Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Comparative analysis of amino acids and amino-acid derivatives in protein crystallization

Len Ito,[a,b]* Kentaro Shiraki[c] and Hiroshi Yamaguchi[b]

[a]Japan Synchrotron Radiation Research Institute (SPring-8), 1-1-1 Kouto, Sayo, Hyogo 679-5198, Japan, [b]School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan, and [c]Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

Correspondence e-mail: l-ito@spring8.or.jp

Received 7 October 2009
Accepted 14 April 2010

© 2010 International Union of Crystallography
All rights reserved

Optimal conditions for protein crystallization are difficult to determine because proteins tend to aggregate in saturated solutions. This study comprehensively evaluates amino acids and amino-acid derivatives as additives for crystallization. This fourth component of the solution increases the probability of crystallization of hen egg-white lysozyme in various precipitants owing to a decrease in aggregation. These results suggest that the addition of certain types of amino acids and amino-acid derivatives, such as Arg, Lys and esterified and amidated amino acids, is a simple method of improving the success rate of protein crystallization.

1. Introduction

X-ray crystallography of proteins is an important biological technique. X-ray crystallographic methods have been developed for the determination of the tertiary structure of proteins and include the development of detector technology and computational methods and the use of synchrotron radiation and improved protein-purification protocols. To date, the crystallization step of proteins has remained a major obstacle in X-ray crystallography. Various crystallization techniques have been developed in an attempt to facilitate and improve crystal nucleation, such as the use of temperature control (Adachi, Takano, Yoshimura *et al.*, 2003), gravity (McPherson *et al.*, 1999), magnetic fields (Sazaki *et al.*, 1997), pressure (Suzuki *et al.*, 1994), ultrasonic irradiation (Luft & DeTitta, 1999), laser irradiation (Adachi, Takano, Hosokawa *et al.*, 2003) and solution flow (Adachi *et al.*, 2002). The current efforts have developed narrow screening cocktails to produce initial crystallization hits, *e.g.* using additives such as metals, nucleants *etc.* However, these kits are unsuitable in many cases owing to the limited conditions under examination.

Although proteins crystallize in solution containing a certain type of precipitant, determining the optimal condition is difficult owing to the formation of amorphous aggregates. The dynamic light-scattering method has revealed that crystal nucleation is initiated by a relatively sharp transition from a monodisperse solution to insoluble small aggregates with increasing precipitant concentration, whereas a continuum of aggregates before precipitation leads to amorphous material (Mikol *et al.*, 1990; Kadima *et al.*, 1990; Thibault *et al.*, 1992). Thus, prevention of protein aggregation plays a key role in the formation of single crystals in aggregation-prone solution conditions (McPherson *et al.*, 1986).

The solution used for protein crystallization usually contains buffer, salts and precipitant. Recently, several studies have proposed that aggregation suppressors be used in the crystallization of proteins and viruses (Sauter *et al.*, 1999; Jeruzalmi & Steitz, 1997; McPherson & Cudney, 2006; Larson *et al.*, 2007). The underlying hypothesis behind this approach was that small molecules would form reversible cross-links in the crystal lattice through intermolecular electrostatic, hydrogen-bonding and perhaps hydrophobic interactions. In this study, we focused on using new types of aggregation suppressors, such as amino acids and their derivatives, as additives. We evaluated the

effect of these additives on initial screening and on optimization of crystallization conditions using hen egg-white lysozyme (HEWL) as a model protein. Guanidine and urea are well known aggregation suppressors that weaken the intermolecular hydrophobic interactions between protein molecules (Buchner & Rudolph, 1991; Rudolph & Lilie, 1996), but these denaturants decrease the stability of the native state of the proteins. Recently, several types of amino-acid derivative have been used as additives to decrease aggregation during refolding and heat-treatment of proteins (Shiraki *et al.*, 2002, 2004, 2005; Taneja & Ahmad, 1994; Sakamoto *et al.*, 2004; Arakawa & Tsumoto, 2003). This study hypothesizes that a new aggregation suppressor that is added as the fourth component could favour protein crystallization by suppressing protein aggregation under supersaturated conditions in the presence of a precipitant.

2. Materials and methods

2.1. Reagents

Lysine, aspartic acid, serine amide, glycine ethyl ester, serine ethyl ester, arginine ethyl ester, lysine ethyl ester and glutamic diethyl ester were obtained from Sigma–Aldrich Co. (St Louis, Missouri, USA). Threonine amide was obtained from Novabiochem (Basel, Switzerland). Lysine glutamate was obtained from MP Biomedicals Inc. (Irvine, California, USA). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). All chemicals used were of analytical grade. Sixfold-crystallized HEWL was obtained from Seikagaku Kogyo Co. (Tokyo, Japan) and was used without further purification. Crystal Screen I (Hampton Research Co., California, USA) was used for sparse-matrix sampling.

Solutions of glycine (Gly), alanine (Ala), serine (Ser), threonine (Thr), proline (Pro), valine (Val), aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), lysine (Lys), glycine amide (GlyAd), threonine amide (ThrAd), proline amide (ProAd), arginine amide (ArgAd), glycine ethyl ester (GlyEE), serine ethyl ester (SerEE), lysine ethyl ester (LysEE), arginine ethyl ester (ArgEE), phenylalanine ethyl ester (PheEE), glutamic diethyl ester (GludiEE) and lysine glutamate (Lys-Glu) were prepared to evaluate the effect of amino acids and amino-acid derivatives on HEWL crystallization. Additives were adjusted to pH 4.5 or 6.5 using a conventional pH electrode for crystallization. Protein solutions were centrifuged at 15 000*g* for 20 min at 292 K.

2.2. Crystallization

Crystallization of HEWL using ammonium sulfate as a precipitant was performed as follows. Protein solutions containing 50, 100 or 150 mg ml^{-1} HEWL and 0.1 *M* sodium acetate pH 4.5 buffer were prepared in 1.5 ml microtubes. The hanging-drop vapour-diffusion method was used for crystallization: 1.5 µl protein solution was combined with 1.5 µl reservoir solution containing an ammonium sulfate concentration of 0.1, 0.25, 0.5, 0.75 or 1.0 *M*, using 500 µl reservoir solution containing 0.2 or 0.5 *M* additive per well. The initial concentration of the additive in each hanging drop was 0.1 or 0.25 *M*. The plate was incubated at 293 K. Crystallization of HEWL using Hampton Research Crystal Screen I was performed as follows. Protein solutions containing 25 or 50 mg ml^{-1} HEWL, 0.1 *M* acetate buffer pH 4.5 and 0.2 *M* of each additive were prepared in 1.5 ml microtubes. Sparse-matrix crystallization screening was performed with Crystal Screen I by hanging-drop vapour diffusion at 293 K. Hanging drops were obtained by mixing 1.5 µl protein solution with 1.5 µl reservoir solution. The volume of the reservoir solution was 600 µl for each setting. The initial concentration of the additives in each hanging droplet was 0.1 *M* per additive.

Crystallization of lysozyme using sodium chloride as precipitant was performed as follows. Protein solution containing 50 mg ml^{-1} HEWL in 0.1 *M* sodium phosphate buffer pH 6.5 was prepared in a 1.5 ml microtube. The hanging-drop vapour-diffusion method was used for crystallization at 293 K. Hanging drops were prepared by mixing 1.5 µl protein solution with 1.5 µl reservoir solution containing sodium chloride at a concentration of 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 or 2.25 *M* and 0.5 *M* additive. The volume of reservoir solution was 500 µl for each setting.

Reproducibility was evaluated by repeating each experiment more than three times. Drops were carefully examined under a stereoscopic microscope every day during the first several weeks and then on a monthly basis.

2.3. Turbidity and solubility measurements

The batch method was used to study the influence of different amino acids on the evolution of the turbidity of supersaturated solutions of HEWL. A solution of 20 mg ml^{-1} lysozyme in 0.1 *M* sodium phosphate pH 6.5 with 0.4 *M* amino acids and amino-acid derivatives and precipitant solutions containing 11 different sodium chloride concentrations (2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0 and

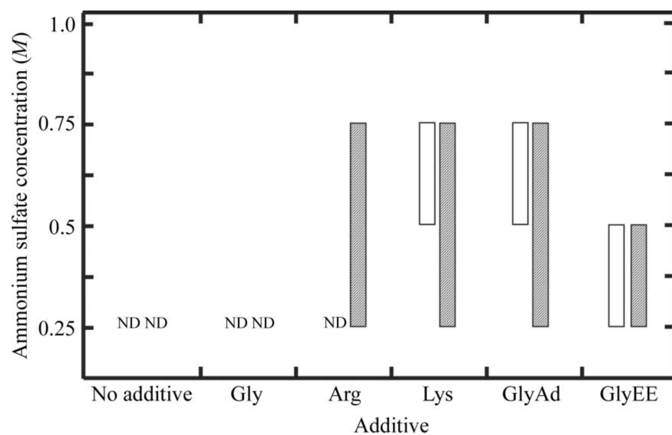


Figure 1
Crystallization of 100 mg ml^{-1} (open bars) or 150 mg ml^{-1} (shaded bars) HEWL using ammonium sulfate as a precipitant in the presence of 0.25 *M* amino acid at pH 4.5.

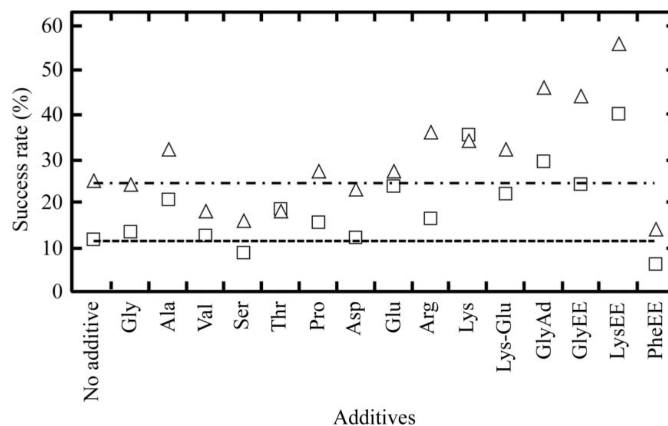


Figure 2
Success rate of crystallization of 25 mg ml^{-1} (squares) or 50 mg ml^{-1} (triangles) HEWL in the presence of 0.1 *M* amino acids at pH 4.5 with 50 unique combinations of sparse-matrix reagents using Crystal Screen I. A reference line across the graph indicates the success rate of the control experiments.

4.2 *M*) were prepared. All additive solutions were passed through disposable 0.2 µm sterile syringe filters and adjusted to pH 6.5 using a conventional pH electrode before mixing with protein solutions. A sample was obtained by mixing 500 µl protein solution with an equal amount of precipitant solution in a 1.5 ml microtube. The final solution contained 0.2 *M* amino acids and amino-acid derivatives, 10 mg ml^{-1} lysozyme and 0.1 *M* sodium phosphate pH 6.5. After 2 d, the samples were monitored using a Jasco spectrophotometer (model V-550, Japan Spectroscopic Company, Tokyo), focusing on turbidity changes measured at 600 nm. After this measurement, all solutions were centrifuged at 15 000*g* for 20 min at 293 K and the concentration of soluble protein was then measured at 280 nm using an ND-1000 UV–Vis spectrometer (NanoDrop Technologies, Wilmington, Delaware, USA).

3. Results and discussion

HEWL is a good model protein as it is difficult to crystallize using the popular salting-out agent ammonium sulfate (Mikol *et al.*, 1990). Fig. 1 shows the crystallization of HEWL with ammonium sulfate at pH 4.5. In the absence of additives HEWL did not crystallize, as previously reported (Riès-Kautt & Ducruix, 1989). When using 0.25 *M* amino acids or their derivatives, HEWL crystals were observed after several days. Under acidic conditions (pH 4.5) in the presence of positively charged amino acids (Arg and Lys) and amino-acid derivatives (GlyEE and GlyAd), HEWL could be crystallized using ammonium sulfate (Fig. 1). We obtained HEWL crystals in the presence of 0.1 *M* solutions of these additives or 50 mg ml^{-1} HEWL (data not shown), but it took a few months. It is thought that HEWL only produces an amorphous precipitate using ammonium sulfate, although Forsythe and coworkers have reported ultrapure HEWL crystallization using a low concentration of ammonium sulfate and a high protein concentration (Forsythe *et al.*, 1997). We obtained HEWL crystals in an expanded range of ammonium sulfate concentrations in the presence of some of these reagents.

We evaluated the effect of amino acids and their derivatives on the commercially available screening kit Crystal Screen I, which contains 50 conditions with respect to salts, precipitants and buffers. HEWL was crystallized in Crystal Screen I with ten kinds of amino acid, one dipeptide and four types of amino-acid derivative as additives. We defined the success rate of crystallization by obtaining well faceted crystals from the 50 conditions by repeating each experiment more than three times. Fig. 2 shows the success rate of crystallization of HEWL in the presence of 0.1 *M* amino acid at pH 4.5. The positively charged amino acid LysEE exhibited the highest crystallization success rate using 25 mg ml^{-1} HEWL of all the amino acids tested. The other additives slightly increased the crystallization success rate upon the addition of a small hydrophilic amino acid (Thr), slightly hydrophobic amino acids (Ala and Pro), a negatively charged amino acid (Glu) and a dipeptide (Lys-Glu). Gly was not effective in the crystallization of HEWL, but GlyEE and GlyAd doubled the success rate. Similarly, LysEE increased the success rate of crystallization twofold compared with Lys. When we investigated the success rate of crystallization using 50 mg ml^{-1} HEWL, the results indicated a definite increase; the superior aggregation suppressors Arg, GlyEE and GlyAd performed well with the higher concentration of HEWL. The success rate of crystallization increased with increasing length of the carbon chain in the additives, as shown by the comparative data for Gly and Ala, for Ser and Thr and for Asp and Glu (Fig. 2). PheEE and Val were unfavourable for HEWL crystallization.

Fig. 3(*a*) shows representative images of HEWL crystals obtained using 0.2 *M* ammonium sulfate and 30% PEG 8000 (condition No. 30 of Crystal Screen I) in the presence or absence of amino acids as additives. Single crystals of HEWL were successfully grown in the presence of positively charged amino acids (Arg and Lys) and a negatively charged amino acid (Glu). In the absence of additives, proteins were prone to forming aggregates and no crystals were obtained as a result of the decreased protein concentration. These results indicate

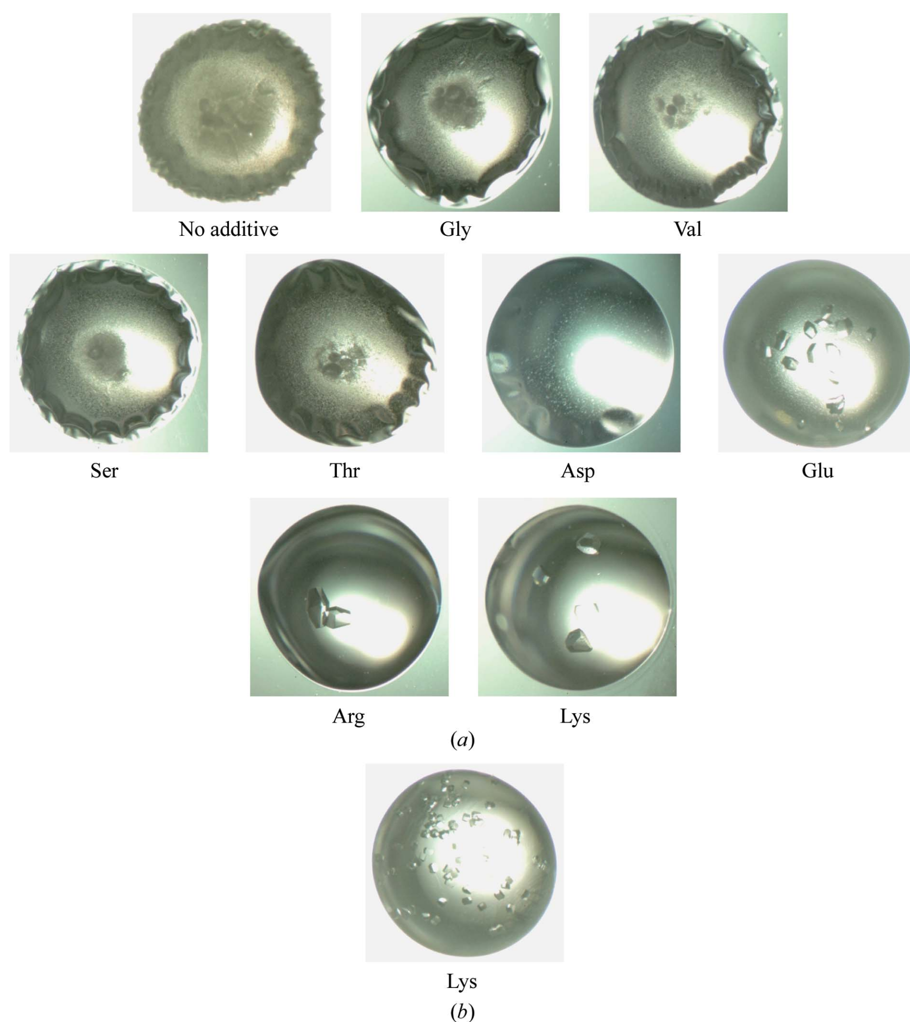


Figure 3
Representative images of HEWL crystals in the presence or absence of amino acids as additives. (*a*) The crystallization condition was 0.2 *M* ammonium sulfate, 30% PEG 8000 pH 4.5 with 0.1 *M* additives. A control crystallization was performed without additives. (*b*) The crystallization condition was 2.0 *M* ammonium sulfate, 2% PEG 400, 0.1 *M* Na HEPES pH 7.5 and 0.1 *M* Lys.

that the addition of amino acids and their derivatives is a simple and valuable method of improving HEWL crystallization. HEWL crystals were obtained using ammonium sulfate as a precipitant. It is known that HEWL aggregates when >18% ammonium sulfate is added at pH 4 (Riès-Kautt & Ducruix, 1989); however, crystals were obtained from a solution containing 2.0 *M* ammonium sulfate (corresponding to about 26.4% ammonium sulfate) in the presence of 0.1 *M* Lys (Fig. 3*b*).

Fig. 4 shows the results of the crystallization of 50 mg ml^{-1} HEWL using sodium chloride as precipitant in the presence of additives at pH 6.5. The vertical axis shows the concentration range of precipitant under which HEWL forms crystals. The experiment with no additive indicated that HEWL crystals were obtained in the concentration range 0.75–1.25 *M* sodium chloride. In 0.1 *M* Gly, similar results were obtained. In the presence of Asp and Glu, crystals were only obtained in an extremely narrow range of precipitant concentrations and at relatively low concentrations. In the presence of Arg and Pro the concentrations under which crystallization was observed expanded to a higher range whereas in the presence of Lys the concentrations expanded to a lower range compared with no additive. All crystals had similar morphology to a tetragonal crystal. At a sodium chloride concentration of 2.25 *M*, HEWL crystallized in the presence of Arg (Fig. 4), which is the most effective of the amino acids in suppressing heat-induced and refolding-induced aggregation (Shiraki *et al.*, 2002). At a high concentration of sodium chloride, HEWL could also be crystallized in the presence of amidated and esterified amino-acid derivatives. In the presence of GludiEE HEWL crystals were only obtained in an extremely narrow range of precipitant concentrations, while in the presence of PheEE HEWL crystals could not be obtained, indicating that amino-acid ethyl esters possessing high hydrophobicity decreased protein crystallization (Figs. 2, 3, 4 and 5). Comparative studies of the relationship between the hydrophobicity of the amino acid and the success rate of crystallization (Fig. 6) and protein aggregation (Fig. 5) showed that appropriate hydrophobicity is required to select an amino acid as an additive to promote protein crystallization (Figs. 5 and 6). These results suggested that the hydrophobicity of additives is one of the key factors in HEWL crystallization.

To estimate the aggregation, the turbidity of the lysozyme solution was evaluated from the optical density at 600 nm of the supersaturated solution of sodium chloride in the presence or absence of 0.2 *M* amino acids and amino-acid

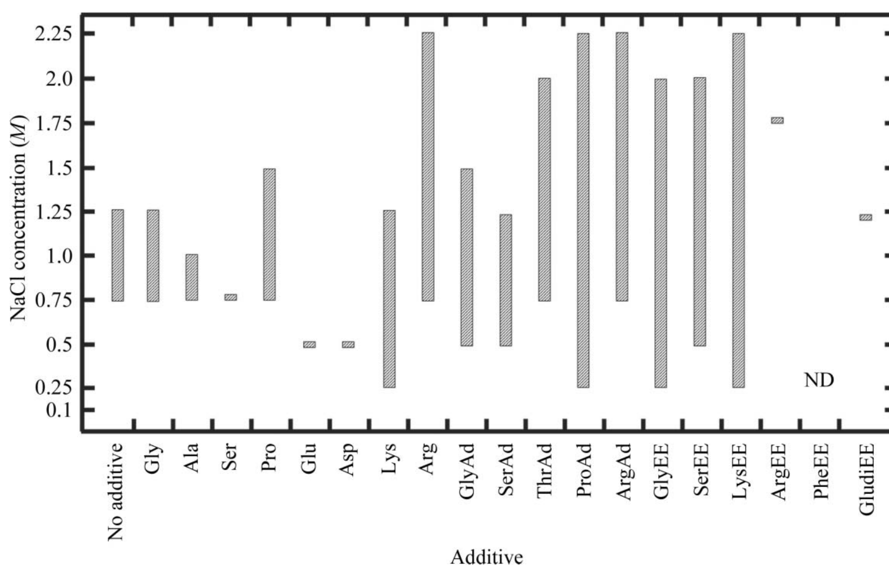
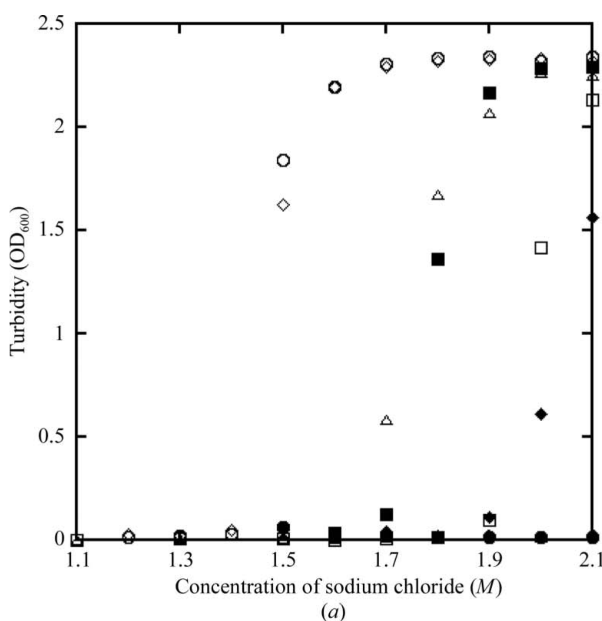


Figure 4
Concentration ranges of sodium chloride as a precipitant in the presence of 0.25 *M* additives at pH 6.5 in which crystals were observed using 50 mg ml^{-1} HEWL after 2 d.



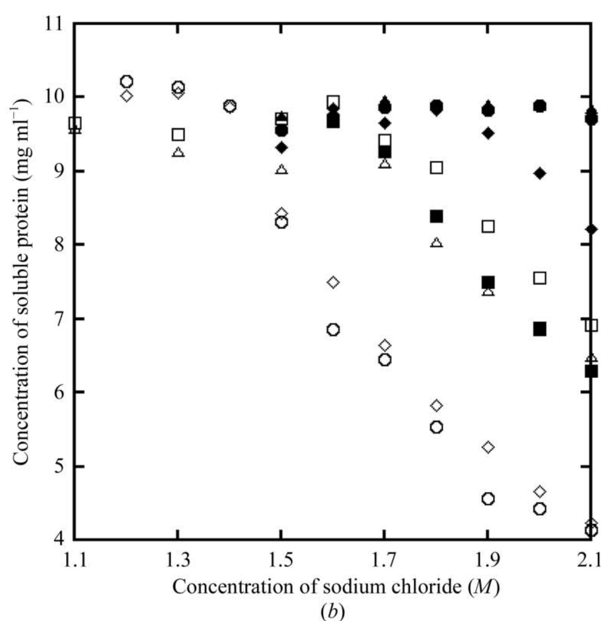


Figure 5
Aggregation of lysozyme under various sodium chloride concentrations in the presence or absence of amino acids and amino-acid derivatives at pH 6.5 and 293 K after 2 d. (*a*) Turbidity at 600 nm. (*b*) Supernatant concentration of lysozyme. No additive, open squares; Gly, open triangles; Asp, open circles; Glu, open diamonds; Lys, full squares; Arg, full triangles; GlyEE, full circles; GlyAd, full diamonds.

derivatives at pH 6.5 (Fig. 5*a*). The turbidity steeply increased with increasing concentrations of NaCl at around 1.9 *M*. In the presence of Gly the turbidity increased similarly, *i.e.* at around 1.7 *M* NaCl. In the presence of GlyAd the turbidity decreased compared with control experiments. Interestingly, in the presence of Arg and GlyEE no turbidity increase was measured above 1.9 *M*. These results show that the side chains of charged amino acids such as Arg and esterification and amidation of the carboxyl group in Gly might be very important for inhibiting the aggregation of lysozyme in supersaturated solutions. Fig. 5(*b*) shows the concentration of supernatant protein after centrifugation, measured by the absorbance at 280 nm. The data showed a similar pattern to the turbidity measurements. With increasing concentrations of NaCl, the amount of soluble protein decreased much earlier in the absence and presence of Gly than in the presence of Arg and GlyEE. These results are in agreement with the crystallization experiments (Fig. 4): aggregation suppressors such as Arg, GlyAd and GlyEE and Asp and Glu that decreased the protein solubility expanded the concentrations of NaCl under which protein crystals were obtained to higher and lower ranges compared with control experiments, respectively. In general, aggregation can be diminished by imposing high ionic strength. These reagents suppress aggregation by hydrophobic interactions between denatured proteins (Shiraki *et al.*, 2004, 2005). Although only HEWL was used for the experiments in this work, it is probable that proteins tend to crystallize with aggregation suppressors using a wide range of precipitants and protein concentrations (Fig. 4).

Fig. 6 shows the relationship between the hydrophobicity of the amino acid and the crystallization success rate of 50 mg ml^{-1} HEWL. Positively charged amino acids such as Arg and Lys, which have negative hydrophobicity values of −1.01 and −0.99, respectively, have high success rates of crystallization. The success rate of HEWL crystallization increased with increasing hydrophobicity of the additives. There was an inverse correlation between the success rate and the hydrophobicity (correlation coefficient of −0.542). These results show that favourable additives for HEWL crystallization possess low hydrophobicity, although other specific factors of the additives may also be involved.

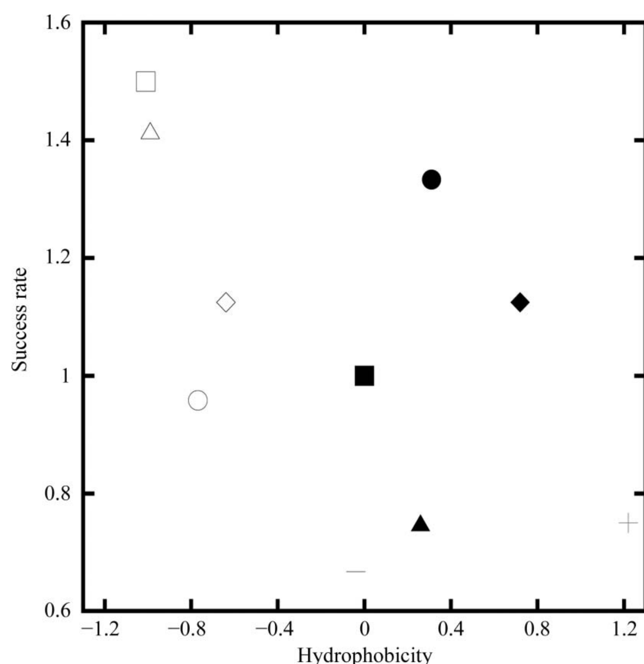


Figure 6
Comparison of the relationship between the hydrophobicity of each amino acid (Fauchere & Pliska, 1983) and the success rate of HEWL crystallization. Arg, open square; Lys, open triangle; Asp, open circle; Glu, open diamond; Ser, line; Gly, full square; Thr, full triangle; Ala, full circle; Pro, full diamond; Val, plus symbol.

4. Conclusions

Protein crystallization is generally performed in a solution containing buffer, salts and precipitant. This study investigated the effects of amino acids and their derivatives as fourth components of the crystallization solution. The additives that were known to be aggregation suppressors increased the possibility of obtaining HEWL crystals. We have reported the successful crystallization of equine haemoglobin and bovine pancreatic ribonuclease A (Ito, Kobayashi *et al.*, 2008), as well as a newly crystallized protein (Ito, Hidaka *et al.*, 2008), using fourth components. These results suggest that aggregation suppressors play a significant role in various types of protein-crystallization protocols. Many researchers have recently applied new crystallization parameters, such as temperature control (Adachi, Takano, Yoshimura *et al.*, 2003), gravity (McPherson *et al.*, 1999), magnetic fields (Sazaki *et al.*, 1997), pressure (Suzuki *et al.*, 1994), ultrasonic irradiation (Luft & DeTitta, 1999), laser irradiation (Adachi, Tanako, Hosokawa *et al.*, 2003) and solution flow (Adachi *et al.*, 2002). These methods require specialized equipment which is expensive, whereas our approach is convenient and versatile.

We thank Dr Hiroshi Komatsu, Dr Takashi Kumasaka and Dr Gen Sazaki for useful discussions. We thank Ms Satomi Uramoto, Mr Toyoaki Kobayashi, Mr Tomohisa Shibano, Mr Makoto Kanoh, Ms Tomoko Miyashita, Ms Akiko Oosuka and Mr Takayuki Sugiyama for conducting many of the crystallization experiments. This work was supported by a Sasagawa Scientific Research Grant from The Japan Science Society.

References

Adachi, H., Takano, K., Hosokawa, Y., Inoue, T., Mori, Y., Matsumura, H., Yoshimura, M., Tsunaka, Y., Morikawa, M., Kanaya, S., Masuhara, H., Kai, Y. & Sasaki, T. (2003). *Jpn J. Appl. Phys.* **42**, L798–L800.
Adachi, H., Takano, K., Yoshimura, M., Mori, Y. & Sasaki, T. (2002). *Jpn J. Appl. Phys.* **41**, L1025–L1027.
Adachi, H., Takano, K., Yoshimura, M., Mori, Y. & Sasaki, T. (2003). *Jpn J. Appl. Phys.* **42**, L384–L385.
Arakawa, T. & Tsumoto, K. (2003). *Biochem. Biophys. Res. Commun.* **304**, 148–152.
Buchner, J. & Rudolph, R. (1991). *Biotechnology*, **9**, 157–162.
Fauchere, J. L. & Pliska, V. (1983). *Eur. J. Med. Chem.* **18**, 369–375.
Forsythe, E. L., Snell, E. H. & Pusey, M. L. (1997). *Acta Cryst.* D**53**, 795–797.
Ito, L., Hidaka, Y., Okumura, M., Konishi, H. & Yamaguchi, H. (2008). *Acta Cryst.* F**64**, 531–532.
Ito, L., Kobayashi, T., Shiraki, K. & Yamaguchi, H. (2008). *J. Synchrotron Rad.* **15**, 316–318.
Jeruzalmi, D. & Steitz, T. A. (1997). *J. Mol. Biol.* **274**, 748–756.
Kadima, W., McPherson, A., Dunn, M. F. & Jurnak, F. A. (1990). *Biophys. J.* **57**, 125–132.
Larson, S. B., Day, J. S., Cudney, R. & McPherson, A. (2007). *Acta Cryst.* D**63**, 310–318.
Luft, J. R. & DeTitta, G. T. (1999). *Acta Cryst.* D**55**, 988–993.
McPherson, A. & Cudney, B. (2006). *J. Struct. Biol.* **156**, 387–406.
McPherson, A., Koszelak, S., Axelrod, H., Day, J., Williams, R., Robinson, L., McGrath, M. & Cascio, D. (1986). *J. Biol. Chem.* **261**, 1969–1975.
McPherson, A., Malkin, A. J., Kuznetsov, Y. G., Koszelak, S., Wells, M., Jenkins, G., Howard, J. & Lawson, G. (1999). *J. Cryst. Growth*, **196**, 572–586.
Mikol, V., Hirsch, E. & Giegé, R. (1990). *J. Mol. Biol.* **213**, 187–195.
Riès-Kautt, M. M. & Ducruix, A. F. (1989). *J. Biol. Chem.* **264**, 745–748.
Rudolph, R. & Lilie, H. (1996). *FASEB J.* **10**, 49–56.

Sakamoto, R., Nishikori, S. & Shiraki, K. (2004). *Biotechnol. Prog.* **20**, 1128–1133.
Sauter, C., Ng, J. D., Lorber, B., Keith, G., Brion, P., Hosseini, M. W., Lehn, J. M. & Giegé, R. (1999). *J. Cryst. Growth*, **196**, 365–376.
Sazaki, G., Yoshida, E., Komatsu, H., Nakada, T., Miyashita, S. & Watanabe, K. (1997). *J. Cryst. Growth*, **173**, 231–234.
Shiraki, K., Kudou, M., Fujiwara, S., Imanaka, T. & Takagi, M. (2002). *J. Biochem. (Tokyo)*, **132**, 591–595.
Shiraki, K., Kudou, M., Nishikori, S., Kitagawa, H., Imanaka, T. & Takagi, M. (2004). *Eur. J. Biochem.* **271**, 3242–3247.
Shiraki, K., Kudou, M., Sakamoto, R., Yanagihara, I. & Takagi, M. (2005). *Biotechnol. Prog.* **21**, 640–643.
Suzuki, Y., Miyashita, S., Komatsu, H., Sato, K. & Yagi, T. (1994). *Jpn J. Appl. Phys.* **33**, L1568–L1570.
Taneja, S. & Ahmad, F. (1994). *Biochem. J.* **303**, 147–153.
Thibault, F., Langowski, J. & Leberman, R. (1992). *J. Mol. Biol.* **225**, 185–191.