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Refolding of Denatured/Reduced Lysozyme Using Weak-Cation Exchange Chromatography

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Abstract: Oxidative refolding of the denatured/reduced lysozyme was investigated by using weak-cation exchange chromatography (WCX). The stationary phase of WCX binds to the reduced lysozyme and prevented it from forming intermolecular aggregates. At the same time urea and ammonium sulfate were added to the mobile phase to increase the elution strength for lysozyme. Ammonium sulfate can more stabilize the native protein than a common eluting agent, sodium chloride. Refolding of lysozyme by using this WCX is successfully. It was simply carried out to obtain a completely and correctly refolding of the denatured lysozyme at high concentration of 20.0 mg/mL.

Keywords: Weak-cation exchange chromatography, lysozyme, protein refolding.

Protein folding remains one of the key unresolved issues in biochemistry. During protein refolding, many proteins tend to aggregate, causing a significant reduction in the yield of active protein[1]. Though much effort has been expended to solve this problem, no universal method has been established. Refolding by liquid chromatography (LC) is a new method of refolding proteins and has been paid much attention in recent years[2, 3].

The decrease in yield at a high concentration of protein has been explained by the kinetic competition of folding and incorrect aggregation[1]. Aggregation of protein would be greatly suppressed when the individual protein molecules are separated from each other. A promising method for achieving the separation of protein molecules during refolding is to immobilize the folding polypeptide onto a solid support. Several studies demonstrated that the attachment of proteins to a solid support eliminated aggregation and facilitated protein renaturation[3]. However, one drawback of such methods is that the affinity tag must be removed after renaturation for obtaining the intact recombinant proteins. At present, by using ion-exchange chromatography (IEC) as a refolding tool[3] exists the same problem.

In this article, lysozyme was chosen as a model protein, and an ion-exchange method was developed to refold the denatured/reduced lysozyme. Refolding by using IEC, denatured protein is reversibly bonded onto the surface of the ion-exchanger. During lysozyme refolding aggregation is strongly depressed and the renaturation yield is

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improved. Our results demonstrated that IEC can be applied to the simultaneous refolding and purification of the target protein from inclusion bodies containing various contaminants.

Experimental

The chromatographic system employed consists of both LC-10ATvp pump and UV-VIS spectrophotometer (Shimadzu, Japan). A 100×4.6 mm I.D. column was packed into the high performance weak-cation exchanger packing, which synthesized by ourselves[4].

Mobile phase consisted of solution A, 0.1 mol/L Tris-HCl (pH=8.0), 1 mmol/L EDTA, 3 mmol/L reduced glutathione (GSH) /0.6 mmol/L oxidized glutathione (GSSG), and 0-5 mol/L urea, and solution B, 1.0 mol/L sodium chloride (or 1.0 mol/L ammonium sulfate), 0.1 mol/L Tris-HCl (pH=8.0), 1 mmol/L EDTA, 3 mmol/L GSH/0.6 mmol/L GSSG, and 0-5 mol/L urea. All chromatograms were run using linear gradient conditions for 20 min at 1 mL/min, and detection at 280 nm.

Lysozyme (10-25 mg/mL) was dissolved in buffer containing 8.0 mol/L urea, 0.1 mmol/L Tris-HCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) at pH 8.5. Reduction of disulphide bonds and denaturation were archived by incubation for three hours at 40°C with a 25-fold molar excess of dithiothreitol (DTT) over lysozyme disulphides. The reduced and denatured lysozyme was precipitated by the addition of ten volumes of water at 4°C, centrifuged, and washed twice with water. The reduced lysozyme was dissolved in formic acid (pH 2.0), dialyzed extensively against 10 mmol/L hydrochloride acid, lyophilized, and stored at -20°C[5].

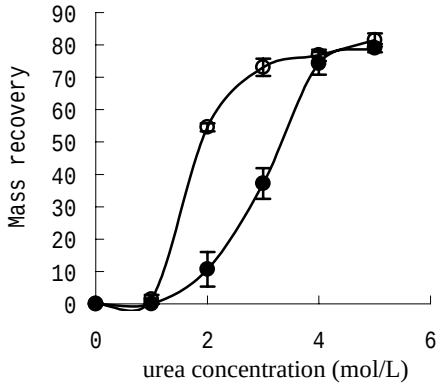
The denatured/reduced lysozyme (dissolved in 8.0 mol/L urea) of 2.0 mg/ml, unless otherwise specified, was directly injected into the WCX column, eluting and the fraction containing lysozyme was collected. For kinetics request, after 4 hours incubation at ambient temperature, the enzymatic activities of the collected fractions were measured[6]. By comparison, dilution method was used at the same time. The denatured lysozyme was rapidly diluted with the renaturation buffer. The pooled fractions of mobile phase, which ran the same gradient except the sample was not loaded, were used as the renaturation buffer of dilution method, and enzymatic activities was measured also after 4 hours incubation for kinetics request.

Results and Discussion

The effects of urea on the IEC of hen egg lysozyme were reported[7]. Generally, the presence of urea decreases lysozyme retention. In other words, urea increases the eluting strength of mobile phase in IEC. On the other hand, urea has often been employed as an effective protein solubilizing agent in IEC[7]. As **Figure 1** shown, the mass recovery of lysozyme increases with the increase of urea concentration in the mobile phase. The changeable trend is the same for the both of sodium chloride and ammonium sulfate as eluting agent. In IEC, sodium chloride is usually used as a commonly eluting agent. Ammonium sulfate is a stronger eluting agent. When the same concentration of urea was used, the mass recovery of using ammonium sulfate as

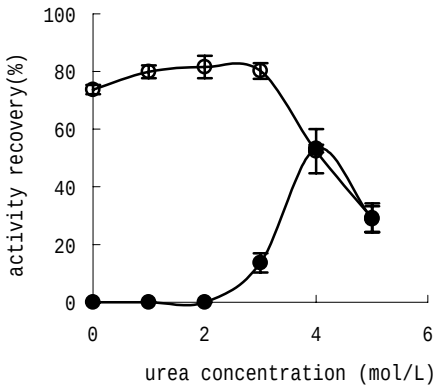
an eluting agent is more than that of using sodium chloride, especially at lower urea concentrations (**Figure 1**).

Figure 1 Effect of sodium chloride and Ammonium sulfate used as eluting agent on the mass recovery of lysozyme in the presence of urea with different concentrations.



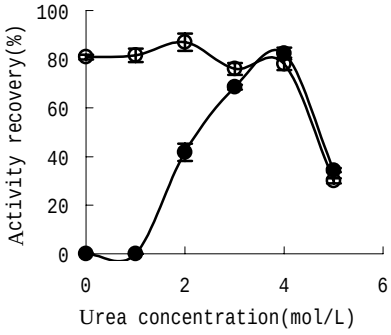
●, sodium chloride; ○, ammonium sulfate

Figure 2 Effect of sodium chloride as elutingagent on the lysozyme refolding in the presence of urea with different concentrations.



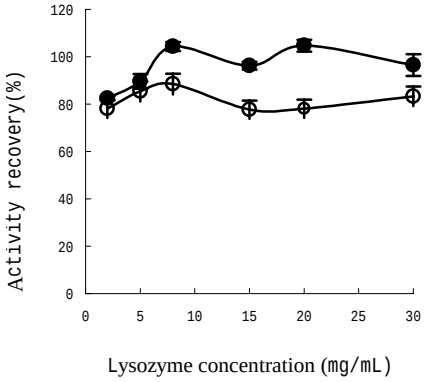
●, refolding by WCX; ○, dilution method

Figure 3 Effect of ammonium sulfate as eluting agent on the lysozyme refolding of in the presence of urea with different concentrations.



●, refolding by WCX; ○, dilution method

Figure 4 Effect of concentration loading of lysozyme on the its refolding.



●, refolding by WCX; ○, dilution method

As shown in **Figures 2** and **3**, the changes in the bioactivity recovery of lysozyme with urea concentration is similar to that of the mass recovery for both ammonium sulfate and sodium chloride as eluted agent, when urea concentration is lower than 4

mol/L. So the loss of bioactivity recovery of the refolded lysozyme is mainly due to the loss of protein mass when the urea concentration is lower than 4 mol/L. In **Figure 2**, it can be seen that the bioactivity recovery of the refolded lysozyme by both WCX and dilution decrease sharply when urea concentration is higher than 4mol/L.

However, **Figure 3** shows that the folded yield of the reduced lysozyme does not decrease sharply with the increasing of urea concentration. In the presence of 4 mol/L urea and ammonium sulfate, the final folding yield of the reduced lysozyme increases. Ammonium sulfate, which thermodynamically stabilizes native lysozyme[8], is effective for the increasing the folded yield of the reduced lysozyme in the presence of high urea concentration.

Due to the kinetic competition between folding and aggregation, aggregation predominates upon refolding at high protein concentrations. Reduced lysozyme, with positive charge, can be bound to the cation-exchanger by means of electrostatic interaction. Aggregation should be eliminated due to the adsorption of the reduced polypeptide on the solid support. As it would be expected, the renaturation of lysozyme by using IEC can be performed at a much higher protein concentration comparing with renaturation in solution under the same conditions (**Figure 4**). The renaturation yield approachs to 100% at the protein concentration of 20.0 mg/mL. For the successful oxidative refolding, the key point is that how to depress the formation of both protein aggregates and intermolecular inappropriate disulfide bonds. The latter is easy to occur if the formations of the tertiary structure and of disulfide bonds start at the same time[9]. If the intermediate states, which have native-like tertiary structure, are separately trapped from each other, the following formation of the disulfide bonds may proceed successfully.

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