Journal of Bioscience and Bioengineering
VOL. 120 No. 6, 720–724, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

Protein-poly(amino acid) precipitation stabilizes a therapeutic protein L-asparaginase against physicochemical stress

Takuya Maruyama,[1,‡] Shunsuke Izaki,[2,‡] Takaaki Kurinomaru,[1] Kenji Handa,[2] Tomoaki Kimoto,[2] and Kentaro Shiraki[1,*]

Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan[1] and Research and Development Center, Terumo Corporation, 1500 Inokuchi, Nakai-machi, Ashigarakamigun, Kanagawa 259-0151, Japan[2]

Received 4 February 2015; accepted 14 April 2015
Available online 13 May 2015

Long-term storage in aqueous solution has been demanded for the practical application of therapeutic proteins. Recently, a precipitation–redissolution method was proposed to prepare salt-dissociable protein–polyelectrolyte complex (PPC). To elucidate the utility of the complex for storage of proteins, we investigated the stress tolerance of PPC precipitates containing L-asparaginase (ASNase) and poly-L-lysine (polyK). PPC precipitate containing ASNase and polyK was prepared by precipitation-redissolution method. The sample was treated to three types of stress, i.e., heat, shaking, and oxidation. The protein concentration, enzyme activity, and CD spectrum of the supernatants of samples were measured after stressed. PPC precipitate consisting of ASNase and polyK showed tolerance against thermal and shaking stress compared to the native solution. In addition, PPC precipitate protected ASNase from inactivation by oxidation. PPC precipitate of ASNase/polyK complex successfully stabilized ASNase against physicochemical stresses. These results suggest that the PPC precipitate has great potential as a storage method in aqueous solution for unstable proteins.

[**Key words:** L-Asparaginase; Precipitation; Protein-polyelectrolyte complex; Redissolution; Stabilization]

Progress in the fields of recombinant DNA technology and biotechnology has markedly increased the numbers of therapeutic proteins available for use, including antibodies and enzymes (1). Despite these significant advances, the storage of therapeutic proteins has been challenging due to their physical and chemical instabilities. Lyophilization has been well-used for the storage of the proteins. A lyophilized formulation can greatly enhance conformational stability of protein by restricting mobility and can minimize chemical reactions in the presence of water (2). At present, 50% of the therapeutic proteins approved for clinical application by the US Food and Drug Administration (FDA) are lyophilized formulations (3). However, lyophilized formulations require a long dissolution time, which places a limitation on their practical usage (4).

Solution formulations are favorable over lyophilized formulations from the viewpoint of convenience, but the proteins are intrinsically unstable in aqueous solution. Simple and effective methods to overcome the problems associated with instability of protein in solution involve the addition of excipients (i.e., solution additives). The proteins are stabilized by long time storage- or thermal-induced inactivation and aggregation in the presence of solution additives, such as arginine (5,6), amino acid alkyl esters (7), oligoamine compounds (8–10), sugars (11–13), and polysorbate 80 (14). However, high concentrations of the solution additives are required to suppress inactivation and aggregation of protein. In addition, most solution additives, with the exception of detergents, cannot inhibit the effects of mechanical stress, including shaking, associated with the degradation of therapeutic proteins during transport.

Precipitation has a great deal of potential as an alternative method for stabilizing proteins. The protein precipitate is separated from the solvent, which is a solid-like state with restricted mobility. Among the protein precipitants, polyelectrolytes are unique in biological and biotechnological methods, such as protein purification (15–19). Polyelectrolytes interact strongly with complementary charged proteins through multiple electrostatic interactions, resulting in the formation of various types of protein–polyelectrolyte complex (PPC) (20), which enables the modulation of protein functions (21–26). PPC is prone to form precipitates depending on several factors, such as stoichiometric ratio, pH, temperature, and ionic strength. In addition, PPC precipitates at low ionic strength are redissolved by the addition of another solution of physiological ionic strength (150 mM), which enhances the utility for medical use.

Previously, we developed a precipitation–redissolution method using poly(amino acid) for preparation of protein formulations at high concentrations (27). At low ionic strength, cationic or anionic proteins formed precipitable PPC by the addition of anionic poly-L-glutamic acid (polyE) and cationic poly-L-lysine (polyK), respectively, and then fully precipitated by centrifugation. The PPC

* Corresponding author. Tel.: +81 29 853 5306; fax: +81 29 853 5215.
E-mail address: shiraki@bk.tsukuba.ac.jp (K. Shiraki).
‡ The first two authors contributed equally to this work.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.04.010

precipitate can be easily redissolved by the exchange of buffer with 150 mM NaCl. It should be noted that the redissolved proteins retain its native activity and structure for various types of proteins (27). However, it remains unclear whether the PPC precipitate stabilizes the protein against physical and chemical stresses in aqueous solution. Knowledge regarding the stabilizing effect of PPC precipitate would facilitate the storage of therapeutic proteins even in aqueous solution.

In the present study, stress tolerance experiments were performed in three states, i.e., native protein, PPC suspension, and PPC precipitate (Fig. 1). Anionic L-asparaginase (ASNase), which is used in treatment of acute lymphoblastic leukemia, was used to form a PPC with cationic polyK. The PPC samples were pretreated with three stresses, i.e., heat, shaking, and oxidation. The samples were redissolved by exchange of other buffer with NaCl and then characterized by measurement of protein concentrations and enzyme activity as well as far-UV circular dichroism (CD) spectroscopy. As expected, the PPC precipitate showed greater resistance to these stresses than the native protein.

MATERIALS AND METHODS

Materials L-Asparaginase (ASNase) from *Escherichia coli* was from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan). Ammonium sulfate, L-asparagine, trichloroacetic acid (TCA), and hydrogen peroxide (H_2O_2) were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium chloride (NaCl) was from Nacalai Tesque, Inc. (Kyoto, Japan). Nessler's reagent, poly-L-lysine hydrobromide with an average molecular weight of 4–15 kDa (polyK) and 3-(*N*-morpholino) propanesulfonic acid (MOPS) were from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used were of high-quality analytical grade and used as received.

Protein concentration The concentration of ASNase was determined from the absorbance at 280 nm using a spetrophotometer (BioSpec-nano; Shimadzu Corp., Kyoto, Japan) with extinction coefficient of 0.646 $(mg/mL)^{-1}\ cm^{-1}$ (28).

Enzyme activity The enzyme activity of ASNase was measured as follows. An aliquot of 50 μL of ASNase solution was mixed with 950 μL of the substrate solution containing 22 mM L-asparagine at 37°C for 15 min. The reaction was stopped by addition of 250 μL of 1.5 M TCA to the assay mixture. Subsequently, the sample was mixed with Nessler's reagent to measure the amount of ammonia released after L-asparagine hydrolysis. The absorbance was monitored spectrophotometrically at 450 nm. The concentration of ammonia produced by the enzyme reaction was determined from a reference curve using ammonium sulfate as the standard.

Circular dichroism Circular dichroism (CD) experiments were performed in a 1-mm path-length quartz cuvette using a spectropolarimeter (J-720; Japan Spectroscopic Co., Ltd., Tokyo, Japan). The spectra of redissolved proteins were measured at 25°C. A buffer sample containing no protein was subtracted from all spectra to account for any background signal.

Thermal stress test Control samples were 40 μL of 5.0 mg/mL ASNase in 10 mM MOPS (pH 7.0). Suspension samples were made by mixing 20 μL of 10 mg/mL ASNase in 10 mM MOPS (pH 7.0) with 20 μL of 0.5 mg/mL polyK in 10 mM MOPS (pH 7.0). Precipitation samples were made by mixing 20 μL of 10 mg/mL ASNase in 10 mM MOPS (pH 7.0) with 20 μL of 0.5 mg/mL polyK in 10 mM MOPS (pH 7.0), and then centrifuged at 15,000 ×*g* for 20 min at 25°C. The samples were heated at 60°C for 0–30 min. After heat treatment, 360 μL of 167 mM NaCl in 10 mM MOPS (pH 7.0) was added and then centrifuged at 15,000 ×*g* for 20 min at 25°C. The protein concentration, enzyme activity, and CD spectrum of the supernatants were measured as described above.

Shaking stress test Control samples were 1200 μL of 0.5 mg/mL ASNase in 10 mM MOPS (pH 7.0). Suspension samples were made by mixing 600 μL of 1.0 mg/

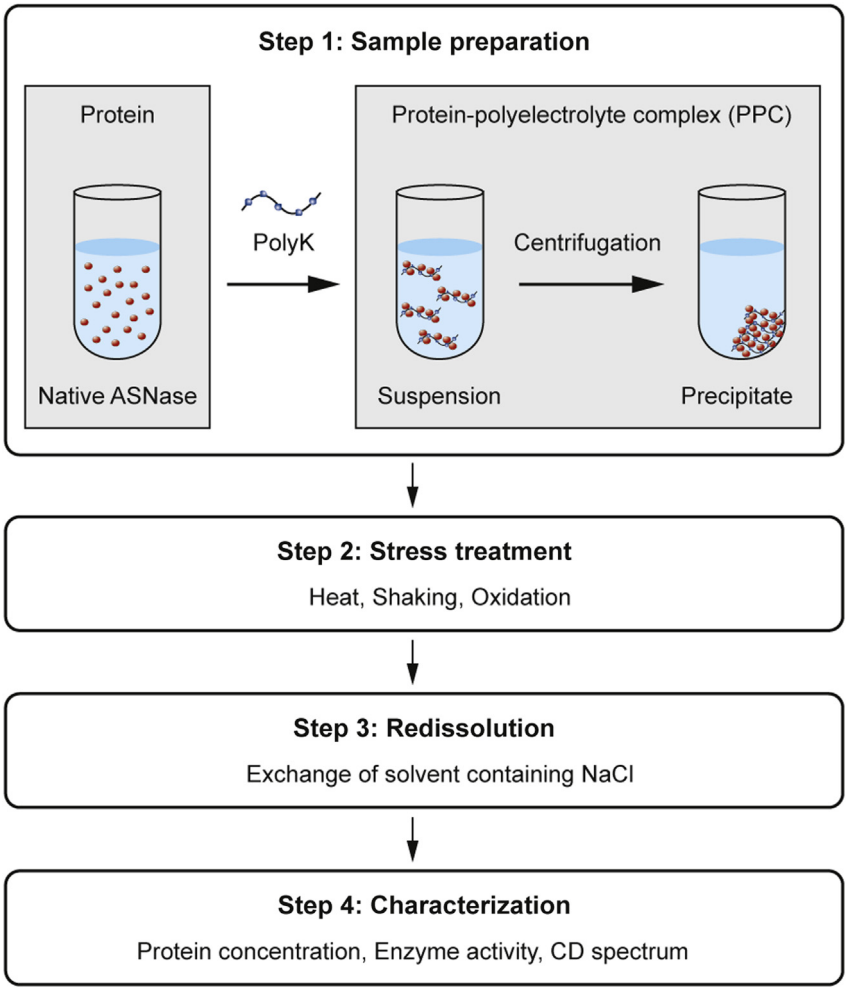

FIG. 1. Procedure of stress tolerance test for PPC precipitate.