative precipitant, it was not possible to grow crystals of both HEWL and ESA. This may suggest that the volatility of MPD is significant enough to affect the composition of droplet through vapor diffusion.

3.3. The heterogeneous vapor diffusion setting

The effects of reservoir solution have been considered to be relatively less important than other factors. It is generally true. However, since protein crystallization is a delicate multiparametic process, even tiny environmental changes may induce significant effects on protein crystallization. Based on our results, the nature of nonvolatile precipitants in reservoir solution is related to equilibration rate. In the case of vapor diffusion method, major factors affecting equilibration rate have been considered to be temperature, initial surface-to-volume of the droplet, and its dilution with respect to the reservoir, etc.[3]. Together with those factors, it is expected that the heterogeneous vapor diffusion setting will provide alternative procedure in protein crystallization : since evaporation rate of water is much higher in aqueous solution of ionic salts than nonionic precipitants such as PEG[3], by using the heterogeneous vapor diffusion technique we may be able to control equilibration rate by adjusting the relative amounts of ionic salts and nonionic precipitants in the reservoir solution.

Aknowlegement

This paper was supported by NON DIRECT RESEARCH FUND, Korea Research Foundation, 1992.

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