Amino Acids (2007) 33: 587–605
DOI 10.1007/s00726-007-0506-3
Printed in The Netherlands

Amino Acids

Biotechnology applications of amino acids in protein purification and formulations

Review Article

T. Arakawa[1], **K. Tsumoto**[2], **Y. Kita**[3], **B. Chang**[4], and **D. Ejima**[5]

[1] Alliance Protein Laboratories, Thousand Oaks, CA, USA
[2] Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, Japan
[3] Department of Pharmacology, KEIO University School of Medicine, Tokyo, Japan
[4] Integrity Biosolution, Camarillo, CA, USA
[5] Amino Science Laboratories, Ajinomoto Co. Inc., Kawasaki, Kanagawa, Japan

Received November 30, 2006
Accepted January 4, 2007
Published online March 16, 2007;

Summary. Amino acids are widely used in biotechnology applications. Since amino acids are natural compounds, they can be safely used in pharmaceutical applications, e.g., as a solvent additive for protein purification and as an excipient for protein formulations. At high concentrations, certain amino acids are found to raise intra-cellular osmotic pressure and adjust to the high salt concentrations of the surrounding medium. They are called "compatible solutes", since they do not affect macromolecular function. Not only are they needed to increase the osmotic pressure, they are known to increase the stability of the proteins. Sucrose, glycerol and certain amino acids were used to enhance the stability of unstable proteins after isolation from natural environments. The mechanism of the action of these protein-stabilizing amino acids is relatively well understood. On the contrary, arginine was accidentally discovered as a useful reagent for assisting in the refolding of recombinant proteins. This effect of arginine was ascribed to its ability to suppress aggregation of the proteins during refolding, thereby increasing refolding efficiency. By the same mechanism, arginine now finds much wider applications than previously anticipated in the research and development of proteins, in particular in pharmaceutical applications. For example, arginine solubilizes proteins from loose inclusion bodies, resulting in efficient production of active proteins. Arginine suppresses protein–protein interactions in solution and also non-specific adsorption to gel permeation chromatography columns. Arginine facilitates elution of bound proteins from various column resins, including Protein-A or dye affinity columns and hydrophobic interaction columns. This review covers various biotechnology applications of amino acids, in particular arginine.

Keywords: Protein purification – Osmolyte – Formulation – Lyophilization – Aggregation suppression

1. Introduction

In living systems, proteins are present in aqueous solutions and are surrounded by various solutes, including macromolecules exceeding 100 mg/ml (e.g., 300–400 mg/ml in *Escherichia coli*) (Zimmerman and Trach, 1991), which by itself stabilizes the macromolecules via excluded volume effects (Wilf and Minton, 1981; Hall et al., 1995; Sasahara et al., 2003; Minton, 2005). The native and functional states of the proteins are stabilized by various other factors, in addition to the excluded volume effects, present in the natural environment. Alternatively, some proteins are made intrinsically unstable and hence are subjected to a degradation pathway (Jubete et al., 1996). In either case, most proteins, when isolated, are often only marginally stable in aqueous solutions (Aune and Tanford, 1969; Brants and Lumry, 1969; Privalov and Khechinashvili, 1974; Pace, 1990) and hence the isolated proteins must be stabilized for characterization and research or for commercial use. Traditionally, sucrose, glycerol and certain amino acids were added at high concentrations to enhance protein stability for biochemical analysis (von Hippel and Wong, 1962, 1965; Back et al., 1979; Gerlsma, 1968, 1970; Arakawa and Timasheff, 1983, 1984, 1985; Taneja and Ahmad, 1994). Therapeutic proteins require more stringent conditions to ensure long-term storage stability (Patro et al., 2002; Wang, 2005). Solvent additives, called excipients, play a major role in stabilizing therapeutic proteins. Proteins are also major targets of pharmaceutical drugs. For example, growth factor receptors, G-protein coupled receptors, nuclear receptors,

and various proteases and kinases are among the major protein targets for therapeutic drugs (Klebe, 2000; Citron, 2002; Bonci and Carezon, 2005). Pharmaceutical drugs or therapeutic antibodies are developed against these target proteins and intervene in their functions. These proteins must be stable in the native, functional state so that such drug development research can be performed.

These proteins, whether therapeutic proteins or targets of pharmaceutical drugs, must be purified, formulated and stored for evaluation, development or marketing. Solvent conditions play a major role in the purification, processing and storage of the proteins. Amino acids are often used in these applications, based on the following two observations:

a) Certain amino acids, including glycine, alanine, proline and other amino acids, are called ''osmolytes'' or ''compatible solutes'', due to the fact that these low molecular weight solutes accumulate in the cells or organisms to raise the osmotic pressure, when they are exposed to high environmental salt concentration (Yancey et al., 1982; Somero, 1986). Selection of these osmolytes over other small molecules is due to their compatibility with the macromolecular structure and function in the cells, and they are thereby called ''compatible solutes'' (Yancey et al., 1982; Somero, 1986). The osmolytes or compatible solutes do not normally interfere with enzyme activities or functional structures of the proteins (Yancey et al., 1982). In addition, not only are they compatible with macromolecular function, they also enhance the stability of macromolecules, in particular proteins (Arakawa and Timasheff, 1983, 1984, 1985). Thus, amino acids can be used to stabilize proteins without affecting their function.
b) The second important observation is that certain small molecules are effective in maintaining the native structure in the dried state. Many organisms survive dehydration, a phenomenon known as anhydrobiosis (Crowe et al., 1992, 1998; Rapoport et al., 1997). These organisms accumulate trehalose, a disaccharide, inside the cells. Understanding the mechanism of the protection of these organisms and cells by trehalose against dehydration has led to the understanding that certain additives are needed to protect proteins against freezing and freeze-drying and to increase the storage stability in the dried state. Amino acids as well as sugars provide a protecting role in the stabilization of proteins during freezing and freeze-drying and in the dried state (Carpenter and Crowe, 1988; Arakawa et al., 1990; Carpenter et al., 1987a, b, 1990, 1993, 1998; Prestrelski et al., 1993a, b; Pyne et al., 2003; Bonacci et al., 2000; Tang and Pikal, 2005; Chang et al., 2005). This review focuses on various biotechnology applications of amino acids to enhance the protein stability in solution and in the dried state.

2. Some observations on amino acids

Amino acids are used for various applications, and these are described in the next section. Amino acids as well as other solutes, which function in vivo and in vitro at high concentrations, are often classified as kosmotropes and chaotropes (Galinski et al., 1997; Moelbert et al., 2002). They have opposite effects on proteins and on water structure, as summarized in Table 1. In this section, the general principle of how small molecule additives, such as amino acids, exert their effects on proteins is described.

2.1 Lessons from nature

As described above, when dealing with proteins in solution or in a dried state, the conditions under which they are produced and stored require manipulation. Lessons from nature show that additives can stablilize the proteins against various stresses, such as high temperature or low water content, which are not encountered under normal conditions. Water stress arises from high salinity, desiccation or freezing of the surrounding environments of the cells or organisms. Surviving water stress can be achieved by amassing highly water-soluble solutes (Csonka and Hanson, 1991; Lucht and Bremer, 1994). In their pioneering work, Yancey et al. (1982) examined the nature of the intracellular low molecular solutes utilized by these organisms living under high ambient salinity or of those cells exposed to high osmolality of the extracellular body fluids. Regardless of the source of high osmolality, in order to raise the osmolality, those cells accumulate in the cytoplasm a few selected classes of low molecular weight metabolic products: polyhydric alcohols (polyols and sugars), amino acids and amino acid derivatives, and methylamines (Yancey et al., 1982; Milner et al., 1987).

Table 1. Classification of solvent additives

	Kosmotropes	Chaotropes
Protein stability	Stabilizer	Destabilizer (or denaturant)
Protein solubility	Salting-out	Salting-in
Water structure	Maker	Breaker
Surface tension	Increase	Decrease

In addition, only a few selected amino acids, amines and amino acid derivatives are utilized, e.g. glutamic acid, proline, glycine, alanine, betaine and trimethylamin N-oxide (TMAO). What is common in these selected solutes? They share a common ability to provide environments of high osmolality, yet are ''compatible'' for macromolecular structure and function (Brown and Simpson, 1972; Brown, 1976). Detailed NMR analysis of protein structure in the presence of two osmolytes, glycine and diglycerol phosphate, showed no effects on the static and dynamic structure of the proteins (Lamosa et al., 2003; Foord and Leatherbarrow, 1998). It is noted that arginine, one of the commonly used amino acids for protein refolding, is not classified as an osmolyte and does interfere with enzymatic function (Yancey et al., 1982; Ishibashi et al., 2005).

Water stress also comes from freezing and dehydration. Organisms which undergo anhydrobiosis (''life without water'') are capable of surviving desiccation, during which they become dormant. Not only can they be dehydrated, they are also highly resistant to environmental stresses, such as high temperature and pressure, in the dried state. Desiccation is thus a consequence of and a survival strategy for the environmental changes (Clegg, 2001; Crowe et al., 1992). Nonreducing sugars, predominantly the disaccharide trehalose (in animals) and sucrose (in plants), are utilized by anhydrobiotic cells. The removal of intracellular water causes drastic changes in inter- and intra-molecular interactions, leading to the loss of hydrogen bonding with water for intracellular proteins and membranes, which is compensated for by hydrogen bonding with other molecules. This results in forced intermolecular interactions between molecules that normally would not interact with each other in the presence of bulk water. In proteins, the loss of hydrogen bonding with water can be compensated for by protein–protein interactions, and may thus lead to protein aggregation and inactivation (Carpenter and Crowe, 1989; Prestrelski et al., 1993a, b; Dong et al., 1995; Carpenter et al., 1987a, b). In membranes, water loss from the phospholipids headgroups leads to transition in phases from the biologically active fluid phase to the gel phase (Crowe et al., 1992), or cell fusions. One of the survival strategies that desiccation-tolerant organisms seem to have in common is the accumulation of high concentrations of small carbohydrates, in particular trehalose (Crowe et al., 1992; Leslie et al., 1994; Eroglu et al., 2000; Guo et al., 2000; Tsvetkova et al., 1998). The role of sugars in desiccation is two-fold. First, sugars play a role in the protection of cellular and macromolecular structure by replacing the water for hydrogen bonding. Second, sugars are involved in the production of the glassy matrix which protects the cells and proteins. Protection of proteins by trehalose against freezing was shown to be replaced by many other protein stabilizers that show preferential hydration in aqueous solution. Protection of proteins by trehalose against dehydration was interpreted in terms of water replacement, as described above. Trehalose also has a high glass transition temperature and hence provides an amorphous glassy environment for dried proteins, which limits the mobility of the molecules and slows any chemical reactions. Hydrogen bonding and high glass transition temperature can be achieved by other disaccharides.

The lesson learned from ''anhydrobiosis'' is that the addition of small molecules can stabilize proteins against freezing and dehydration stresses and against stresses that may be encountered in the dried state. This is exactly the case for lyophilization of proteins, in which successful freeze-drying requires the presence of protein stabilizers, such as sucrose and trehalose. Once the proteins are successfully dried, then these stabilizers protect the proteins from various stresses during storage in the dried state.

2.2 How do amino acids stabilize proteins?

The stabilization of proteins by the additives, such as amino acids, is manifested in the increased melting temperature of the proteins. An example is given in Table 2, in which the change in the melting temperature by the addition of several amino acids is shown. As shown in Table 2, Na glutamate and other amino acids at neutral pH stabilize proteins, here bovine serum albumin (BSA) and lysozyme, against thermal stresses (Arakawa and Timasheff, 1984; Diamant et al., 2003). Na glutamate stabilizes tubulin at a level of 0.1–0.2 M (Wilson, 1970). Similarly, the stability of maize leaf phosphoenolpyruvate

Table 2. Melting temperature of proteins in aqueous amino acid solutions[a]

Protein and condition	Increase in melting temperature °C
Lysozyme	
1 M Na glutamate	9
1 M Lysine HCl	6
0.9 M Proline	4
0.9 M Serine	7
0.9 M Alanine	4
0.9 M Glycine	5
BSA	
1 M Glycine	13

[a] Data are taken from Arakawa and Timasheff (1983, 1984, 1985)

carboxylase and pig heart mitochondrial malate dehydrogenase can be increased by Na glutamate, aspartate and glycinate (Jensen et al., 1996, 1997). In addition to protein stabilization, Na glutamate, in the concentration range of 1 M, enhances self-association of tubulin (Hamel and Lin, 1981a, b; Hamel et al., 1982). Preferential hydration was proposed as the mechanism of stabilization and increased self-association by Na glutamate, as described below (Arakawa and Timasheff, 1984).

How, then, do the small molecule additives work in stabilizing proteins in solution and in the dried state? First, we consider the solution state. Proteins are marginally stable in aqueous solutions and readily lose their structure and functional activity upon isolation from natural environments, as described above. Such structural and functional loss of the proteins can occur via both thermodynamic (reversible) and kinetic (irreversible) mechanisms. The thermodynamic stability of the proteins is determined by the differences in the free energy of the native (functional) and unfolded (denatured, non-functional) states, as schematically shown in Fig. 1 (middle panel), where ΔG_u is the free energy of unfolding. The greater the quantity of the free energy, more stable the native protein is. There are two mechanisms to increase the free energy of unfolding. One is to decrease the free energy of the native state as shown in Fig. 1A. In this case any ligands, which preferentially bind to the native state, should decrease the free energy of the native state, thereby increasing the value of ΔG_u. Such ligands are specific to the native protein and include, for example, the substrates, inhibitors or co-factors of the enzymes, metals for metal-binding proteins and binding partners of cell signaling proteins (Zhang et al., 1995b). However, these are protein-specific, meaning that a factor stabilizing a particular protein may not stabilize other proteins and may even destabilize some proteins. The second mechanism is to increase the free energy of the protein, more so for the unfolded state (Fig. 1B).

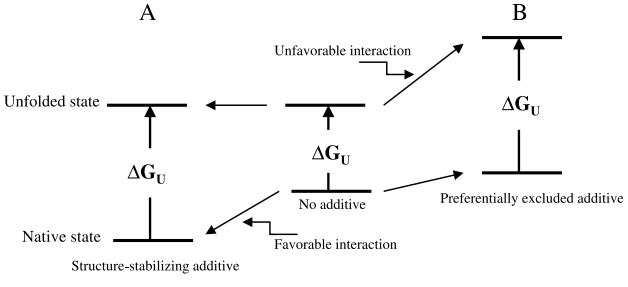

Fig. 1. Free energy diagram of different states of proteins in the absence and presence of the structure-stabilizing additives

There is great interest in understanding why a variety of small molecules, such as polyols, sugars and amino acids, stabilize the proteins, since they were commonly used to stabilize proteins during purification and for long-term storage. There are three important contributions to understanding the mechanism of the effect of additives on protein stability: preferential interaction of solvent components with the proteins; the effects of additives on water structure e.g. surface tension of water; and the solubility of amino acids and peptides in aqueous solutions of amino additives. Preferential interaction measurements, which are based on equilibrium dialysis, showed that these stabilizing additives (solutes) do not bind to the native proteins. Binding of these is actually negative; that is, there is a deficiency of these additives in the vicinity of the protein molecules (Arakawa and Timasheff, 1983, 1984, 1985). Such a situation is schematically shown in Fig. 2 (as depicted by the arrow for solute exclusion). Regardless of the cause of the apparent repulsion of the stabilizing solutes (preferentially excluded additives) from the protein surface, the thermodynamic consequence of solute deficiency (exclusion) around the protein surface is the increase in the free energy of the proteins (Fig. 1B). Thus, the addition of the stabilizing solutes increases the free energy of the native state protein. Since the surface area increases upon unfolding (Fig. 2B), solute repulsion and negative binding will be greater for the unfolded state. This corresponds to an even greater increase in the free energy of the unfolded state, as shown in Fig. 1B. As a consequence, the free energy of the unfolded state increases in the presence of the stabilizing solutes and hence the native structure is stabilized (Fig. 1B and Fig. 2B). A major difference between the first (ligand binding) and second (solute exclusion) mechanism is that the preferential interactions of the stabilizing solutes are protein-

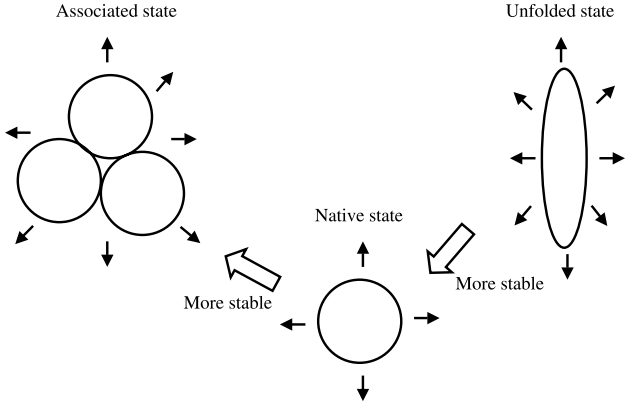

Fig. 2. Schematic illustration of solute exclusion

independent. Thus, these solutes do not bind to the surface of any proteins that is, they are universally effective as a protein stabilizer.

Such unfavorable free energy also leads to enhanced self-association or protein–protein interaction, as has been observed with Na glutamate. This is illustrated in Fig. 2A. In a similar way to the unfolding reaction, protein–protein interaction results in decreased surface area per molecule and hence reduces the unfavorable interaction of free energy by the additives, with a consequence of enhanced protein association.

One property shared by these additives (polyols and sugars) is the ability to increase the surface tension of aqueous solutions. Glycine, alanine and other amino acids also increase the surface tension of water (Pappenheimer et al., 1936; Kita et al., 1994). Gibbs binding isotherm (Gibbs, 1878; Arakawa and Timasheff, 1983) shows that those solutes which increase the surface tension of water should be deficient in the interface between water and air. Extending this relation to the protein surface, amino acids such as glycine, alanine, aspartic acid and glutamic acid are expected to be deficient in the vicinity of the protein surface. Such a relation between surface tension and solute exclusion was formalized as a cavity theory, whereby the ability of the surface free energy of the formation of a cavity to accommodate solute molecules, such as proteins and nucleic acids, in a solvent plays an important role in the stabilization and self-association of macromolecules (Sinanoglu and Abdulnur, 1964, 1965). The cavity theory was expanded to include the contribution of surface tension to protein solubility and hydrophobic interaction chromatography, or a measure of hydrophobicity scale (Melander and Horvath, 1977; Nicholls et al., 1991; Sharp et al., 1991). Preferential interaction measurements of these amino acids showed that they are excluded from the protein surface, similar to the observation made for polyols and sugars (Arakawa and Timasheff, 1983, 1984, 1985). As expected from the negative interactions, these amino acids do stabilize proteins, regardless of the chemical and physical properties (Table 2). Arginine is different from other amino acids, as described in the next section.

Surface tension is a reflection of the effects of the additives on the water structure and hence does not correlate with the effects of the additives when they show affinity for the protein surface. Preferential interaction measurements reflect both the effects of the additives on the water structure and the affinity of them for the protein surface. However, neither measurement gives us insight into the molecular details of the additive interaction with the amino acid residues on the protein surface. The effects of the additives on the solubility of 20 amino acids and model peptides show how the additives interact with the side chains and peptide group. Unfortunately, except for arginine (K. Tsumoto, unpublished results), no solubility measurements of the 20 amino acids are available in aqueous solutions of amino acids. Although the mechanism of stabilization by small molecules has been described above for proteins in solution, it has been shown that the principle that can be used for the protein solution can be applied to the proteins in a frozen state (Carpenter and Crowe, 1988; Carpenter et al., 1990). As is evident, the ability of small molecules to exert their stabilizing effects depends on the presence of water, and water still remains both during and after the freezing process. There is always non-freezable water around the protein surface, remaining bound to the proteins (Kuntz et al., 1969). The same principle no longer applies to the proteins in the dried state, due to the removal of water.

The presence of small molecules is essential for proteins during the freeze-drying process and in the dried state. Compared with many small molecule additives that work in solution or against freezing, a limited number of small molecules are effective in the dried state. How do they accomplish such stabilization? One mechanism is water replacement. Proteins bind many water molecules, which are removed during the drying process, and hence those residues involved in water binding undergo inter- or intra-molecular hydrogen-bonding with other residues, which result in unfolding or aggregation. Sugars can replace water for hydrogen bonding, resulting in hydrogen bonding between sugars and the protein surface. In the dried state, the rate of the chemical reaction and unfolding is determined by the mobility of the protein and the surrounding molecules. Such mobility is greatly reduced in the glassy state of the dried state, which can be more stable in the presence of sugars.

In addition to thermodynamic stabilization, the solutes may affect the rate of conformational changes or aggregation of the proteins. Namely, the intrinsically (or natively unstable) proteins may be kinetically stabilized in the natural environments and, upon isolation, undergo conformational changes or aggregation, as schematically depicted in Fig. 3. In this mechanism, binding of ligands (L) to the native protein (N) kinetically traps them to the more stable state (N^*), or raises the activation energy. Only when the bound ligand dissociates from the protein does it assume a functional structure, but only in a transient manner. Alternatively, it must dissociate before undergoing conformational changes. Upon isolation, the natural

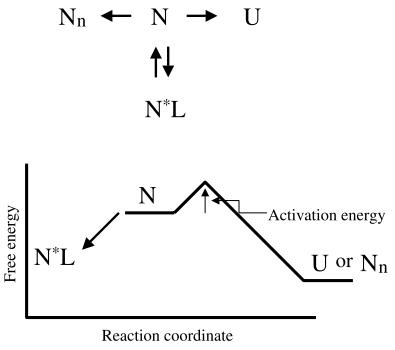

Fig. 3. Free energy diagram of kinetic stabilization of proteins

ligand will be lost and hence the protein has to be kinetically stabilized.

2.3 Unique properties of arginine

Arginine differs from other amino acids, although the reason for the difference is not well understood. Arginine does not stabilize proteins, in contrast to other structure-stabilizing amino acids. Arginine shows a number of interesting properties in its effects on proteins, as summarized below.

Arginine is not a protein-denaturant

Arginine, like other amino acids, does not affect the native structure of the proteins (Arakawa et al., 2006a). However, it does not stabilize them either, which is different from certain amino acids (Arakawa and Tsumoto, 2003; Shiraki et al., 2002). Thus, arginine barely affects the melting temperatures of the proteins: Table 3 summarizes the effects of arginine on the melting temperature of ribonuclease and lysozyme. The melting temperatures of these proteins change little below 0.5 M arginine and slightly decrease above 0.5 M. Thus, arginine is not a protein-stabilizer.

Table 3. Melting temperature of proteins in aqueous arginine solutions

Arginine concentration, M	Thermal transition temperature, °C	
	Ribonuclease	Lysozyme[a]
0	63	71
0.1	63	71
0.5	62	70
1.0	60	70
1.5	59	–
2.0	60	71

[a] The onset temperature of melting is shown

Table 4. Aggregation temperature of bovine serum albumin

Arginine concentration, M	Temperature at the onset of turbidity occurrence, °C
0	67
0.1	73
0.5	75
1.0	78
2.0	83

Arginine suppresses aggregation against heat-induced unfolding

Both ribonuclease and lysozyme show little change in melting temperature as affected by arginine, as summarized in Table 3 (Arakawa and Tsumoto, 2003). Aggregation of the thermally unfolded state is greatly reduced and the reversibility of the melting is increased in the presence of arginine. Thermal unfolding of BSA is irreversible even in the presence of arginine (Arakawa et al., 2003). BSA aggregates at around 67 °C in the absence of arginine (see Table 4). This temperature gradually increases with increasing arginine concentration, reaching 83 °C at 2 M arginine. This may be explained by the suppression of aggregation of unfolded BSA by arginine, rendering unfolded protein more soluble. Even in the presence of 2 M arginine, however, BSA does aggregate at about 83 °C.

Lysozyme was heat-denatured in the absence and presence of arginine at neutral pH, where lysozyme normally aggregates and precipitates. Arginine, as well as guanidine hydrochloride (GdnHCl), increased the soluble fraction concentration-dependently, while protein-stabilizing additives, such as TMAO, betaine and NaCl, were ineffective (Kudou et al., 2003). Arginine at 0.2–0.5 M also reduced the aggregation of reduced-carboxymethylated unfolded lysozyme when diluted from an 8 M urea solution (Shiraki et al., 2002).

α-Crystallin suppresses heat-induced aggregation of proteins (Horwitz, 1992). The addition of arginine at 50–200 mM enhances the ability of α-crystallin to suppress the aggregation of insulin (Horwitz, 1992; Srinivas et al., 2003). Arginine has been shown to be effective in suppressing the aggregation of proteins during refolding. This is reflected in its ability to increase the second virial coefficient of proteins (Ho et al., 2003). During lysozyme refolding, 0.5 M arginine suppressed its aggregation and enhanced the refolding efficiency. A loop domain of tight junction protein, claudin-5, shows dimerization at the μM protein concentration range, which is suppressed by arginine even in mM concentrations. The addition of 0.4 M

arginine fully suppressed the dimerization (Blasig et al., 2006).

A short arginine-rich peptide efficiently transports proteins across cell membranes

A variety of arginine-rich (but not lysine-rich) peptides mediate efficient translocation of proteins through biological membranes (Futaki, 2002). Even β-amino acids carrying the guanidinium group work for this purpose (Umezawa et al., 2002), indicating the importance of the guanidinium group for membrane interaction. The guanidinium group can form hydrogen-bonding with the phosphate backbone of RNA (Calnan et al., 1991).

Interaction with amino acid side chains and peptide bond

The guanidinium group of arginine binds the π-electron cloud of aromatic compounds (Pellequer et al., 2000). Solubility measurements of 20 amino acids and model peptides showed that a majority of amino acid side chains, in particular aromatic side chains, favorably interact with arginine (Tsumoto, K., unpublished results), a similar observation to that of their interactions with urea and GdnHCl (Nozaki and Tanford, 1970; Lee and Timasheff, 1974; Prakash et al., 1981).

Interaction with protein surface

The preferential interaction measurements showed that the interaction of arginine with proteins is qualitatively different from the interactions with other structure-stabilizing amino acids (Kita et al., 1994; Lin and Timasheff, 1996). The structure-stabilizing amino acids are preferentially excluded from the protein surface as described above, meaning that they do not favorably interact with the amino acid side chains or peptide groups on the protein surface. On the contrary, though, arginine does bind to the proteins. However, the interaction of arginine with the proteins was also greatly different from that of urea and GdnHCl (Prakash et al., 1981; Lee and Timasheff, 1974; Timasheff and Xie, 2003). There was a limited degree of arginine binding to the native protein surface (Kita et al., 1994; Lin and Timasheff, 1996).

As described above, there is insufficient information on arginine to elucidate its unique properties. The existing data on both surface tension and preferential interaction cannot provide a definite answer to the questions concerning why arginine is unique. More detailed analysis of preferential interactions of arginine with proteins and of the effects of arginine on amino acid solubility should shed light on this question. The affinity of arginine for aromatic amino acids is at least one factor contributing to the observed unique effects of arginine on proteins.

3. Biotechnology applications

3.1 Recombinant protein production

Recombinant production of protein reagents and pharmaceuticals is a major step forward in the development of biotechnology products. The production of large quantities of proteins became possible with the advent of recombinant DNA technologies (Itakura et al., 1977). When *Escherichia coli* (*E. coli*) is used for protein expression, two different approaches are taken; i.e., soluble expression and expression of proteins as inclusion bodies. Soluble expression is not always possible due to insufficient folding machinery in *E. coli*. Osmolytes are often used to increase the expression of soluble folded proteins. Glycerol enhances the expression of the ATP-binding cassette transport proteins (Higgins, 1992; Figler et al., 2000). Betaine is also frequently used to assist folding and expression of soluble proteins when the recombinant *E. coli* cells were subjected to osmotic stress (Barth et al., 2000; Bourot et al., 2000), thereby increasing soluble expression. Low physiological levels of proline, glycerol and glycine betaine induce protein chaperones and suppress aggregation of the intracellar proteins, which also help increase the expression of soluble, folded proteins (Diamant et al., 2001). Glycine betaine exogenously supplied induced accumulation of intra-cellular glycine betaine, which in turn assisted a conversion of the inactive enzyme to an active conformation (Bourot et al., 2000).

Expression of foreign proteins can be directed to the periplasmic space through the cytoplasmic membrane export machinery, leading to the correctly folded proteins (Michaelis and Beckwith, 1982; Pugsley, 1993; Wickner et al., 1991). Barth et al. (2000) showed that the periplasmic expression of recombinant immunotoxins is enhanced in the presence of 10 mM glycine betaine. Although the periplasmic expression is designed for correct folding, there are a number of exceptions, resulting in the accumulation of incorrectly folded aggregated structures in the periplasmic space of *E. coli* cells. Schaffner et al. (2001) showed that the addition of arginine up to 0.4 M increased the periplasmic expression of recombinant proteins. Although the mechanism of the increase was not clear, it is likely that arginine penetrates the peptideglycan outer layer of *E. coli* cells and prevents the secreted proteins from aggregating.

During cell culture of CHO cells expressing recombinant erythropoietin, the following nine amino acids cysteine, isoleucine, leucine, tryptophan, valine, asparagine, aspartic acid, glutamine and glutamic acids were observed to be depleted after 5 days of cell culture. The higher initial concentration of these amino acids resulted in a modest but significant increase in the protein production, but with a disproportionate increase in the lower sialylated forms of the protein (Crowell et al., 2007). This shows that manipulation of culture media nutrients, such as amino acids, can boost expression of recombinant proteins in mammalian cells, although it may alter the glycosylation.

Heterologous expression of foreign proteins in *E. coli* leads to the frequent formation of insoluble proteins into a form of refractile body called inclusion bodies (reviewed in Singh and Panda, 2005). No matter what causes inclusion body (IB) formation, the protein in IBs must be solubilized for any applications. To gain functional proteins, the solubilized protein must be refolded into a stable, active conformation, which is not a trivial process and requires a case-by-case approach. It is also possible that proteins expressed as a soluble form in *E. coli* are misfolded and need to be disaggregated and refolded.

Refolding of proteins is initiated by decreasing the concentration of solubilizing agents, such as urea, guanidine hydrochloride or strong detergents. There have been many attempts to increase the refolding efficiency using folding enhancers or aggregation suppressors, including sugars, amino acids, surfactants and polymers (Cleland and Wang, 1990; Cleland, 1993). Arginine at neutral pH has been used as a solvent additive to facilitate the refolding process (Buchner and Rudolph, 1991; Rudolph et al., 1992; Rinas et al., 1990; Ahn et al., 1997; Arora and Khanna, 1996; Buchner et al., 1992). However, most of the studies do not compare refolding yield in the absence and presence of this reagent. A 10-fold increase in the yield of gamma interferon was observed with 0.5 M arginine (Arora and Khanna, 1992). Rudolph et al. (1992) proposed that arginine is chaotropic and hence destabilizes incorrectly folded structures, allowing the misfolded molecules to proceed down the pathway to correct folding. Substantial evidence exists to show that arginine suppresses aggregation of misfolded or partially folded structures by weakly binding to these structures, as described above.

While among 20 amino acids arginine is the most frequently used amino acid for assisting protein refolding, proline also assists refolding of proteins by suppressing aggregation above 1.5 M (Samuel et al., 2000). The observed suppression of aggregation was attributed to the formation of a higher order structure of proline at high concentrations, which presents detergent-like properties.

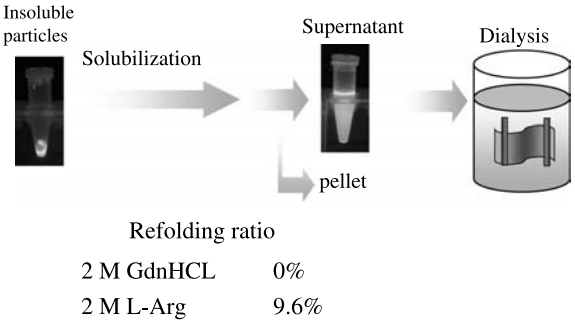

Fig. 4. Solubilization of GFP IBs by arginine

IBs are generally solubilized by denaturing solvents, as described above. Alternatively, certain types of IBs can be solubilized by arginine, which makes the solubilized proteins native or native-like. This approach takes advantage of the native-like secondary structure of the IBs (Speed et al., 1996; Fink, 1998; Umetsu et al., 2004). When IBs consist more of the native-like proteins, they often appear as flocculate-like, loose particulates and are difficult to centrifuge. These IBs can be solubilized by 0.5–2 M arginine, which results in the native-like structure of the solubilized proteins. This approach has been successfully used to obtain the native structure of green fluorescent protein (Tsumoto et al., 2003) and β2-microglobulin (Umetsu et al., 2005). Since arginine does not denature proteins, the native proteins are already present in the aqueous arginine solution used for solubilization. This was readily demonstrated using GFP, as shown in Fig. 4, in which the solubilized GFP exhibited green fluorescence, an indication of the folded protein structure. If arginine is unwanted in the final protein solutions, it can simply be dialyzed or buffer-exchanged out. In fact, this resulted in recovery of 9.6% active GFP of the total expressed GFP, as shown in Fig. 4. When the solubilization was done in 2 M GdnHCl, there was no recovery of the active GFP, indicating that 2 M GdnHCl at least partially unfolded the GFP and simple dialysis is not adequate for restoring the fully native structure.

A systematic screening approach of protein refolding has been developed, which combines different parameters affecting the refolding efficiency of proteins from IBs. In such an approach, arginine at 0.5–1 M appears to always have a positive impact on protein refolding (Armstrong et al., 1999; Lindwall et al., 2000). The solubilization power of arginine is also demonstrated using wheat flour gluten, as shown in Fig. 5. Gluten is essentially insoluble even in the presence of GdnHCl or arginine. However,

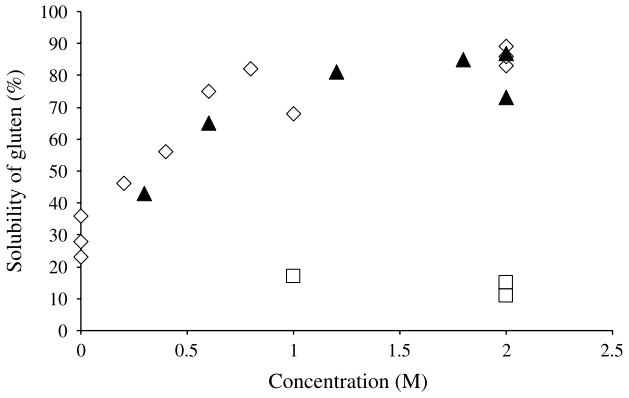

Fig. 5. Solubilization of gluten proteins by arginine. The solubility in 6 M GdnHCl was taken as 100% (◇ in arginine, 20 mM phosphate, pH 7.0; ▲ in GdnHCl; □ NaCl). At both 1 and 2 mg/ml gluten, 6 M GdnHCl gave an apparent transparent solution

these reagents can disperse the protein so that low-speed centrifugation is insufficient to sediment the suspended proteins. Figure 5 shows the dispersed protein as a percentage of the supernatant protein in 6 M GdnHCl. Arginine and GdnHCl are parallel in their ability to disperse the protein as a function of concentration. Conversely, NaCl decreased the amount of the protein dispersed.

3.2 Protein liquid formulation

Proteins for pharmaceutical applications must be stable over the course of ∼2 years or longer against various stresses encountered during storage, shipping and handling. Various additives (called excipients for formulation work) are used to enhance stability and reduce aggregation of the proteins against these stresses. Ciliary neurotrophic factor is an unstable protein and susceptible to both temperature and agitation stresses. While amino acids such as arginine and lysine are effective against heat stress, detergents and organic solvents are effective against agitation (Arakawa et al., 2003a). Combinations of both additives resulted in the formulation of a protein suitable for long-term storage.

Keratinocyte growth factor (KGF), one of the tissue growth factors with marketing approval, has a great tendency to aggregate. The aggregation of KGF against heat stress and during storage at pH 7.0 can be inhibited by histidine, glycine, aspartate, glutamate and lysine (Zhang et al., 1995a) or by aspartate and glutamate during 37 °C storage (Chen et al., 1994). Glycine and proline above 0.2 M increase the melting temperature of KGF, but only glycine is effective against 37 °C storage stability (Chen and Arakawa, 1996).

During storage, proteins are subjected to chemical modification as well as structural damage. Among chemical changes, oxidation is one of the common modifications of therapeutic proteins. Methionine is often added as an anti-oxidant; e.g. methionine and histidine are used as an anti-oxidant, through, for the case of histidine, formation of Cu-histidine complex (Kanazawa et al., 1996).

β-Lactoglobulin (β-LG) aggregates at the pH of its isoelectric point due to the large dipole moment of the protein. Glycine greatly increases the solubility of β-LG by binding to the protein, neutralizing the dipole moment (Arakawa and Timasheff, 1987). Since glycine is normally excluded from the proteins and enhances protein–protein interactions (Arakawa and Timasheff, 1983), the observed binding is a special case of glycine effects, but may be applicable to those proteins which have low solubilities in water at isoelectric pH.

In protein formulations, amino acids are used to increase solubility, to reduce aggregation or as a tonicity modifier or a bulking agent (Chang and Hershenson, 2002). Histidine stabilized a monoclonal antibody against heat stress, resulting in less aggregation in solution as well as in the dried state (Chen et al., 2003).

Frozen formulations are used as an alternative to liquid or freeze-dried formulation. Freezing can cause damage to the proteins (Koseki et al., 1990). Chimeric L6 antibody is sensitive to freeze-thaw cycles and undergoes dimerization, that can be reversed at elevated temperatures (Paborji et al., 1994). At 5%, several amino acids, i.e., glycine, lysine, arginine and alanine, reduced the reversible dimerization conferred by freeze-thaw cycles.

Aggregation suppression is fundamental to formulation development of pharmaceutical proteins and versatile protein reagents for drug screening. Recently, a combination of 50 mM arginine and 50 mM glutamic acid resulted in increased solubility and stability of several intra-cellular proteins (Golovanov et al., 2004) which would be potential candidates for small molecule or antibody-based drug development. The authors attributed these effects of the combination to ionic binding to the charges on the proteins.

3.3 Lyophilization formulations

Freeze-drying, or lyophilization, is a critical technology of therapeutic proteins (Pikal, 1990a, b). During lyophilization, both the freezing and drying can be very damaging. The insight learned from anhydrobiotic organisms has been adapted to this problem. In lyophilization, freezing and drying are fundamentally different stress vectors

that both have to be taken into consideration to stabilize proteins during the freeze-drying process. Stabilization of proteins during freezing, during which water is still present, requires additives that are preferentially excluded from the protein surface (Arakawa and Timasheff, 1982; Carpenter and Crowe, 1988). A wide range of molecules, including amino acids, can be used with similar effect. In contrast to freezing, the requirements for solute specificity are more stringent for drying; only certain sugars are effective and stabilization requires direct interaction between the sugar and proteins (Crowe et al., 1990). While in small-scale operation, sugars are sufficient for successful drying of the proteins, a large-scale freeze-drying of drug proteins in the real pharmaceutical world requires not only sugars, but also a bulking agent for better performance of the lyophilization process and formation of acceptable cakes.

Successful freeze-drying requires a proper mixture of additives, including amino acids. A number of amino acids are used as bulking agents for freeze-dried formulations. A bulking agent is essential to generate a pharmaceutically acceptable dried cake of lyophilized products. Glycine is a commonly used bulking agent. Crystallization of glycine during freezing and drying results in successful lyophilization and formation of visually acceptable cake, although it does not provide stabilization for the dried proteins (Cleland et al., 2001). Without bulking agents, so-called product blowout may occur, when the total solid is low: the streaming water vapor disrupts, and results in unacceptable, cake structure (Pikal, 1990b). Glycine crystallizes during freeze-drying, providing adequate space for ice sublimation (Akers et al., 1995; Pikal, 1994). While the free basic form of lysine, arginine and histidine was amorphous after freeze-drying, all other amino acids were crystalline (Mattern et al., 1999). The salt form of arginine, in particular the phosphate salt, was more fully amorphous after freeze-drying (Mattern et al., 1999). It is well known that the crystalline state facilitates ice sublimation, while the amorphous state protects proteins against conformational changes and chemical degradation. The use of glassy carriers such as sucrose, trahalose or arginine is known to reduce aggregation of the proteins during spray-drying (Broadhead et al., 1994; Mumenthaler et al., 1994; Andya et al., 1999).

Histidine protects an antibody against lyophilization-induced structural perturbation and increases the stability of the antibody during subsequent storage (Sane et al., 2004). During the freeze-drying process, histidine, arginine and aspartic acid became partially amorphous, whereas glycine remained crystalline (Tian et al., 2006). It was suggested that both histidine and arginine interact with proteins via hydrogen bonding (or ion-dipole) during freeze-drying, contributing to the observed stabilization of the antibody in the dried state. Those interactions between arginine or histidine and proteins, which cannot be seen well in solution, can be detected in the dried state. Since the crystalline form of excipients after lyophilization cannot lead to hydrogen bonding with the proteins, the tendency of arginine and histidine to become amorphous may be consistent with their ability to bind to the proteins.

Aggregation of lyophilized proteins can be reduced by reconstitution conditions. Certain amino acids, including lysine, glutamic acid and glycine, showed suppression of aggregate formation of lyophilized interleukin-2 and keratinocyte growth factor, when included in the reconstitution media (Zhang et al., 1995a, 1996). As a consequence, the amount of the native, active proteins increased in the presence of various additives, including certain amino acids; note that arginine was not tested in these studies.

In addition to freeze-drying, proteins can be dried, without going through the freezing step, by spray-drying, in which the additives are also required for stabilizing the proteins. However, the spray-dried EPO powders showed the storage stability independent of the addition of stabilizing excipients such as mannitol or amino acids (Mehta et al., 1996).

3.4 Protein purification: HIC, protein-A, hydroxyapatite, Ni-resin

Amino acids have rarely been used, until recently, to facilitate purification or to stabilize proteins during purification. However, it has become clear that arginine is highly effective in improving the performance of various column chromatographies, as summarized below.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) uses a weakly hydrophobic ligand to bind the native proteins so that the proteins to be purified maintain the native structure upon elution. Due to the weak hydrophobicity, binding of proteins is induced by a salting-out salt. Ammonium sulfate is commonly used for HIC due to its strong salting-out effects and high solubility. However, with this salt, HIC cannot be carried out at alkaline pH. For this, Na glutamate was successfully used, in which proteins were bound to phenyl-sepharose in 2 M Na glutamate and

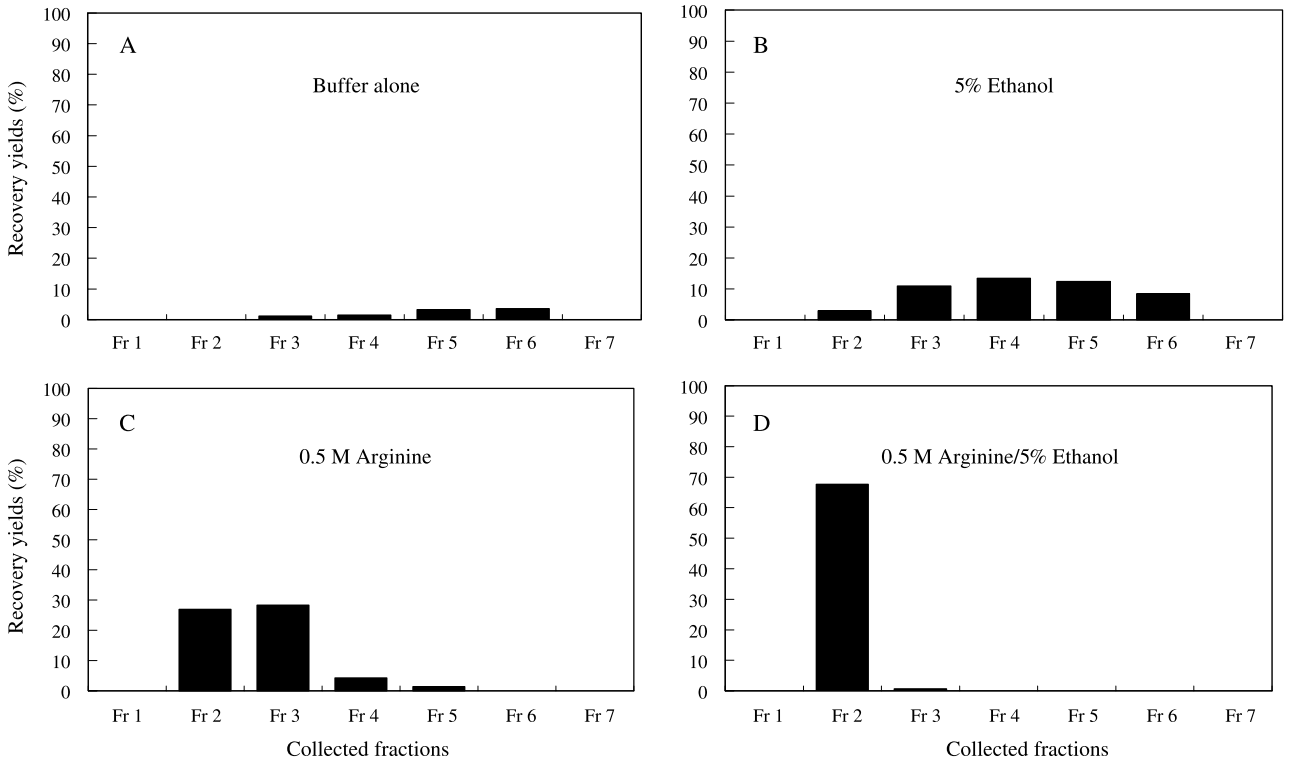

Fig. 6. Elution recovery of activin from butyl-separose by arginine. Activin-A in 1 M ammonium sulfate was loaded on HiTrap butyl-sepharose FF, and the bound activin-A was eluted with: **A** no addition; **B** 5% ethanol; **C** 0.5 M arginine; **D** 5% ethanol and 0.5 M arginine

eluted with a descending gradient of Na glutamate, just the way ammonium sulfate works (Narhi et al., 1989).

Due to hydrophobic binding of the proteins, the elution is often compromised, meaning that the proteins, once bound under salting-out conditions, cannot be eluted simply by reducing the salt concentrations. A typical example is found in activin during HIC. This protein is so hydrophobic that the HIC purification has not been successful until recently (e.g., see Fig. 6A). Using butyl-sepharose, activin was bound to the HIC column in the presence of 1 M ammonium sulfate and the bound protein eluted with 0.2–1 M arginine with reasonable yield (Fig. 6C). The addition of ethanol, which by itself was marginally effective (Fig. 6B), further improved the elution behavior in terms of yield and sharpness of elution peak (Fig. 6D). A similar elution effect of arginine was observed with the use of phenyl-sepharose for other proteins, including interleukin-6 and monoclonal antibodies (manuscript submitted).

In addition to arginine, the arginine derivatives were also effective in HIC. Acetyl-arginine, in which the amino group of arginine is acetylated, has no net charge at neutral pH. Agmatine, in which the carboxyl group is replaced with proton, has two positive charges at neutral pH. Both arginine derivatives enhanced elution of activin from butyl-sepharose, similarly to arginine (Ejima et al., 2005a).

Gel permeation chromatography

Non-specific interactions between the proteins to be analyzed or purified and the column resin cause loss of the proteins, error in determination of molecular size and damage on the column in gel permeation chromatography (GPC). GPC is also a workhorse for analysis of pharmaceutical protein products for regulatory approval, and hence reduction of non-specific protein binding is essential for this application. Such non-specific binding occurs more frequently for aggregated proteins, resulting in an underestimated amount of aggregated species in the samples. The addition of salts or organic solvents has been commonly used, but with limited success. Arginine at 0.2–0.7 M is highly effective in suppressing such non-specific binding. A typical result is shown using activin (Fig. 7-2, Ejima et al., 2005b) and a monoclonal antibody (Fig. 8). Activin was so sticky to the column resin that its elution in GPC was delayed to a position identical to that of salts in the absence of arginine (even in the presence of 0.75 M NaCl, Fig. 7-1) and the addition of 0.7 M arginine restored the normal elution, resulting in a peak of activin elution separated from the salt peak (Fig. 7). As described above, GPC is the routine analytical technology for analyzing the level of aggregation in the pharmaceu-

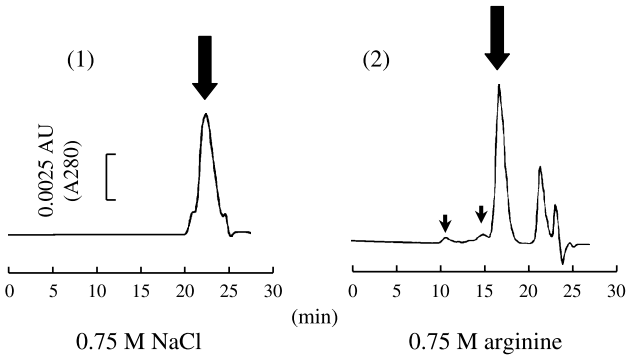

Fig. 7. GPC analysis of activin by Superdex-75HR in 0.1 M Na phosphate, pH 7.3. (*1*) plus 0.75 M NaCl, (*2*) plus 0.75 M Arginine

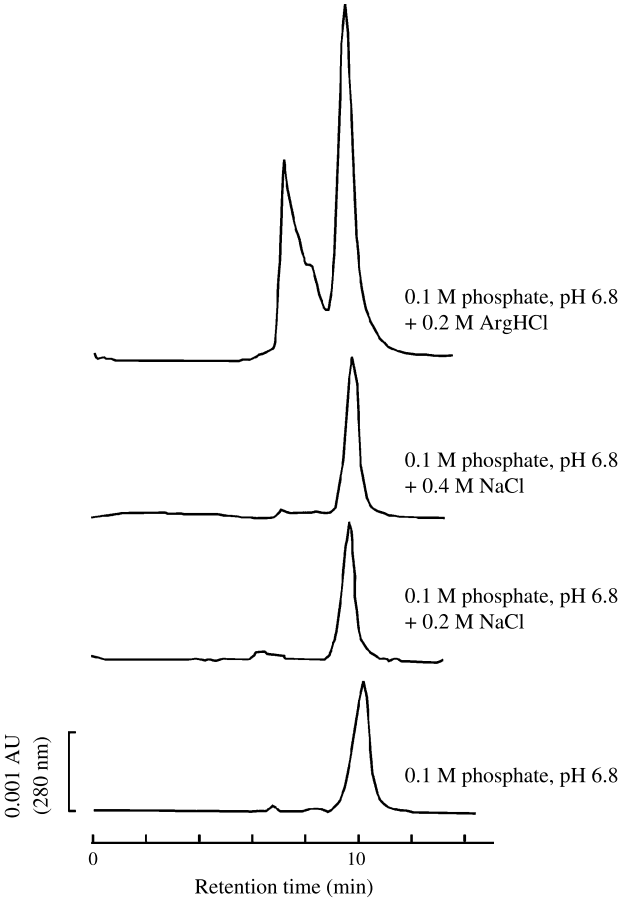

Fig. 8. The effect of NaCl and arginine on the recovery of the antibody from GPC. Mouse monoclonal antibodies containing soluble aggregates were analyzed by GPC in the presence of NaCl or arginine as indicated

tical protein products. A major concern about using this method is in regard to the preferential loss of aggregates to the column resin, as shown in Fig. 8. In this experiment, the mAb sample containing soluble aggregates was subjected to the GPC analysis in the presence of either NaCl or arginine. The elution of monomeric mAb was not affected by 0.2 M arginine, while the recovery of aggregated species was much higher in the presence of arginine. The addition of 0.2–0.4 M NaCl had no significant impact on the reduction of non-specific binding of aggregates to the resin. The aggregate content determined in the presence of arginine is consistent with the data from sedimentation velocity analysis.

Ion exchange chromatography

Arginine is ionic above ~pH 4 and hence is not suitable as an additive for ion exchange chromatography (IEC). The addition at high concentrations may interfere with binding of proteins to the IEC resin. Nevertheless, the advantage of including 0.2 M arginine in the loading sample that has been observed is that it suppresses aggregation of interleukin-6 eluted from the cation exchange columns (D. Ejima, unpublished observation).

Affinity chromatography

Affinity chromatography uses specific interactions between column ligands and proteins. Such ligands include Protein-A/G, antigen, enzyme inhibitor and substrate, dyes, and peptides. Affinity recognition of ligands for proteins in general provides high affinity and selectivity and hence often leads to one-step purification of complex protein mixtures. However, such high affinity is often disadvantageous for elution; i.e., it is often difficult to elute. Protein-A/G is used to purify antibodies, which can be eluted from the columns at low pH. The elution is more efficient at lower pH, which alters conformation

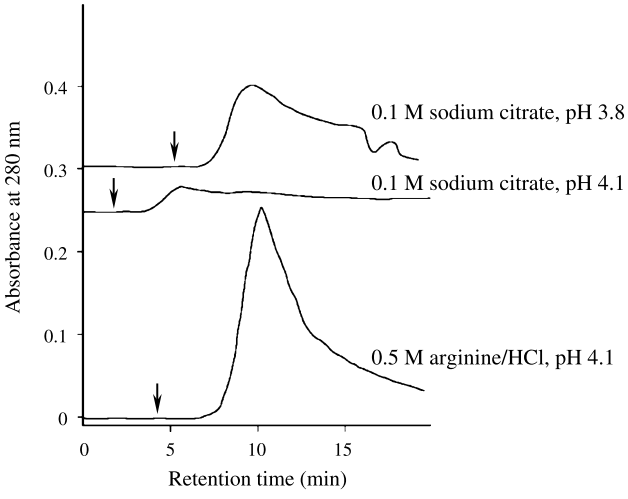

Fig. 9. Elution profile of mAb from Protein-A columns. A small elution peak with extensive tailing was observed with 0.1 M citrate at both pH 3.8 and 4.1. At pH 4.1, 0.5 M arginine resulted in much higher elution

of the antibodies eluted and frequently leads to aggregation (Ejima et al., 2006). We have shown that antibodies can be efficiently eluted at higher pH (above 4) when arginine is included in the elution solvent. Figure 9 shows the elution profile of an mAb from Protein-A columns. Above pH 4, a conventional elution buffer, citrate or glycine, was not effective, as the elution profile using 0.1 M citrate, pH 3.8 or 4.1, was extremely broad with little recovery of bound mAbs. On the contrary, 0.5 M arginine, pH 4.1 resulted in a much sharper elution profile. Higher arginine concentration further raised the pH to achieve effective elution.

Arginine was also effective in Protein-G chromatography. In this experiment, mouse IgG1 was applied to the Protein-G column and the bound protein eluted with citrate or arginine at different pH. As shown in Fig. 10, this particular antibody required 0.1 M citrate, pH 2.9 for >80% recovery (triangle). The recovery sharply decreased as the pH was increased to 3.5 (~10%). Arginine at 0.1 M was only slightly more effective than 0.1 M citrate. Above pH 3.5, citrate was ineffective independent of the concentration. On the contrary, 0.7 M arginine resulted in 40–60% recovery at pH 3.5–4.1, and even greater recovery was obtained with 1.1 M arginine. Above pH 4.3, 1 and 2 M arginine showed ~60% recovery. In general, elution of this mouse IgG1 was more difficult with Protein-G than with Protein-A and even under such conditions, aqueous arginine solutions were effective.

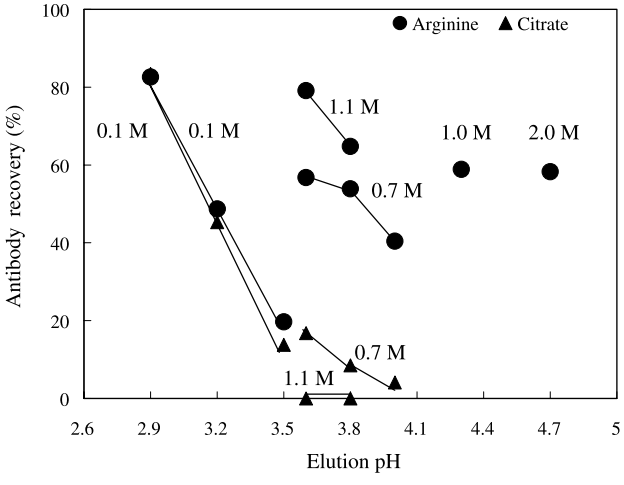

Fig. 10. Elution recovery of mouse mAb from Protein-G columns

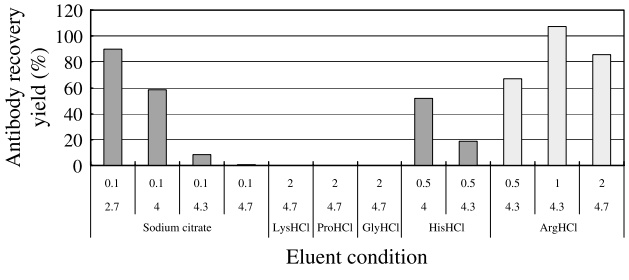

Fig. 11. Elution recovery of mAb from protein with different amino acids

Other amino acids, such as histidine, are also, but rarely, used for elution from Protein-A. Figure 11 compares some amino acids in eluting humanized IgG4 from protein-A above pH 4.0. While 2 M arginine showed greater that 80% recovery at pH 4.7, lysine, proline and glycine at 2 M were ineffective, as was citrate. At pH 4.3, arginine at both 0.5 and 1 M resulted in 70–100% recovery and 0.5 M histidine was marginally effective; however, the recovery was better (with ~50% recovery) at pH 4.0 using 0.5 M histidine. Thus, only histidine showed an ability to elute the antibody above pH 4.0, but with much less effectiveness than arginine.

Antigen chromatography

Polyclonal antibodies are versatile reagents and are also used for the detection of therapeutic markers in diagnostic assays. The polyclonal antibodies used for such applications must be specific for target antigens. Specific antibodies can readily be purified by antigen-affinity chromatography (Cuatrecasas, 1969). The bound antibodies are eluted by acid, however, often with low recovery (Casey et al., 1995). This is because the affinity of antibodies for antigens is too high, arising from a multitude of interaction forces as described above (Smith-Gill et al., 1982; Sakurabayashi, 1995). Various elution conditions for antigen chromatography, such as different pH (acid or alkaline elution), high salt concentrations and different types of the buffer, have been developed (Thompson et al., 1990, 1992; Berry et al., 1991). In a series of papers, Narhi et al. (1997a, b) and Caughey et al. (1999) pointed out the danger of using a single-step elution of polyclonal antibodies, as high-affinity antibodies could be eluted from the antigen columns only with progressively chaotropic conditions.

The above study demonstrated a proof of principle that the binding of antibodies to the antigen columns could be too strong to dissociate by mildly acidic solvents and might only be dissociated by denaturing conditions. It would be better if a milder, but more efficient, elution condition could be developed. Toward this goal, Ejima et al. (2005a) attempted to use aqueous arginine solution to elute polyclonal antibodies raised against interleukin-6,

as arginine has been shown to be effective in suppressing protein–protein interactions without destabilizing or denaturing the proteins (Arakawa et al., 2006b). Thus antisera against interleukin-6 was applied to the antigen column and the bound protein eluted in the presence of 2 M arginine, with the descending pH gradient from 5.4 to 2.4 utilizing the buffer action of arginine itself. Figure 12 shows the elution profile of the bound antibodies (bold solid curve, absorbance at 280 nm) as well as the pH change. The absorbance showed multiple peaks with the elution of the antibodies even at pH 5.4. The eluted fractions were collected into three pools. These pooled antibodies were highly pure in antibodies and exhibited binding to IL-6. Although the cause of the difference in the elution pH was not investigated, the results demonstrate that antibodies can be effectively eluted by arginine, consistent with its ability to suppress protein–protein interactions. Since arginine does not denature proteins and antibodies, nor destabilize under normal solvent conditions (Arakawa et al., 2006a), it should be a better alternative to more acidic or denaturing solvents for dissociating antigen–antibody interactions.

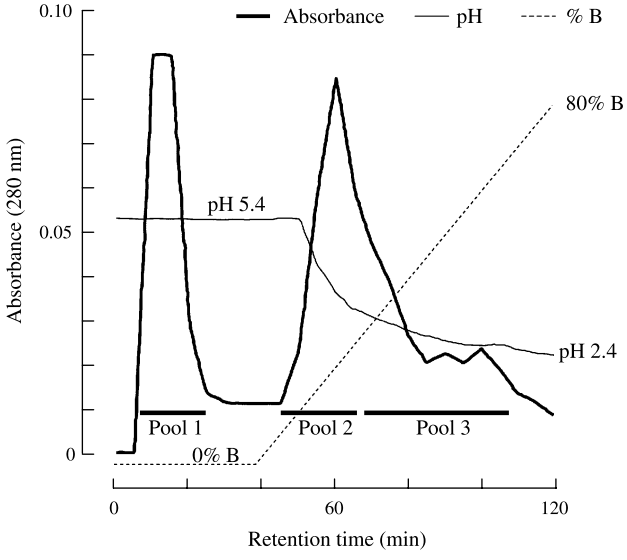

Fig. 12. Elution profile of polyclonal antibody from antigen columns using arginine

Ligand chromatography

Ligand-affinity chromatography is a convenient way to purify proteins, in particular for protein research. Since a ligand binds the specific protein, a protein of interest can be purified in one step. Ligands encompass dye, substrate, inhibitor and many other small molecules, which specifically bind target proteins. Among them, dye-affinity column chromatography offers a robust, rapid purification of proteins (Sii and Sadana, 1991). Due to the relatively high

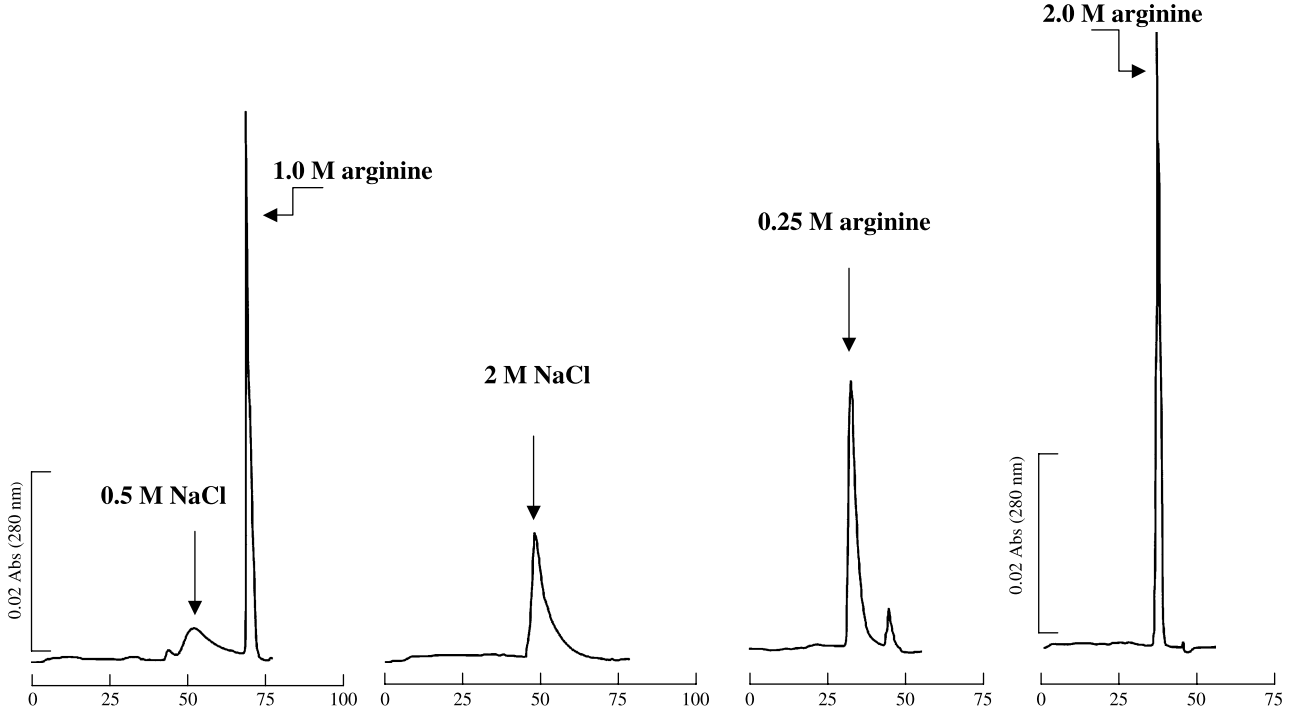

Fig. 13. Elution profile of LDH from blue-sepharose columns with NaCl or arginine

affinity and specificity of the proteins for dye columns, a large volume of samples can be applied without prior treatment such as buffer-exchange and concentration. For dye-affinity chromatography, covalently attached group-specific ligands are used for the purification of enzymes, such as dehydrogenases and kinases (Stead, 1991). Blue-dye columns bind enzymes requiring adenyl-containing cofactors such as NAD and NADP. Lactate dehydrogenase (LDH) binds to blue-dye columns and the bound enzyme can be eluted by salts, but with low recovery and a broad elution peak (Mattiasson et al., 1996; Kumar et al., 2000). We have investigated the ability of arginine to elute LDH from blue-sepharose (Arakawa et al., 2006b). As shown in Fig. 13 (first panel), 0.5 M NaCl was ineffective in eluting the protein, with the recovery of only ∼30%; the second large peak is elution with 1 M arginine after 0.5 M NaCl elution. Even 2 M NaCl showed a skewed elution peak, with the recovery of less than 60% (second panel). On the contrary, 0.25 M arginine showed a sharp elution pattern (third panel), with the higher recovery of ∼65%. Increasing arginine concentration to 2 M resulted in nearly quantitative recovery of the bound LDH (last panel). It is evident that arginine is much more effective than NaCl in eluting the bound LDH.

Arginine can have an impact on other ligand-affinity chromatography as well as on the dye-affinity chromatography described above. For example, arginine was applied to elute nucleoside diphosphate kinase (NDK) bound to its substrate, ATP, conjugated to agarose resin (Arakawa et al., 2007). Competition with free ATP is the elution procedure conventionally used. In this experiment, 0.5 M arginine effectively eluted the bound NDK (data not shown).

4. Conclusion

Here we have described a variety of amino acid applications for biotechnology products and technologies. While amino acids have been used in protein pharmaceuticals as an excipient, as summarized in Table 5, their applications will undoubtedly expand in protein production and chromatography as well as in formulation of protein pharmaceuticals. As described here, amino acids do modulate protein binding and elution during affinity chromatography. However, applications of solvents to modulate protein–protein or protein–ligand interactions are not limited to affinity chromatography. Affinity chromatography is used not only for protein purification, but also for characterization of protein–protein or protein–ligand interactions in the batch-mode as well as in the flow-mode. In the batch-mode, affinity chromatography is equivalent to many other biochemical assays, such as immuno-precipitation, ELISA, pull-down, immuno-staining, protein expression profiling and inhibitor screening. Thus, the same solvents that worked in affinity chromatography may be used to improve the performance of these different assays. When the interactions to be studied are too weak to be detected, solvents may be used to enhance these weak interactions. When the background is too high, those solvents, which are effective in eluting bound proteins, may be used to reduce the background to such an extent that the interactions being studied are retained. Affinity is also used in various instruments, such as Biacore and SELDI, where the protein–protein or protein–ligand interactions are measured on the chip surface. Although solvents may interfere with these analyses, they may find applications with certain limitations, such as extensive washing before the analysis of the bound proteins.

Table 5. Approved protein pharmaceuticals containing amino acids as an excipient

Amino acids	Concentration	Product	Drug substance	Company
L-Arginine	55 mg/ml	TNKase®	Human tissue plasminogen activator (tPA)	Genentech
L-Arginine	35 mg/ml	Activase®	Human tissue plasminogen activator (tPA)	Genentech
L-Glycine	21–25 mM	Kogenate® FS	Recombinant antihemophilic factor (rAHF)	Bayer
L-Glycine	3 mM	Synagis®	Humanized monoclonal antibody	MedImmune
L-Glycine	260 mM	BeneFIX®	Coagulation factor IX	Wyeth
L-Glycine	0.17–0.34 mg/ml	Nutropin®	Human growth hormone (hGH)	Genentech
L-Glycine	23 mg/mL	Neumega®	Interleukin-11	Wyeth
L-Glutamate	1 mg/ml	VariVax®	Varicella virus vaccine live	Merck
L-Glutamate	5 mg/ml	Streptase®	Streptokinase	Aventis
L-Histidine	55 mM	Recombinate	Recombinate antihemophilic factor (rAHF)	Baxter
L-Histidine	18–23 mM	Kogenate® FS	Recombinant antihemophilic factor (rAHF)	Bayer
L-Histidine	47 mM	Synagis®	Humanized monoclonal antibody	MedImmune
L-Histidine	5 mM	Herceptin®	Humanized monoclonal antibody	Genentech
L-Histidine	10 mM	BeneFIX®	Coagulation factor IX	Wyeth

References

Ahn JH, Lee YP, Rhee JS (1997) Investigation of refolding condition for *Pseudomonas fluorescens* lipase by response surface methodology. J Biotechnol 54: 151–160

Akers M, Milton N, Byrn S, Neil S (1995) Glycine crystallization during freezing: the effects of salt form, pH, and ionic strength. Pharmacol Res 12: 1457–1461

Andya JD, Maa YF, Costantino HR, Nguyen PA, Dasovich N, Sweeney TD, Hsu CC, Shire SJ (1999) The effect of formulation excipients on protein stability and aerosol performance of spray-dried powders of a recombinant humanized anti-IgE monoclonal antibody. Pharmacol Res 16: 350–358

Arakawa T, Carpenter JF, Kita Y, Crowe JH (1990) Basis for toxicity of certain cryoprotectants: an hypothesis. Cryobiology 27: 410–415

Arakawa T, Dix DB, Chang BS (2003a) The effects of protein stabilizer on aggregation induced by multiple-stresses. Yakugaku Zasshi 123: 957–961

Arakawa T, Ejima D, Tsumoto K, Ishibashi M, Tokunaga M (2007) Improved performance of column chromatography by arginine: dye-affinity chromatography. Protein Expr Purif 52: 410–414

Arakawa T, Katsuyama I, Kumagai I, Tsumoto K (2003b) A magic agent in protein refolding: arginine. Tanpakushitsu Kakusan Koso 48: 2310–2317

Arakawa T, Kita Y, Tsumoto K, Ejima D, Fukada H (2006b) Aggregation suppression of proteins by arginine during thermal unfolding. Protein Pept Lett 13: 921–927

Arakawa T, Timasheff SN (1982) Stabilization of protein structure by sugars. Biochemistry 21: 6536–6544

Arakawa T, Timasheff SN (1983) Preferential interactions of proteins with solvent components in aqueous amino acid solutions. Arch Biochem Biophys 224: 169–177

Arakawa T, Timasheff SN (1984) The mechanism of action of Na glutamate, lysine HCl, and piperazine-N,N′-bis(2-ethanesulfonic acid) in the stabilization of tubulin and microtubule formation. J Biol Chem 259: 4979–4986

Arakawa T, Timasheff SN (1985) The stabilization of proteins by osmolytes. Biophys J 47: 411–414

Arakawa T, Timasheff SN (1987) Abnormal solubility behavior of β-lactoglobulin: salting-in by glycine and NaCl. Biochemistry 26: 5147–5153

Arakawa T, Tsumoto K (2003) The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. Biochem Biophys Res Commun 304: 148–152

Armstrong N, de Lencastre A, Gouaux E (1999) A new protein folding screen: application to the ligand binding domains of a glutamate and kainite receptor and to lysozyme and carbonic anhydrase. Protein Sci 8: 1475–1483

Arora D, Khanna N (1996) Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies. J Biotechnol 52: 127–133

Aune KC, Tanford C (1969) Thermodynamics of the denaturation of lysozyme by guandine hydrochloride. II. Dependence on denaturation concentration at 25 °C. Biochemistry 8: 4586–4590

Back JF, Oakenfull D, Smith MB (1979) Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry 18: 5191–5196

Barth S, Huhn M, Matthey B, Klimka A, Galinski EA, Engert A (2000) Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. Appl Environ Microbiol 66: 1572–1579

Berry MJ, Davies J, Smith CG, Smith I (1991) Immobilization of Fv antibody fragments on porous silica and their utility in affinity chromatography. J Chromatogr 587: 161–169

Blasig IE, Winkler L, Lassowski B, Mueller SL, Zuleger N, Krause E, Krause G, Gast K, Kolbe M, Piontek L (2006) On the self-association potential of transmembrane tight junction proteins. Cell Mol Life Sci 63: 505–514

Bonacci G, Sanchez MC, Gonzalez M, Ceschin D, Fidelio G, Vides MA, Chiabrando G (2000) Stabilization of homogeneous preparations of pregnancy zone protein lyophilized in the presence of saccharose. Structural and functional studies. J Biochem Biophys Methods 46: 95–105

Bonci A, Carezon WA Jr (2005) Ion channels and intracellular signaling proteins as potential targets for novel therapeutics for addictive and depressive disorders. Pharmacol Ther 108: 65–75

Bourot S, Sire O, Trautwetter A, Touze T, Wu LF, Blanco C, Bernard T (2000) Glycine betaine-assisted protein folding in a lysA mutant of *Escherichia coli*. J Biol Chem 275: 1050–1056

Brants JF, Lumry R (1969) The thermodynamics of protein denaturation. IV. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. J Am Chem Soc 91: 4256–4264

Broadhead J, Rouan SK, Hau I, Rhodes CT (1994) The effect of process and formulation variables on the properties of spray-dried beta-galactosidase. J Pharm Pharmacol 46: 458–467

Brown AD (1976) Microbial water stress. Bacteriol Rev 40: 803–846

Brown AD, Simpson JR (1972) Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J Gen Microbiol 72: 589–591

Buchner J, Pastan I, Brinkmann U (1992) A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies. Anal Biochem 205: 263–270

Buchner J, Rudolph R (1991) Renaturation, purification and characterization of recombinant Fab-fragments produced in *Escherichia coli*. Biotechnology 9: 157–162

Calnan BJ, Tidor B, Biancalana S, Hudson D, Frankel AD (1991) Arginine-mediated RNA recognition: the arginine fork. Science 252: 1167–1171

Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25: 244–255

Carpenter JF, Crowe JH (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28: 3916–3922

Carpenter JF, Crowe JH, Arakawa T (1990) Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. J Dairy Sci 73: 3727–3736

Carpenter JF, Crowe LM, Crowe JH (1987a) Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations. Biochem Biophys Acta 923: 109–115

Carpenter JF, Martin B, Crowe LM, Crowe JH (1987b) Stabilization of phosphofructokinase during air-drying with sugars and sugar/transition metal mixtures. Cryobiology 24: 455–464

Carpenter JF, Prestrelski SJ, Arakawa T (1993) Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. Arch Biochem Biophys 303: 456–464

Carpenter JF, Prestrelski SJ, Dong A (1998) Application of infrared spectroscopy to development of stable lyophilized protein formulations. Eur J Pharm Biopharm 45: 231–238

Casey JL, Keep PA, Chester KA, Robson L, Hawkins RE, Begent RHJ (1995) Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography. J Immunol Methods 179: 105–116

Caughey DJ, Narhi LO, Kita Y, Meng SY, Wen D, Chen W, Ratzkin BJ, Fujimoto J, Iwahara T, Yamamoto T, Arakawa T (1999) Fractionation of polyclonal antibodies to fragments of a neuroreceptor using three increasing chaotropic solvents. J Chromatogr B 728: 49–57

Chang BS, Hershenson S (2002) Rational design of stable protein formulations. Theory and practice. In: Carpenter JF, Manning MC (eds) Pharmaceutical biotechnology, vol 13. Plenum Press, New York, pp 1–25

Chang LL, Shepherd D, Sun J, Tang XC, Pikal MJ (2005) Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: Implications for the mechanism of protein stabilization in the solid state. J Pharmacol Sci 94: 1445–1455

Chen B, Bautista R, Yu K, Zapata GA, Mulkerrin MG, Chamow SM (2003) Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. Pharmacol Res 20: 1952–1960

Chen BL, Arakawa T (1996) Stabilization of recombinant human keratinocyte growth factor by osmolytes and salts. J Pharmcol Sci 85: 419–426

Chen BL, Arakawa T, Hsu E, Narhi LO, Tressel TJ, Chien SL (1994) Strategy to suppress aggregation of recombinant keratinocyte growth factor during liquid formulation development. J Pharmacol Sci 83: 1657–1661

Citron M (2002) Emerging Alzheimer's disease therapies: inhibition of beta-secretase. Neurobiol Aging 23: 1017–1022

Clegg JS (2001) Cryptobiosis. A peculiar state of biological organization. Comp Biochem Physiol B 128: 613–624

Cleland JL (1993) Impact of protein folding on biotechnology. In: Cleland JL (ed) Protein folding: in vivo and in vitro. American Chemical Society, Washington DC, pp 1–21

Cleland JL, Lam X, Kendrick B, Yang J, Yang TH, Overcashier D, Brooks D, Hsu C, Carpenter JF (2001) A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharmacol Sci 90: 310–321

Cleland JL, Wang DI (1990) Cosolvent assisted protein refolding. Biotechnol 8: 1274–1278

Crowe JH, Carpenter JF, Crowe LM (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60: 73–103

Crowe JH, Carpenter JF, Crowe LM, Anchordoguy TJ (1990) Are freezing and dehydration similar stress vector. A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27: 219–231

Crowe JH, Hoekstra FA, Crowe LM (1992) Anhydrobiosis. Annu Rev Physiol 54: 579–599

Crowell CK, Grampp GE, Rogers GN, Miller J, Scheinman RI (2007) Amino acid and manganese supplementation modulates the glycosylation state of erythropoietin in a CHO culture system. Biotech Bioeng 96: 538–549

Csonka LN, Hanson AD (1991) Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol 45: 569–606

Cuatrecasas P (1969) Insulin-sepharose: immunoreactivity and use in the purification of antibody. Biochem Biophys Res Commun 35: 531–537

Diamant S, Eliahu N, Rosenthal D, Goloubinoff P (2001) Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stress. J Biol Chem 276: 39586–39591

Diamant S, Rosenthal D, Azem A, Eliahu N, Ben-Zvi AP, Goloubinoff P (2003) Dicarboxylic amino acids and glycine-betaine regulate chaperone-mediated protein-disaggregation under stress. Mol Microbiol 49: 401–410

Dong A, Prestrelski SJ, Allison SD, Carpenter JF (1995) Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J Pharmacol Sci 84: 415–424

Ejima D, Tsumoto K, Fukada H, Yumioka R, Nagase K, Arakawa T, Philo JS (2006) Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. Proteins 66: 954–962

Ejima D, Yumioka R, Arakawa T, Tsumoto K (2005b) Arginine as an effective additive in gel permeation chromatography. J Chromatogr A 1094: 49–55

Ejima D, Yumioka R, Tsumoto K, Arakawa T (2005a) Effective elution of antibodies by arginine and arginine derivatives in affinity chromatography. Anal Biochem 345: 250–257

Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H, Toner M (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nat Biotechnol 18: 163–167

Figler RA, Omote H, Nakamoto RK, Al-Shawi MK (2000) Use of chemical chaperones in the yeast Saccharomyces cerevisiae to enhance heterologous membrane protein expression: high-yield expression and purification of human P-glycoprotein. Arch Biochem Biophys 376: 34–46

Fink AL (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des 3: R9–R23

Foord RL, Leatherbarrow RJ (1998) Effect of osmolytes on the exchange rates of backbone amide protons in proteins. Biochemistry 37: 2969–2978

Futaki S (2002) Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. Int J Pharm 245: 1–7

Galinski EA, Stein M, Amendt M, Kinder M (1997) The kosmotropic (structure-forming) effect of compatible solutes. Comp Biochem Physiol 117A: 357–365

Gerlsma SY (1968) Reversible denaturation of ribonuclease in aqueous solution as influenced by polyhydric alcohols and some other additives. J Biol Chem 243: 957–961

Gerlsma SY (1970) The effects of polyhdric and monohydric alcohols on the heat induced reversible denaturation of chymotrypsinogen A. Eur J Biochem 14: 150–153

Gibbs JW (1878) On the equilibrium of heterogeneous substances. Conn Acad Arts Sci 3: 343–524

Golovanov AP, Hautbergue GM, Wilson SA, Lian LY (2004) simple method for improving protein solubility and long-term stability. J Am Chem Soc 126: 8933–8939

Guo N, Puhlev I, Brown DR, Mansbridge J, Levine F (2000) Trehalose expression confers desiccation tolerance on human cells. Nat Biotechnol 18: 168–171

Hall DR, Jacobsen MP, Winzor DJ (1995) Stabilizing effect of sucrose against irreversible denaturation of rabbit muscle lactate dehydrogenase. Biophys Chem 57: 47–54

Hamel E, del Campo AA, Lowe MC, Waxman PG, Lin CM (1982) Effects of organic acids on tubulin polymerization and associated guanosine 5′-triphosphate hydrolysis. Biochemistry 21: 503–509

Hamel E, Lin CM (1981a) Glutamate-induced polymerization of tubulin: characteristics of the reaction and application to the large-scale purification of tubulin. Arch Biochem Biophys 209: 29–40

Hamel E, Lin CM (1981b) Interaction of tubulin with ribose-modified analogs of GTP and GDP: evidence for two mutually exclusive exchangeable nucleotide binding sites. Proc Natl Acad Sci USA 78: 3368–3372

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8: 67–113

Ho JGS, Middelberg APJ, Ramage P, Kocher HP (2003) The likelihood of aggregation during protein renaturation can be assesses using the second virial coefficient. Protein Sci 12: 708–716

Horwitz P (1992) α-Crystallin can function as a molecular chaperone. Proc Natl Acad Sci USA 89: 10449–10453

Ishibashi M, Tsumoto K, Ejima D, Arakawa T, Tokunaga M (2005) Characterization of arginine as a solvent additive: a halophilic enzyme as a model protein. Protein Pept Lett 12: 649–653

Itakura K, Hirose T, Crea R, Riggs AD, Heyneker HL, Bolivar F, Boyer HW (1977) Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. Science 198: 1056–1063

Jensen WA, Armstrong JM, De Giorgio J, Hearn MT (1996) Stability studies on pig heart mitochondrial malate dehydrogenase; the effect of salts and amino acids. Biochem Biophys Acta 1296: 23–34

Jensen WA, Armstrong JM, De Giorgio J, Hearn MT (1997) Thermodynamic analysis of the stabilisation of pig heart mitochondrial malate dehydrogenase and maize leaf phosphoenolpyruvate carboxylase by different salts, amino acids and polyols. Biochem Biophys Acta 1338: 186–198

Jubete Y, Maurizi MR, Gottesman S (1996) Role of the heat shock protein DnaJ in the lon-dependent degradation of naturally unstable proteins. J Biol Chem 271: 30798–30803

Kanazawa H, Fujimoto S, Ohara A (1996) Effect of radical scavengers on the inactivation of papain by ascorbic acid in the presence of cupric ions. Biol Pharm Bull 17: 476–481

Kita Y, Arakawa T, Lin TY, Timasheff SN (1994) Contribution of the surface free energy perturbation to protein-solvent interactions. Biochemistry 33: 15178–15189

Klebe G (2000) Recent development in structure-based drug design. J Mol Med 78: 245–246

Koseki T, Kitabatake N, Doi E (1990) Freezing denaturation of ovalbumin at acid pH. J Biochem 107: 389–394

Kudou M, Shiraki K, Fujiwara S, Imanaka T, Takagi M (2003) Prevention of thermal inactivation and aggregation of lysozyme by polyamines. Eur J Biochem 270: 4547–4554

Kumar A, Galaev IY, Mattiasson B (2000) Polymer displacement/shielding in protein chromatography. J Chromatogr B 741: 103–113

Kuntz ID Jr, Brassfield TS, Law GD, Purcell GV (1969) Hydration of macromolecules. Science 163: 1329–1331

Lamosa P, Turner DL, Maycock C, Santos H (2003) Protein stabilization by compatible solutes. Effects of diglycerol phosphate on the dynamics of *Desulfovibrio gigas* rubredoxin studied by NMR. Eur J Biochem 270: 4606–4614

Lee JC, Timasheff SN (1974) Partial specific volumes and interactions with solvent components of proteins in guanidine hydrochloride. Biochemistry 13: 257–265

Leslie SB, Teter SA, Crowe LM, Crowe JH (1994) Trehalose lowers membrane phase transition in dry yeast cells. Biochem Biophys Acta 1192: 7–13

Lin TY, Timasheff SN (1996) On the role of surface tension in the stabilization of globular proteins. Protein Sci 5: 372–381

Lindwall G, Chau M, Gardner SR, Kohlstaedt LA (2000) A sparse matrix approach to the solubilization of over expressed proteins. Protein Eng 13: 67–71

Lucht JM, Bremer E (1994) Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. FEMS Microbiol Rev 14: 3–20

Mattern M, Winter G, Kohnert U, Lee G (1999) Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. Pharm Dev Tech 4: 199–208

Mattiasson B, Galaev IY, Garg N (1996) Polymer-shielded dye-affinity chromatography. J Mol Recog 9: 509–514

Mehta D, Corbo D, Iqbal K (1996) Spray dried erythropoietin. International patent WO 96/18647

Melander W, Horvath C (1977) Salt effect on hydrophobic interactions in precipitation and chromatography of proteins: an interpretation of the lyotropic series. Arch Biochem Biophys 183: 200–215

Michaelis S, Beckwith J (1982) Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Annu Rev Microbiol 36: 435–465

Milner JL, McClellan DJ, Wood JM (1987) Proline as a osmoprotectant. J Gen Microbiol 133: 1851–1860

Minton AP (2005) Influence of macromolecular crowding upon the stability and state of association of proteins: prediction and observation. J Pharm Sci 94: 1668–1675

Moelbert S, Normand B, Rios PDL (2002) Kosmotropes and chaotropes: modeling preferential exclusion, binding and aggregate stability. Biophys Chem 112: 45–57

Mumenthaler M, Hsu CC, Pearlman R (1994) Feasibility study on spray-drying protein pharmaceuticals: recombinant human growth hormone and tissue-type plasminogen activator. Pharm Res 11: 12–20

Narhi LO, Caughey DJ, Horan T, Kita Y, Chang D, Arakawa T (1997a) Effect of three elution buffers on the recovery and structure of monoclonal antibodies. Anal Biochem 253: 236–245

Narhi LO, Caughey DJ, Horan TP, Kita Y, Chang D, Arakawa T (1997b) Fractionation and characterization of polyclonal antibodies using three progressively more chaotropic solvents. Anal Biochem 253: 246–252

Nicholls A, Sharp KA, Honig B (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins 11: 281–296

Nozaki Y, Tanford C (1963) The solubility of amino acids and related compounds in aqueous urea solutions. J Biol Chem 238: 4074–4081

Nozaki Y, Tanford C (1970) The solubility of amino acids, diglycine, and triglycine in aqueous guanidine hydrochloride solutions. J Biol Chem 242: 4731–4735

Paborji M, Pochopin NL, Coppola WP, Bogardus JB (1994) Chemical and physical stability of chimeric L6, a mouse-human monoclonal antibody. Pharmacol Res 11: 764–771

Pace CN (1990) Conformational stability of globular proteins. Trends Biochem Sci 15: 14–17

Pappenheimer JR, Lepie MP, Wyman J (1936) The surface tension of. aqueous solutions of dipolar ions. J Am Chem Soc 58: 1851–1855

Patro SY, Freund E, Chang BS (2002) Protein formulation and fill-finish operations. Biotechnol Annu Rev 8: 55–84

Pellequer JL, Zhao B, Kao HI, Bell CW, Li K, Li QL, Karu AE, Roberts VA (2000) Stabilization of bound polycyclic aromatic hydrocarbons by a π-cation interactions. J Mol Biol 302: 691–699

Pikal MJ (1990a) Freeze-drying of proteins. Part I: process design. BioPharm 3: 18–26

Pikal MJ (1990b) Freeze-drying of proteins. Part II: formulation selection. BioPharm 3: 27–30

Pikal MJ (1994) Freeze-drying of proteins: process, formulation, and stability. In: Cleland J, Langer R (eds) Formulation and delivery of proteins and peptides. American Chemical Society, Washington DC, pp 120–133

Prakash V, Loucheux C, Scheufele S, Gorbunoff MJ, Timasheff SN (1981) Interactions of proteins with solvent components in 8 M urea. Arch Biochem Biophys 210: 455–464

Prestrelski SJ, Arakawa T, Carpenter JF (1993a) Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. Structural studies using infrared spectroscopy. Arch Biochem Biophys 303: 465–473

Prestrelski SJ, Tedeschi N, Arakawa T, Carpenter JF (1993b) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys J 65: 661–671

Privalov PL, Khechinashvili NN (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J Mol Biol 86: 665–684

Pugsley AP (1993) The complete general secretory pathway in gram-negative bacteria. Microbiol Rev 57: 50–108

Pyne A, Chatterjee K, Suryanarayanan R (2003) Solute crystallization in mannitol-glycine systems – implications on protein stabilization in freeze-dried formulations. J Pharmcol Sci 92: 2272–2283

Rapoport AI, Khroustalyova GM, Kuklina EN (1997) Anhydrobiosis in yeast: activation effect. Braz J Med Biol Res 36: 9–13

Rinas U, Risse B, Jaenicke R, Abel KJ, Zettlmeissl G (1990) Denaturation-renaturation of the fibrin-stabilizing factor XIII a-chain isolated from human placenta. Properties of the native and reconstituted protein. Biol Chem Hoppe Seyler 371: 49–56

Rudolph R, Opitz U, Hesse F, Rießland R, Fischer S (1992) Reactivation of microbially produced human tissue-type plasminogen activator. In: North K (ed) Biotechnology international. Century Press, London, pp 321–325

Sakurabayashi I (1995) Dynamics of antigen–antibody interaction – an introduction. Nippon Rinsho 53: 2126–2133

Samuel D, Kumar TK, Ganesh G, Jayaraman G, Yang PW, Chang MM, Trivedi VD, Wang SL, Hwang KC, Chang DK, Yu C (2000) Proline inhibits aggregation during protein refolding. Protein Sci 9: 344–352

Sane SU, Wong R, Hsu CC (2004) Raman spectroscopic characterization of drying induced structural changes in a therapeutic antibody: correlating structural changes with long term stability. J Pharmacol Sci 93: 1005–1018

Sasahara K, McPhie P, Minton AP (2003) Effect of dextran on protein stabilization and conformation attributed to macromolecular crowding. J Mol Biol 326: 1227–1237

Schaffner J, Winter J, Rudolph R, Schwarz E (2001) Co-secretion of chaperones and low-molecular-size medium additives increases the yield of recombinant disulfide-bridged proteins. Appl Environ Microbiol 67: 3994–4000

Sharp KA, Nicholls A, Fine RF, Honig B (1991) Reconciling the magnitude of the microscopic and macroscopic hydrophobic effects. Science 252: 106–109

Shiraki K, Kudou M, Fujiwara S, Imanaka T, Takagi T (2002) Biophysical effect of amino acids on the prevention of protein aggregation. J Biochem 132: 591–595

Sii D, Sadana A (1991) Bioseparation using affinity techniques. J Biotechnol 19: 83–98

Sinanoglu O, Abdulnur S (1964) Hydrophobic stacking of bases and the solvent denaturation of DNA. Photochem Photobiol 3: 333–342

Sinanoglu O, Abdulnur S (1965) Effect of water and other solvents on the structure of biopolymers. Fed Proc 24: S12–S23

Singh SM, Panda AK (2005) Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng 99: 303–310

Smith-Gill SJ, Wilson AC, Potter M, Prager EM, Feldmann RJ, Mainhart CR (1982) Mapping the antigenic epitope for a monoclonal antibody against lysozyme. J Immunol 128: 314–322

Somero GN (1986) Protons, osmolytes, and fitness of internal milieu from protein function. Am J Physiol 251: R197–R213

Speed MA, Wang DI, King J (1996) Specific aggregation of partially folded polypeptide chains: the molecular basis of inclusion body composition. Nat Biotechnol 14: 1283–1287

Srinivas V, Raman B, Rao KS, Ramakrishna T, Rao CM (2003) Structural perturbation and enhancement of the chaperone-like activity of α-crystallin by arginine hydrochloride. Protein Sci 12: 1262–1270

Stead CV (1991) The use of dyes in protein purification. Bioseparation 2: 129–136

Taneja S, Ahmad F (1994) Increased thermal stability of proteins in the presence of amino acids. Biochem J 303: 147–153

Tang XC, Pikal MJ (2005) The effect of stabilizers and denaturants on the cold denaturation temperatures of proteins and implications for freeze-drying. Pharmacol Res 22: 1167–1175

Timasheff SN, Xie G (2003) Preferential interactions of urea with lysozyme and their linkage to protein denaturation. Biophys Chem 105: 421–448

Thompson NE, Aronson DB, Burgess RR (1990) Purification of eukaryotic RNA polymerase II by immunoaffinity chromatography. J Biol Chem 265: 7069–7077

Thompson NE, Hager DA, Burgess RR (1992) Isolation and characterization of a polyol-responsive monoclonal antibody useful for gentle purification of *Escherichai coli* RNA polymerase. Biochemistry 31: 7003–7008

Tian F, Sane S, Rytting JH (2006) Calorimetric investigation of protein/amino interactions in the solid state. Int J Pharmacol 310: 175–186

Tsumoto K, Umetsu M, Kumagai I, Ejima D, Arakawa T (2003) Solubilization of active green fluorescent protein from insoluble particles by guanidine and arginine. Biochem Biophys Res Commun 312: 1383–1386

Tsvetkova NM, Phillips BL, Crowe LM, Crowe JH, Risbud SH (1998) Effect of sugars on headgroup mobility in freeze-dried dipalmitoylphosphatidylcholine bilayers: solid-state 31P NMR and FTIR studies. Biophys J 75: 2947–2955

Umetsu M, Tsumoto K, Ashish K, Nita S, Tanaka Y, Adschiri T, Kumagai I (2004) Structural characteristics and refolding of in vivo aggregated hyperthermophilic archaeon proteins. FEBS Lett 557: 49–56

Umetsu M, Tsumoto K, Nitta S, Adschiri T, Ejima D, Arakawa T, Kumagai I (2005) Nondenaturing solubilization of beta2 microglobulin from inclusion bodies by L-arginine. Biochem Biophys Res Commun 328: 189–197

Umezawa N, Gelman MA, Haigis MC, Raines RT, Gellman SH (2002) Translocation of a beta-peptide across cell membranes. J Am Chem Soc 124: 368–369

von Hippel PH, Wong KY (1962) The effect of ions on the kinetics of formation and the stability of the collagen fold. Biochemistry 1: 664–674

von Hippel PH, Wong KY (1965) On the conformational stability of globular proteins. J Biol Chem 240: 3909–3923

Wang W (2005) Protein aggregation and its inhibition in biopharmaceuticals. Int J Pharmacol 289: 1–30

Wickner W, Driessen AJ, Hartl FU (1991) The enzymology of protein translocation across the *Escherichia coli* plasma membrane. Annu Rev Biochem 60: 101–124

Wilf J, Minton AP (1981) Evidence for protein self-association induced by excluded volume. Myoglobin in the presence of globular proteins. Biochem Biophys Acta 670: 316–322

Wilson L (1970) Properties of colchicine binding protein from chick embryo brain. Interactions with vinca alkaloids and podophyllotoxin. Biochemistry 9: 4999–5007

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217: 1214–1222

Zhang J, Peng X, Jonas A, Jonas J (1995b) NMR study of the cold, heat, and pressure unfolding of ribonuclease A. Biochemistry 34: 8631–8641

Zhang MZ, Pikal K, Nguyen T, Arakawa T, Prestrelski SJ (1996) The effect of the reconstitution medium on aggregation of lyophilized recombinant interleukin-2 and ribonuclease A. Pharmacol Res 13: 643–646

Zhang MZ, Wen J, Arakawa T, Prestrelski SJ (1995a) A new strategy for enhancing the stability of lyophilized protein: the effect of the reconstitution medium on keratinocyte growth factor. Pharmacol Res 12: 1447–1452

Zimmerman SB, Trach SOJ (1991) Estimation of macromolecular concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*. J Mol Biol 222: 599–620

Authors' address: Tsutomu Arakawa, President of Alliance Protein Laboratories, 3957 Corte Cancion, Thousand Oaks, CA 91360, U.S.A., Fax: +1-805-388-7252, E-mail: tarakawa2@aol.com