Aggregative protein polyelectrolyte complex for high-concentration formulation of protein drugs

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Aggregative protein–polyelectrolyte complex for high-concentration formulation of protein drugs

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Keywords

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Abstract

Aggregative protein-polyelectrolyte complex (PPC) has been proposed as a concentrated state of protein with a great potential for biopharmaceutical application. In this review article, we introduce a unique concentration method of protein formulation using PPC for a dozen types of pharmaceutical antibodies, hormones, and enzymes. Aggregative PPC can be obtained only by mixing poly(amino acid)s with proteins under low salt concentration conditions at an ambient temperature. The aggregative PPC is in a stabilized state against shaking, heating, and oxidation. More importantly, the aggregative PPC can be fully redissolved by the addition of physiological saline without denaturation and activity loss for many proteins. In addition, the general toxicity and pharmacokinetic profiles of the aggregative PPC are identical to those of the control antibody formulation. Thus, the protein formulation produced by aggregative PPC would be applicable for biomedical use as a kind of concentrated-state protein.

1 Introduction

As the development of therapeutic antibodies increases at a remarkable rate [1, 2], many researchers are interested in high-concentration protein solutions. The subcutaneous injection of high-concentration antibody solution has been demanded as a promising route of administration of these therapeutic antibodies because of the convenience it affords [3–7]. One of the key steps in the successful subcutaneous administration is the preparation of a protein solution of a high concentration (>100 mg/mL) [5, 6, 8, 9]. The redissolution of lyophilized protein at low volumes and ultrafiltration are the most common methods of high concentration protein formulation. Another advantage of using ultrafiltration is that it allows continuous processing of the simple in contrast of batch concentration. However, lyophilization is also harmful to the protein structure by nature of the processes of freezing, drying, and redissolution. Thus, novel concentration methods have been demanded for high-concentration protein solutions, such as gelation [10, 11], crystallization [12], liquid–liquid separation [13], and spray drying [14, 15].

Protein aggregation is one of the most challenging problems in protein formulations. In general, the native structure of a protein is affected by physical and chemical stresses in solution, such as heat, pH, pressure, organic solvents, agitation, and proteases, resulting in the denaturation of its three-dimensional structure. The denatured proteins not only lose their functions but also form irreversible aggregates because of the exposure of hydrophobic amino acid residues to the aqueous medium. A protein solution at a high concentration tends to form undesirable aggregates due to the distance between protein molecules, which can be on the same order of magnitude as the size of the proteins [16]. Because of their immunogenicity, the aggregates of therapeutic proteins are inevitably unacceptable as protein drugs [17].

Despite the disadvantage of aggregation state protein, we have proposed a unique approach to the concentration of protein solutions using protein aggregation with polyelectrolytes [18–21]. Polyelectrolytes, charged linear polymers, have great potential as protein stabilizers [22–29] and functionalizing agents [30–33]. As described below, polyelectrolytes bind to complementary charged proteins, resulting in the formation of a protein–polyelectrolyte complex (PPC). The

polyelectrolyte of a PPC can be easily stripped off by physiological saline. In this review article, we introduce the aggregative PPC of a dozen pharmaceutical proteins as a novel technology for the preparation and stabilization of high concentration protein solutions.

2 Formation of aggregative PPC

As multiple charge units, a polyelectrolyte can interact with oppositely charged proteins at a low salt concentration and ambient temperature, resulting in the formation of a PPC. The complex formation between proteins and polyelectrolytes occurs spontaneously because the driving force is electrostatic interactions. Thus, PPC solutions can be prepared by mixing protein and polyelectrolyte solutions without additional equipment. The states of a PPC also depend on numerous solution factors, such as pH, temperature, pressure, ionic strength, stoichiometric ratio, and the size of the protein and polyelectrolyte. PPCs are roughly classified into soluble PPCs and aggregative PPCs. A soluble PPC has an excess charge associated with dispersion in aqueous medium and a size of <100 nm. In contrast, aggregative PPCs assemble in larger sizes of >100 nm, resulting in precipitates, coacervates, and gel formation [34, 35].

The complementary charge combination should be selected for the preparation of PPCs, i.e., cationic proteins are used with anionic polyelectrolytes. In general, four types of charged amino acids are exposed on the protein surface, i.e., cationic lysine (Lys), arginine (Arg), anionic aspartic acid (Asp), and glutamic acid (Glu). As charged amino acids are exposed on the protein surface, folding protein molecules have positive or negative charges at the respective solution pH. The surface charge of a protein is mainly determined by the isoelectric point (*p*I) and pH: when pH < *p*I, the protein has a positive charge and when pH > *p*I, the protein has a negative charged. Note that aggregative PPC of antibody was found to be empirically favorable in the pH range of $|\mathrm{pH} - \mathrm{pI}| \approx 2$ [18, 36]. In addition, the aggregative PPC prepared by this condition more salt-soluble than that of other conditions (i.e. $|\mathrm{pH} - \mathrm{pI}| \approx 4$). It may be suggested that antibodies are more suitable for the precipitation–redissolution method than other proteins.

Figure 1 shows the soluble–insoluble transition of a protein by precipitants. The mechanism of aggregative PPC formation is different from those of the so-called “aggregation” and “precipitation.” In the case of a common precipitant, such as ammonium sulfate, the proteins assemble with increasing salt concentrations, and then the proteins are completely insoluble at the high concentration of salts (typically >100 M) (Figure 1A). The salting-out behavior by kosmotropic

ion is analogous to the precipitation appearing in another precipitant-containing neutral polymer and organic solvent [37–40]. In contrast, PPC precipitation occurs at low concentrations of polyelectrolyte; the PPC is solubilized by the increasing polyelectrolyte concentration (Figure 1B). The insolubilization by polyelectrolyte is explained as follows: At a low polyelectrolyte concentration, an intra-PPC protein cross-linkage forms between PPCs, resulting in inter-PPC attraction and then the formation of PPC aggregates. At low polyelectrolyte concentrations, the interaction between PPCs is favorable due to charge neutralization of proteins and polyelectrolytes, resulting in the formation of PPC aggregates. At high concentrations of polyelectrolytes, an excess amount of polyelectrolyte decreases the attraction between PPCs due to a decline in the number of protein molecules per unit of polyelectrolyte.

PPC formation is usually reversible. A driving force in the formation of PPC is multiple non-covalent interactions between protein and polyelectrolyte, mainly electrostatic interactions. Electrostatic interactions are shielded in the presence of high ionic strength and PPCs subsequently release protein molecule (Figure 1B). The salt responsibility of PPC is generally independent of the state of the PPC. The release of protein molecules from a PPC is influenced not only by the incremental increase in ionic strength but also by the addition of other electrolyte, including polyelectrolytes and proteins, and hence are used in several applications, such as purification [36, 41–45], enzyme switch [24, 30–32], biosensors [46–48], and drug delivery systems [25, 49, 50].

3 Concentration of therapeutic proteins by aggregative PPC

Protein precipitation in an aqueous solution is not only a conventional separation technique but also a concentration method for proteins. The precipitate contains a high amount of protein molecules, in some cases without denaturation. If protein precipitates can be fully dissolved by a small amount of a solvent, it is quite easy to obtain a high concentration protein solution. Matheus and co-workers have reported a pioneering example of the concentration of IgG1 by this precipitation–redissolution process using well-used precipitants, such as ammonium sulfate, sodium citrate, and polyethylene glycol [51]. However, the high dose of these precipitants that is required to completely precipitate proteins is unfavorable owing to the concern about several practical limitations, such as cytotoxicity [52].

In order to apply the precipitation–redissolution process for therapeutic proteins, we focused on the aggregative PPC as an alternative strategy for high concentration formulation, which has several advantages described above. Figure 2 shows a general procedure of the concentration method by the precipitation–redissolution process using aggregative PPC. A protein solution (Step 1) is mixed with an oppositely charged polyelectrolyte solution without salt, and then the sample becomes immediately clouded (Step 2). The suspension of aggregative PPC is centrifuged and subsequently precipitated (Step 3). Thereafter, the supernatant is removed (Step 4). This is a concentrated state. The precipitated PPC can be dissolved by NaCl due to the electrostatic shield between protein and polyelectrolyte (Step 5).

From the viewpoint of protein refolding technology, the inhibition of protein aggregation is a challenging issue because of the difficulty of refolding from aggregation to native structure [53, 54]. Such protein aggregation can be initially dissolved under a non-physiological condition such as high concentration of denaturant (e.g., 8 M urea or 6 M guanidine hydrochloride). In contrast, PPC aggregation is easily dissolved at a physiological ionic strength (typically 150 mM NaCl). The difference in the solubility of aggregate is attributed to the difference in the driving force of stabilities, i.e., common protein aggregates are formed by the hydrophobic interaction between denatured proteins, whereas PPC aggregates are formed by the electrostatic interaction between

proteins and polyelectrolytes. Furthermore, the protein released from the PPC aggregate retains its native structure and original function, because the aggregative PPC remains in its native structure [18, 19].

As almost all proteins are regarded as charged colloid particles, it is thought that concentration by aggregative PPC formation is applied to various types of therapeutic proteins. Actually, concentration by aggregative PPC has been reported for a dozen therapeutic proteins using two types of poly(amino acid), cationic poly-L-lysine (polyK) and anionic poly-L-glutamic acid (polyE) [18]. The yields of this technique are > 85 %, depending on the kind of proteins. Typical images are shown in Figure 3; high concentration (150 mg/mL) antibody formulation of adalimumab is prepared by this method from low concentration stock solution (30 mg/mL) [19].

The concentration method by aggregative PPC offers another advantage for pharmaceutical applications. Actually, the precipitation and redissolution of adalimumab were achieved under conditions ranging from micro scale (0.1 mL) to large scale (1.0 L) [19]. In comparison with conventional concentration methods, including lyophilization, evaporation, and ultrafiltration, the concentration method by aggregative PPC revealed significant advantages with regard to the time required and yield [19]. Furthermore, *in vivo* experiments indicated that the general toxicity and pharmacokinetic profiles of redissolved antibodies corresponded to those of a conventional antibody formulation [19]. Therefore, the protein formulation produced by the concentration method by aggregative PPC would be applicable for biomedical use.

4 Stabilization of therapeutic proteins by aggregative PPC

Proteins in solution tend to be inactivated by mechanical stresses during manufacturing and transportation processes [55]. Inactivated proteins not only lose biological functions but also subsequently form irreversible aggregates, which cause serious problems in biopharmaceutical products [17]. To protect proteins from the threat of protein aggregation induced by mechanical stress, solution additives that inhibit unfavorable protein-protein interactions are useful [56, 57]. Most solution additives are low molecular weight compounds that stabilize a protein through preferential interaction. However, high concentrations of low molecular weight solution additives are required to suppress inactivation and aggregation of protein (typically several hundred mM). These high concentrations of additives frequently lead to high viscosity and cytotoxicity. In addition, most solution additives other than detergents cannot protect against the effects of mechanical stress.

Agitation is one of the major mechanical stresses associated with the production and transportation processes, which causes irreversible inactivation and aggregation of proteins. We have reported that the aggregative PPC protected unfavorable inactivation and aggregation of therapeutic proteins from the agitation stresses [20, 21]. The stabilization effects of aggregative PPC have been tested as follow: (i) suspension (step 2 in Figure 2) and precipitation (step 3 in Figure 2) of therapeutic proteins and poly(amino acid) complexes were prepared; (ii) the mechanical stress including thermal and shaking were loaded on the sample; (iii) a salt solution (final concentration of 150 mM) was added into the sample; and then (iv) the residual activity and protein concentration of the redissolved proteins were determined. The residual activities and concentration of L-asparaginase (ASNase) redissolved from ASNase/polyK precipitation were higher than those of native ASNase and ASNase/polyK suspensions (Figure 4A, B) [20]. A similar stabilization effect was obtained for antibodies (adalimumab and omalizumab); SEC and MFI revealed that the oligomerization of antibody-induced agitation was reduced by the formation of aggregative PPC [21]. Agitation-induced protein aggregation is primarily attributed to the contact of proteins with the air-water interface [17, 58], indicating that the protein–polyelectrolyte interaction is inhibited by contact between the antibody and the air-water interface.

Thermal treatment is an acceleration test to evaluate the stability of protein against long-term storage. The residual activities and concentration of ASNase redissolved from ASNase/polyK precipitation and suspension are higher than that of native ASNase (Figure 4C, D). In contrast, the heat-induced aggregation of antibody was not reduced by the formation of aggregative antibody/polyE precipitation and suspension, whereas the residual activities of the antibodies remained [21]. This difference in thermo-stability between ASNase and antibodies may arise from the following properties: (i) the thermo-stabilization activity of the amino group of cationic polyK is stronger than that of the carboxyl group of anionic polyE, (ii) the tertiary structures of proteins may be due to the differences in thermal-stability of PPC; ASNase contains assembly homo-subunits, whereas antibodies are roughly Y-shaped with a disulfide bond between two heavy chains and two light chains, and (iii) pH change by heating may affect the state of the PPC precipitate, which is attributed to the unfavorable conformational change of the proteins. Therefore, it will be necessary to determine the long-term stability of PPC precipitates under milder conditions (i.e., 25 °C–40 °C).

Oxidation is one of the major chemical stresses that cause protein degradation [59]. Recently, Jakob and co-workers have reported that the polyphosphate shows the chaperone activity for protection of oxidation-induced protein aggregation *in vitro* and *in vivo* [26]. Similarly, PPC precipitate has protective effects against oxidative stress [20]. The residual activities of ASNase redissolved from ASNase/polyK precipitation are higher than those of native ASNase (Figure 4E). These results suggest that the polyelectrolyte has a potential ability to stabilize protein against chemical degradation.

5 Viscosity of high concentration protein formulation prepared by several methods

Finally, it is interesting to mention the comparison of viscosity of several high concentration protein solutions (Table 1). A high viscosity (typically >50 cP) of a high concentration protein solution hampers an efficient subcutaneous injection. The reduction of viscosity in high concentration antibody formulation was achieved by the addition of compounds with small molecular weights, such as hydrophobic salt [60] and arginine hydrochloride [61, 62]. However, small-molecular weight additives are required at high concentrations to decrease the viscosity of high concentration protein solutions. In contrast, aggregative PPC decreases the viscosity of protein by addition of a low dose of poly(amino acid). Aggregative PPC containing an omalizumab/polyE mixture was lower in viscosity than a free omlaizumab solution (unpublished results). Similar results were obtained using the intentional assembly techniques of protein molecules, such as crystallization [12] and nanocluster [63]. These results suggest that aggregative PPC is a useful approach to reduce the viscosity of high concentration protein solutions.

6 Conclusion

In this review article, we have highlighted the concept of using aggregative PPC as a concentration and stabilization method for therapeutic proteins. A precipitation–redissolution process using aggregative PPC provides various types of high-concentration protein formulation without being time-consuming and expensive. It could be emphasized that the aggregative PPC is more stable than the native protein alone. Thus, aggregative PPC is one of the simplest storage states that can protect protein against various stresses in aqueous solution. We believe that this technique would contribute to the development of a novel type of protein formulation.

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Table and Figure Legends

Table 1. Brief summation of high concentration states of therapeutic proteins.

	Viscosity	**Assembly**	**Ref**
Solution alone	High	No	-
Low molecular weight compounds	Low	No	[59–61]
Aggregative PPC	Low	Yes	[18, 19, 21]
Nano cluster	Low	Yes	[62]
Crystal	Low	Yes	[12]

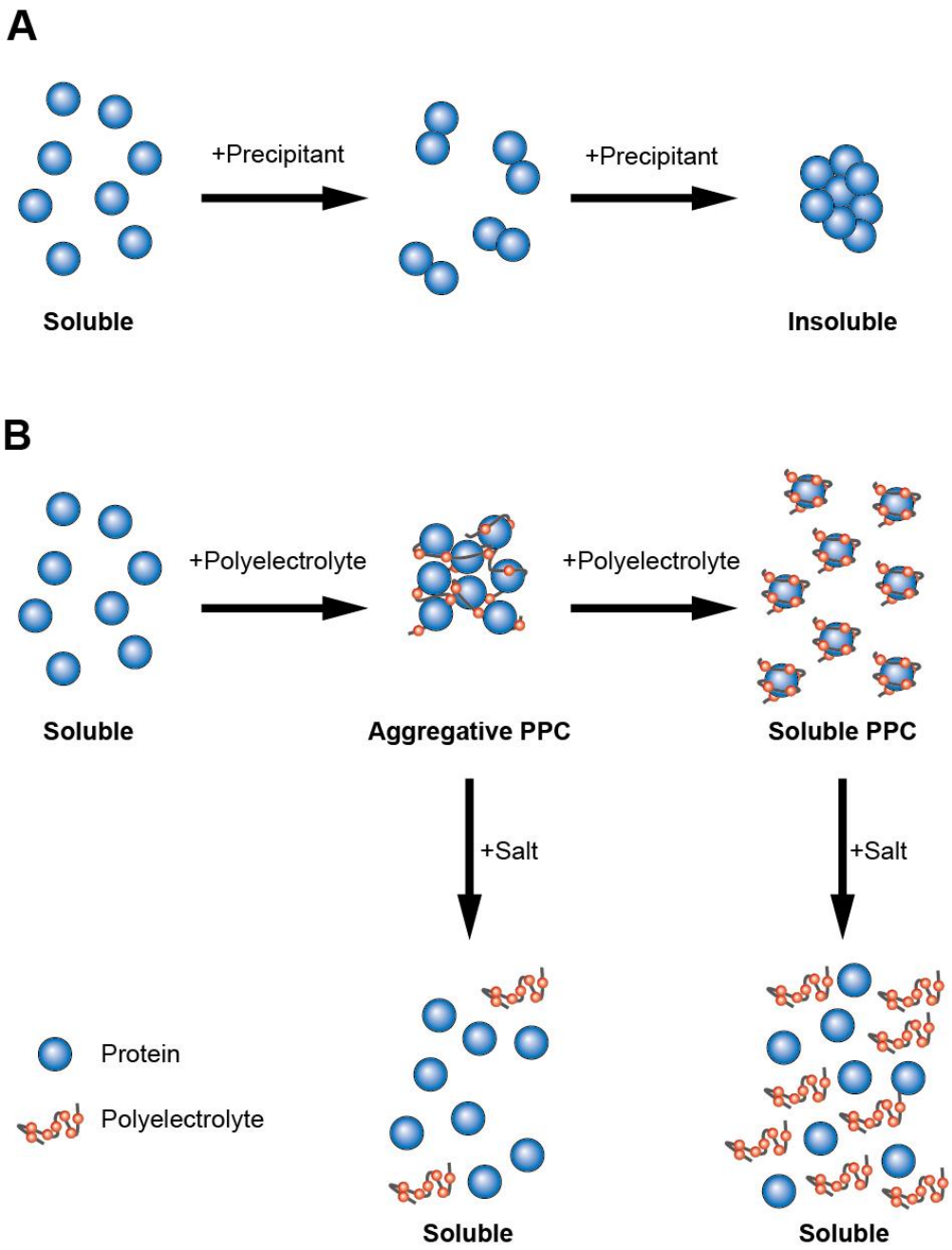

Figure 1. Soluble-insoluble transition of a protein by addition of conventional precipitant (e.g., ammonium sulfate) (**A**) or polyelectrolyte (**B**).

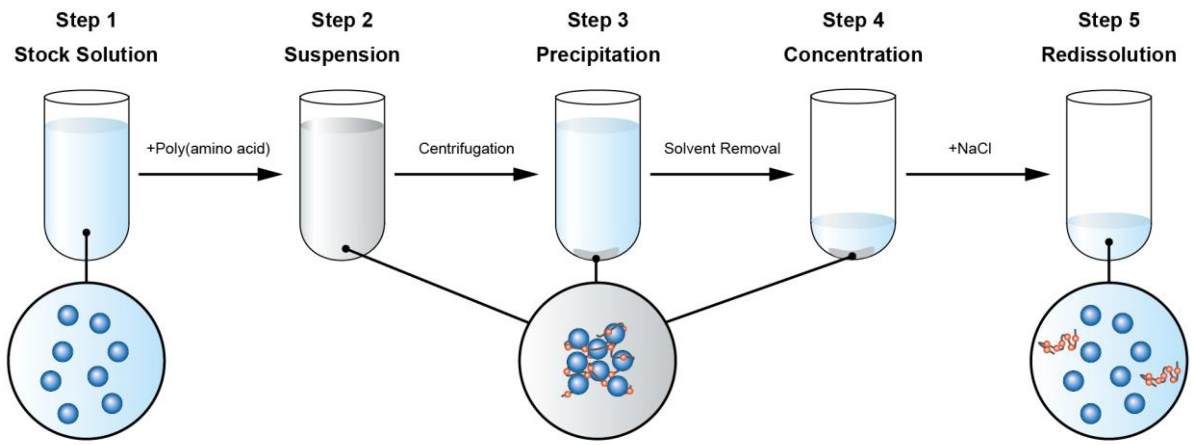

Figure 2. Concentration method of a protein by precipitation–redissolution process using aggregative PPC.

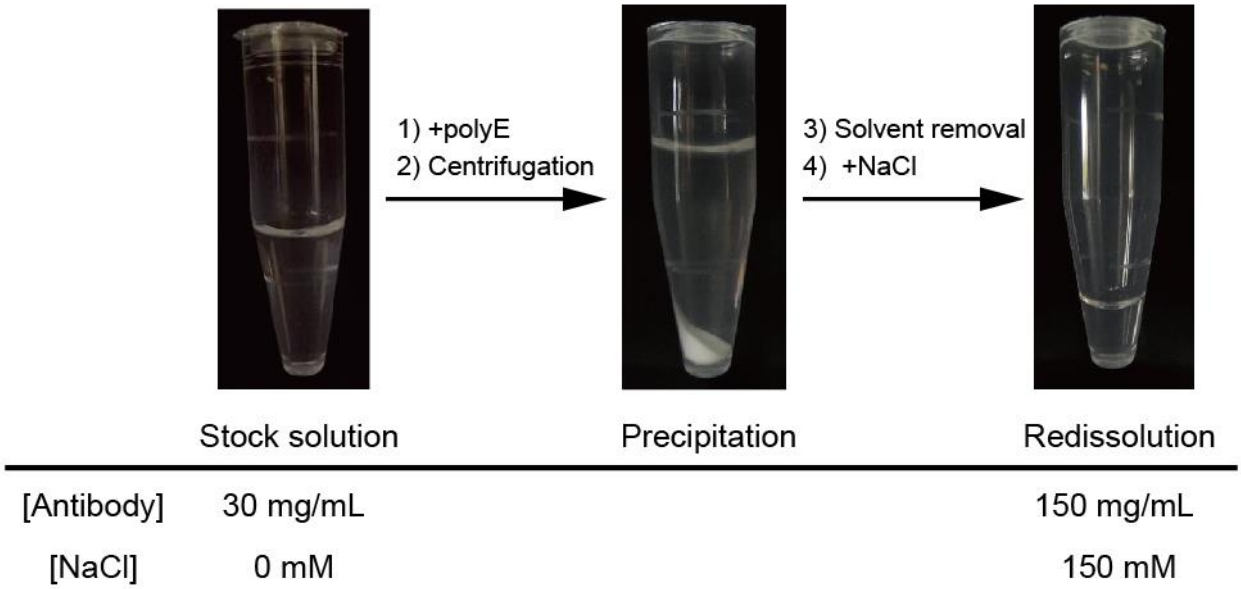

Figure 3. Concentration of antibody by aggregative PPC. Aliquots of 200 μL of polyE solution in 10 mM buffer were added to 200 μL of stock solution containing 30 mg/mL monoclonal antibody (adalimumab) in the same buffer, and then the antibody/polyE complex suspension was centrifuged. Thereafter, 30 μL of supernatant were removed and the precipitate was dissolved by addition of 10 μL buffer containing NaCl. Antibody solution (150 mg/mL) was obtained by this procedure. A portion of the data is from reference [19].

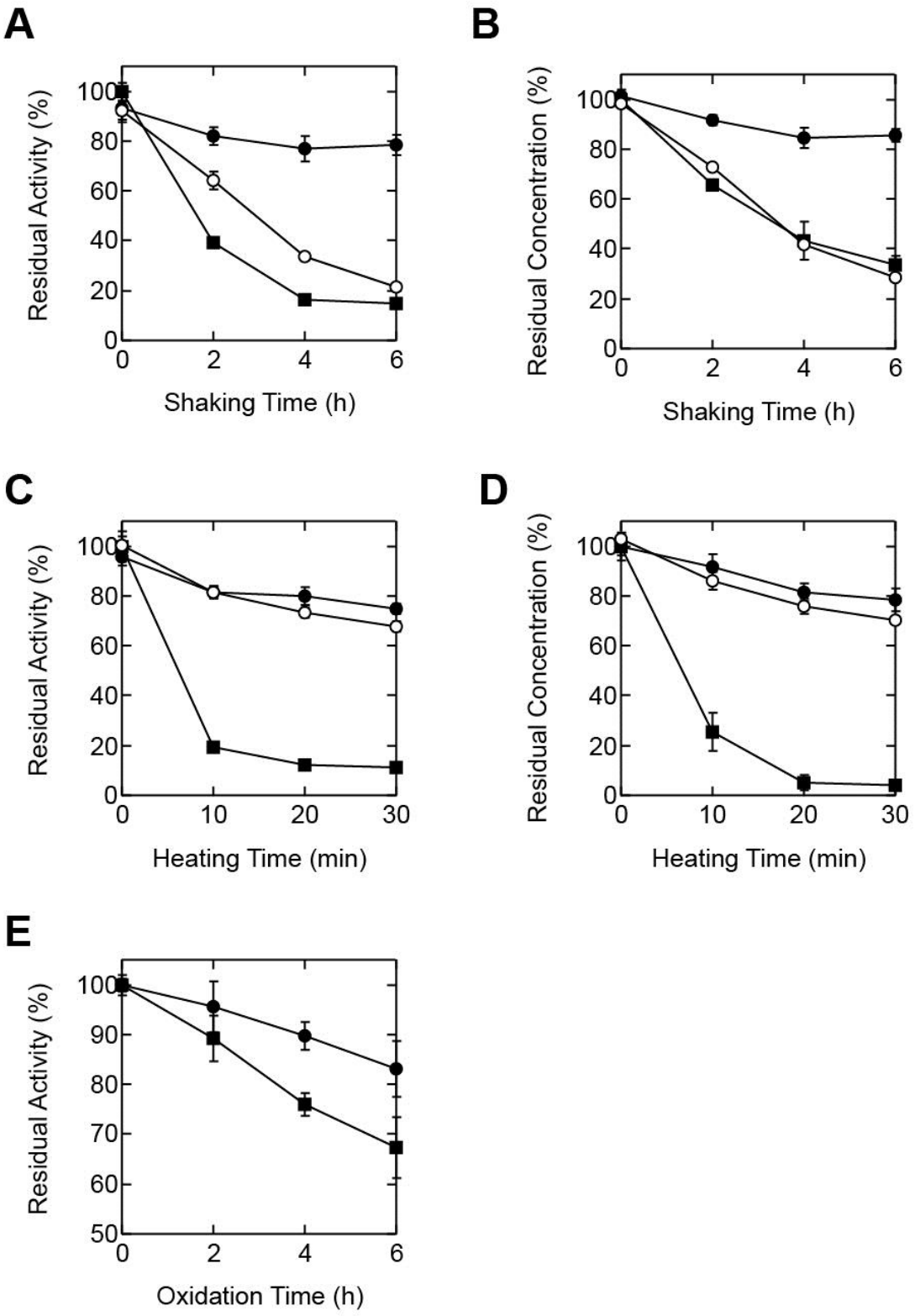

Figure 4. Stress tolerance tests of aggregative PPC containing L-asparaginase (ASNase) and polyK. Residual enzyme activity (A, C, E) and concentration (B, D) of redissolved ASNase after shaking at 500 rpm (A, B), heating at 60 °C (C, D), and treatment with 0.1% H_2O_2 (E) for various periods.

Closed circles, PPC precipitate; open circles, PPC suspension; closed squares, native ASNase. A portion of the data is from reference [20].