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Selective extraction of histidine derivatives by metal affinity with a copper(II)–chelating ligand complex in an aqueous two-phase system

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ABSTRACT

Affinity extraction based on the interaction between a target molecule and a specific affinity ligand offers a novel separation system for biomolecules in an aqueous two-phase system, however, most of affinity ligands are expensive. In the present study, metal affinity extraction of histidine (His) derivatives using a complex between Cu(II) and a commercially available chelating ligand was studied in a poly(ethylene glycol) (PEG)/Li_2SO_4 ATPS. Alizarin complexone (3-[*N*,*N*-bis(carboxymethyl)amino methyl]-1,2-dihydroxy anthraquinone, AC) was selected as the chelating ligand because of the good extractability of Cu(II) into the upper PEG-rich phase. On the basis of coordinate bonding with Cu(II), the extraction of His in the presence of the Cu(II)–AC complex under neutral condition was 73%, which was much higher than that under Cu(II) free condition (13%). Among a series of divalent transition metal ions (Cu(II), Ni(II), Co(II), and Zn(II)), Cu(II) was the most effective for the extraction of His. Derivatives of His were selectively extracted in the presence of many other amino acids because of the specificity of the interaction between Cu(II) and imidazole group of His. Extracted His was quantitatively stripped from the Cu(II)–AC complex using competitive complexation with agents such as iminodiacetic acid and imidazole.

1. Introduction

Aqueous two-phase systems (ATPS), which are formed by combining certain inorganic salts and aqueous solutions of water-soluble polymers, have been studied for the separation of biomolecules because of their mild and non-denaturing conditions [1–3]. Bioprocesses for the recovery and purification of many biological products, such as proteins, genetic material, bionanoparticles, cells, and organelles, have been developed using ATPS [4,5]. The extraction mechanism of biomolecules in an ATPS is complex and not easy to predict. However, the extraction generally depends on physico-chemical properties such as the isoelectric point, surface hydrophobicity, and molecular weight.

Affinity extraction is based on the specific interaction between a target molecule and an affinity ligand, and could effectively improve the selectivity of ATPS extraction [6–9]. Bioaffinity, such as that between an enzyme and substrate, has been used for separation of proteins [10,11]. Andrews et al. reported purification of thaumatin and trypsin using glutathione and trypsin inhibitor as affinity ligands [12]. The affinity extraction of pre-purified plasmid DNA using poly(ethylene glycol) (PEG) derivatized zinc finger–glutathione *S*-transferase fusion protein was also examined [13]. Reactive dyes have been used as affinity ligands in ATPS [14]. In many cases, the affinity ligands are covalently bound to one of the phase-forming polymers. However, free affinity ligands, which preferentially distribute in the extracting phase, can also be used for affinity extraction [15]. We recently developed a selective extraction system for lysine-rich protein cytochrome *c* using a macrocyclic compound, dicyclohexano-18-crown-6, in ATPS [16]. Cytochrome *c* was selectively extracted from other cationic proteins in a hydrophobic PEG-rich phase by complexation between amino groups of the lysine residues and the crown ether.

Metal affinity, which is based on coordinate bonding between functional groups in biomolecules and metal ions, is effective for increasing specificity to a target biomolecule. Immobilized metal affinity chromatography (IMAC) uses metal ions adsorbed on chelating adsorbents for separation, and has been developed as a purification method for recombinant proteins [17–20]. Separation with IMAC is based on differences in the affinities between immobilized metal ions and the functional groups of biomolecules. Typically, histidine (His) residues show strong affinity for transition metal ions such as Cu(II), Ni(II), Co(II), and Zn(II) immobilized on chelating adsorbents, and this affinity is based on the principles of hard and soft acids and bases [21]. Metal affinity can also be used for the separation of smaller His derivatives. For

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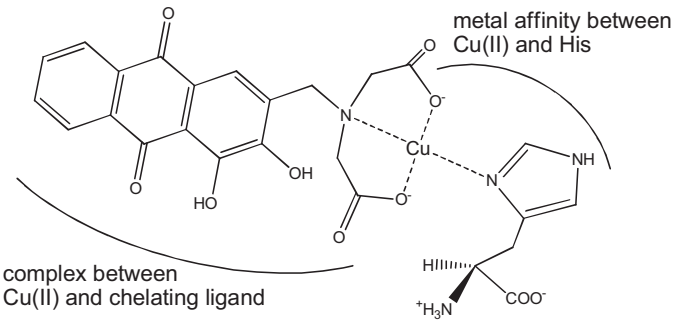

Fig. 1. Schematic for the extraction of His by metal affinity using a chelating ligand in ATPS.

instance, immobilized metal affinity adsorption was effective for the selective recovery of His-containing dipeptides such as carnosine (Car, β-alanyl-L-histidine) and anserine (Ans, β-alanyl-L-1-methylhistidine) [22–25]. These dipeptides are physiologically important because they act as cytosolic buffering agents, metal ion chelators, and anti-oxidants, and can be selectively adsorbed using Cu(II) immobilized chelating resins. Metal affinity can be applied for the separation of proteins in ATPS. Arnold and coworkers developed an iminodiacetic acid (IDA) derivative of PEG for metal affinity extraction of proteins [26–28]. Addition of Cu(II)-immobilized IDA-PEG improved the protein extraction, and the level of improvement was related to the number of His residues. Various proteins can be extracted using Cu(II)-immobilized IDA-PEG in ATPS [29–32]. Recently, Lu et al. developed a recyclable affinity ligand for immobilized metal affinity separation using a temperature-induced effect [33]. A random copolymer of ethylene oxide and propylene oxide that introduced IDA groups was used for metal affinity extraction of nattokinase. These metal affinity extractions have been conducted using a phase-forming polymer bearing chelating groups such as IDA. However, preparation of a chelating polymer is not easy, and the chelating groups are introduced only at the terminal ends of the polymer. A relatively large chelating polymer should be used for metal affinity extraction of proteins because the resulting complex should be much more hydrophobic than the native protein. By contrast, smaller chelating ligands can be used for the metal affinity extraction of smaller His derivatives such as Car and Ans. No previous research for metal affinity extraction in ATPS using commercially available chelating ligands must be published.

In the present study, a novel metal affinity extraction for His and His derivatives using the complex between an intermediate metal ion and a commercially available chelating ligand in ATPS was studied. Fig. 1 shows the extraction of His using the metal complex. An intermediate metal ion such as Cu(II) forms a complex with a chelating ligand bearing an IDA group in PEG/salt ATPS. If the resulting complex is hydrophobic, the complex should distribute in the hydrophobic PEG-rich phase. By contrast, His in the ATPS will coordinate with Cu(II) at residual coordination sites. The resulting ternary metal complex (chelating ligand-Cu(II)-His) can be distributed in the hydrophobic PEG-rich phase. The metal affinity extraction system could be used to separate His derivatives from other amino acids.

Initially, the chelating ligand for the extraction of Cu(II) was investigated using commercially available chelating ligands. Extraction of His using the complex between Cu(II) and AC was studied in detail to clarify the optimal conditions for His extraction. The extraction selectivity for His and His derivatives using the Cu(II)-AC complex over other amino acids on the basis of metal affinity was also studied. Furthermore, back extraction of His extracted by the Cu(II)–AC complex in the PEG-rich phase was examined by adding competitive complexation reagents.

2. Experimental

2.1. Materials

The chelating ligands used in this study (Fig. S1) were all commercially available and had one or two IDA groups. For use as chelating ligands, analytical grade diethylene triamine pentaacetic acid (DTPA), ethylene glycol tetraacetic acid (EGTA), xylenol orange (3,3-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-*o*-cresolsulfonephthalein tetrasodium salt, XO), 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), and trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CyDTA) were purchased from Dojindo Laboratories (Kumamoto, Japan) and used as received. Analytical grade AC (3-[*N*,*N*-bis(carboxymethyl) aminomethyl]-1,2-dihydroxyanthraquinone) was purchased from Tokyo Kasei Co. Ltd (Tokyo, Japan).

The following analytical grade amino acids, dipeptides, and their derivatives (Fig. S2) for the extraction tests were purchased and employed without further purification: glycine (Gly), L-alanine (Ala), Ans, His, L-leucine (Leu), L-valine (Val), (Wako Pure Chemical Industries, Osaka, Japan), Car, Ala-His, L-histidine methyl ester (Sigma-Aldrich Japan K.K., Tokyo, Japan), and 1-trityl-L-histidine (H-His(Trt)-OH, Merck, Hohenbrunn, Germany). An aqueous mixed solution containing 17 amino acids (Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Cys, Met, Ile, Leu, Tyr, Phe, His, Lys, and Arg) was prepared using an amino acids standard solution (Wako Pure Chemical Industries). PEG8000 with an average molecular weight of 8000 g mol^{-1} was purchased from Wako Pure Chemical Industries. All other reagents were of reagent grade and were used as received.

The logarithm of the partitioning coefficient between n-octanol and water (logP) is widely accepted as a quantitative indicator of the hydrophilic-lipophilic balance. The logP values of the chelating ligands, amino acids, and His derivatives were estimated using MarvinSketch 6.2.1 (ChemAxon Ltd., Budapest, Hungary) by the KLOP method [34]. As the chelating ligands deprotonate depending on the pH, the apparent logP values of the chelating ligands were calculated as logD values at pH 4.5 using this program.

2.2. Extraction of Cu(II) in ATPS containing the chelating ligand

Extraction tests were performed in an ATPS composed of 11.0% (mass fraction) Li_2SO_4 and 10.0% (mass fraction) PEG 8000 [15,16]. An aqueous solution containing $CuSO_4$ (0.3 mmol/L) and a chelating ligand (3.75 mmol/L) was prepared using stock solutions and the pH was adjusted with 100 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. The aqueous solution containing Cu(II) and the chelating ligand was mixed with 3.0 mL of 26.5% (mass fraction) PEG8000 and 1.00 g of Li_2SO_4. The volume of the resulting solution was adjusted to 8.0 mL by adding distilled water and the solution was transferred to a polypropylene centrifuge tube. The solution was shaken vigorously (1500 rpm) using a mixer (CM-1000, EYELA, Tokyo, Japan) for 30 min, then centrifuged at 6000 rpm for 3 min to induce phase separation. The upper phase (PEG-rich phase) and lower phase (salt-rich phase) were separated and their volumes measured. After diluting using distilled water, the concentrations of Cu(II) in the aqueous solutions were analyzed using an atomic absorption spectrophotometer (AAnalyst 100, Perkin Elmer, Waltham, MA, USA).

The amounts of Cu(II) ($n_{Cu(II)}$ [mmol]) in the salt-rich phase and the PEG-rich phase were determined using the following equation:

$n_{Cu(II)} = C_{Cu(II)} \times V$, where $C_{Cu(II)}$ is the concentration of Cu(II) in each phase [mmol/L], and V is the volume of each phase after