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Mono-sized microspheres modified with poly(ethylenimine) facilitate the refolding of like-charged lysozyme

Chun-Yan Yang, Lin-Ling Yu, Xiao-Yan Dong, Yan Sun *

Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

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ABSTRACT

Three mono-sized poly(glycidyl methacrylate) (PGMA) microspheres of 0.8, 1.8 and 2.5 μm in particle sizes were prepared via dispersion polymerization. Poly(ethylenimine)s (PEIs) of different molecular weights (60000 and 1200) were coupled to the PGMA bead surface to prepare cationic beads of different charge densities and ligand (cationic group) structures. The cationic microspheres were used to explore the effect of solid phase properties on like-charged lysozyme refolding. The refolding yield increased with increasing bead concentration and charge density. At low bead concentration range, it increased more significantly with bead concentration with the microspheres of higher charge density. This indicates that the microspheres of high charge density are beneficial in facilitating protein refolding. The refolding yield was independent of the cationic group structure. However, PEI of higher molecular weight was favorable in the preparation of microspheres of higher charge density. Smaller-sized particles have higher specific surface area, so they facilitated lysozyme refolding more significantly. By addition of the charged beads, about 90% refolding yield of 4 mg/mL lysozyme could be achieved. The studies provided more insight into the effects of like-charged solids on protein refolding, which would help design more efficient charged media for facilitated protein refolding applications.

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1. Introduction

Bacterial expression systems have been widely used for the production of recombinant proteins because of their high level of expression and ease of scale-up. The heterologous proteins expressed by the systems often form inactive insoluble aggregates, the so-called inclusion bodies (IBs) [1]. The formation of IBs can make the subsequent separation and purification of target proteins easy, if the proteins can be readily refolded *in vitro* [2]. However, since most proteins expressed as IBs have poor refolding yield *in vitro*, it is challenging to obtain active proteins from IBs by efficient *in vitro* refolding, especially at high protein concentrations. Generally, a protein refolding process is determined by the kinetic competition between refolding and other side reactions, including misfolding and aggregation of the denatured protein [3–5]. It is well recognized that unfolded proteins and/or their folding intermediates are prone to aggregation by hydrophobic interactions [6–8], leading to low refolding yields. Therefore, the key strategy to enhance protein refolding is to find a way to the inhibition of aggregation in the refolding process [9,10].

To inhibit protein aggregation, chemical compounds that can suppress protein–protein interactions are often employed as refolding additives [5,11,12]. In recent years, some functional polymers or polymer particles have also been used to enhance protein refolding [13]. Ge et al. fabricated a novel type of particles by grafting thermosensitive poly(*N*-isopropylacrylamide) (PNIPAM) onto polystyrene (PS) cores [14]. The hydrophobic groups on the solid surface could interact with protein molecules and prevent the intermolecular hydrophobic interactions, which suppressed the aggregation of denatured proteins. Copolymeric particles derived from PNIPAM could suppress protein aggregation more effectively through moderate hydrophobic interactions between the copolymers and protein molecules [15]. Eudragit, as a pH-sensitive polymer comprising negatively charged carboxyl groups could form an electrostatic complex with the oppositely charged denatured protein, and the electrostatic protein–polymer interaction could shield hydrophobic residues on the unfolded polypeptide surface, thus reducing the hydrophobic interaction induced aggregation [16].

Recently, our group has found that like-charged resin particles in a refolding solution can greatly enhance the refolding yield at high protein concentrations [17]. It was considered that the electrostatic repulsion between the like-charged resin and protein induced the oriented alignment of protein molecules near the charged solid surface, which maximized the electrostatic repulsion

* Corresponding author. Tel.: +86 22 27404981; fax: +86 22 27406590.
E-mail address: ysun@tju.edu.cn (Y. Sun).

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between unfolding protein molecules, leading to the suppression of protein aggregation. To explore more details of the facilitating effect, Yu et al. studied the effects of like-charged solid properties on protein refolding with Sepharose FF-based resins [18]. It was found that charge density had significant contribution to the enhancing effects on lysozyme refolding. At low resin concentration range (<0.04–0.1 g/mL), the refolding yield increased with increasing charge density up to 1200 mmol/L. At the highest charge density studied (1200 mmol/L), the effective porosity of the resin for lysozyme was almost zero. It implies that the outer surface of the particles played the dominant role in facilitating protein refolding. The result thus suggests that non-porous microspheres that afford high specific surface area and high charge density would be most favorable for the facilitated refolding. Consequently, we have herein synthesized non-porous mono-sized microspheres of 0.8–2.5 μm in particle sizes with different charge densities and ligand structures to study their effects on protein refolding. The research is expected to help develop more efficient media for protein refolding applications.

2. Experimental

2.1. Materials

Chicken egg white lysozyme, *Micrococcus lysodeikticus*, poly(ethylenimine) (PEI) solutions (50% (w/w) solution in water) with mean molecular weights (M_n) of 60000 and 1200, glycidyl methacrylate (GMA), trimethylolpropane trimethacrylate (TRI) and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Glutathione oxidized (GSSG), glutathione reduced (GSH), urea, tris(hydroxymethyl)aminomethane (Tris) and dithiothreitol (DTT) were from Dingguo Biotech (Beijing, China). Cystamine dihydrochloride was from Alfa Aesar (Lancashire, UK). Ethylenediaminetetraacetic acid disodium (EDTA), polyvinylpyrrolidone (PVP K-30) and azodiisobutyronitrile (AIBN) were purchased from Guangfu Fine Chemical Research Institute (Tianjin, China).

2.2. Fabrication of mono-sized microspheres

Mono-sized poly(glycidyl methacrylate) (PGMA) microspheres were prepared by dispersion polymerization as described previously [19] with minor modifications in the concentrations of the monomer and stabilizer in polymerization. Briefly, PVP K-30 was dispersed in 90% ethanol solution, which is used as the continuous phase. Predetermined amounts of GMA and AIBN were dissolved in the solution under nitrogen atmosphere. The polymerization system was kept under stirring (200 rpm) and reflux condensation at 70 °C for 3 h. Then, TRI (crosslinker) was fed into the mixture, and the polymerization was kept going for another 21 h. After cooling, PGMA microspheres were collected by washing with ethanol and distilled water to remove any unreacted monomers or other organic matter.

2.3. Charge modification of microspheres

The PEI grafted microspheres were prepared following the method reported previously [20] with minor modifications. In brief, 1 g of PGMA beads was added into an aqueous solution (20 mL) made of 2 mol/L Na_2CO_3 and various concentrations of PEI of different molecular weights (60000 and 1200). The mixture was kept under agitation at 25 °C and 170 rpm for 120 h. Then, the modified microspheres were washed with distilled water to remove excess PEI. In this work, the charge density of the PEI grafted beads was adjusted by the initial PEI concentrations and PEI molecular weights in the reaction mixture [21].

2.4. Characterization of mono-sized microspheres

The morphology of microspheres was characterized by a S4800 scanning electron microscopy (SEM) from Hitachi (Tokyo, Japan). Particle size distribution, which was represented by the volume-weighted size range of 5–95%, was assayed by Mastersizer 2000U particle size analyzer from Malvern Instruments (Worcestershire, UK), and the mean diameter was represented by volume-weighted average diameter. The structural characterization of the beads was performed by a Nexus Fourier transform infrared spectroscopy (FTIR) from Thermo Nicolet (Madison, WI, USA).

Assays of the charge density and protein adsorption capacity of the PEI grafted microspheres were performed with wet beads. The charge density of the beads was assayed by the acid–base titration [20]. The protein adsorption capacity of the beads was characterized by BSA binding experiments in the batch adsorption. The PEI grafted beads (0.05 g) were added into 5 mL BSA solution (1 mg/mL) at pH 8.5 (20 mmol/L Tris–HCl buffer). The system was kept under agitation at 25 °C and 170 rpm for 24 h. Then the mixture was centrifuged at 5000 rpm for 10 min, and the supernatant was collected. The amount of BSA in the solution was calculated by absorbance measurements at 280 nm. The adsorption capacity of the PEI grafted beads was calculated from the differences in the BSA contents of initial and final solutions.

2.5. Denaturation and refolding of lysozyme

Lysozyme was denatured and reduced by dissolving in the denaturation buffer (20 mmol/L Tris–HCl, 1 mmol/L EDTA, 8 mol/L urea and 20 mmol/L DTT, pH 8.5) to a final concentration of 20 mg/mL. The protein solution was incubated at 40 °C for 3 h [22]. Lysozyme was completely reduced and denatured under this condition.

The equilibrium experiments of lysozyme refolding were performed in 1.5 mL microcentrifuge tubes, which were kept in a shaking incubator at 170 rpm and 25 °C for 3 h. The denatured lysozyme solution was diluted by refolding buffer (20 mmol/L Tris–HCl, 1 mmol/L EDTA, and predetermined concentrations of urea, GSSG, GSH, and wet PEI grafted beads, pH 8.5) to a final solution containing 1 mg/mL lysozyme, 0.6 mol/L urea, 2 mmol/L GSSG, 6 mmol/L GSH, 0–200 mg/mL PEI grafted beads (dry weight), 20 mmol/L Tris–HCl and 1 mmol/L EDTA (pH 8.5).

To compare with the literature data [17], we performed lysozyme refolding at 4 mg/mL with cystamine as the oxidizing agent and DTT as the reducing agent. In this case, the denatured lysozyme solution (60 mg/mL) was diluted by the refolding buffer to a final solution containing 4 mg/mL lysozyme, 1.2 mol/L urea, 5.4 mmol/L cystamine (or GSSG), 4.4 mmol/L DTT, 20 mmol/L Tris–HCl and 1 mmol/L EDTA (pH 8.5). Refolding experiments were carried out in the absence and presence of 200 mg/mL PEI grafted beads (dry weight). The reaction was carried out by 3-h incubation to ensure lysozyme refolding to reach equilibrium [17,18]. The refolding experiments were performed in triplicate and the average value was adopted.

The kinetic refolding experiments were also carried out in 1.5 mL microcentrifuge tubes, which were kept in a shaking incubator at 170 rpm and 25 °C. The denatured lysozyme solution was diluted by the refolding buffer to a final solution containing 1 mg/mL lysozyme, 1.2 mol/L or 0.6 mol/L urea, 2 mmol/L GSSG, 6 mmol/L GSH, 20 mmol/L Tris–HCl and 1 mmol/L EDTA (pH 8.5). Refolding experiments were carried out in the absence and presence of 100 mg/mL PEI grafted beads (dry weight). During the refolding process, small aliquots were withdrawn from the refolding mixture at different time intervals, and the enzyme activity was measured immediately. The kinetic experiments were

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