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Protein microarray using α-amino acids as metal tags on chips

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Abstract—Procedures for synthesizing α-amino acids on a chip for coordination with transitional metal ions and a His-Tagged protein have been successfully developed as a stable protein microarray. Using the recombinant His-Tagged 3CL-protease ($3CL^{pro}$) as a model for attachment to chips containing D-/L-Glu, Asp, Orn, Ser via different transitional metal ions, it was found that the Orn chip was the best of affinity binding and stability by which Zn^{2+} was the best metal ion for affinity while Co^{2+} was the best metal ion for stability. Thus, this protein microarray can be alternatively used as a high throughput screening method for rapid detection against SARS CoV $3CL^{pro}$ and/or efficient purification of other Tagged proteins.

Immobilized metal affinity chromatography (IMAC) is a separation technique that uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which can serve as affinity ligands for various proteins.[1–5] Although IMAC is widely used for the separation of proteins having exposed histidine groups, most of used ligands are immobilized on the solid supports as resins or acrylamides in the columns.[6–13] Recently, microarray has been developed as a high throughput technique for proteomic and genomic analysis; for example, proteins bound to ligands on surfaces have been used to detect protein–protein interactions, enzymatic substrates, and protein–small molecule interaction.[14–19] However, development of this approach for site-specific immobilization of proteins onto chip surfaces still represents a significant challenge. In this study, we describe a new procedure to prepare arrays for proteomic analysis, which combines two techniques between IMAC and microarray, using the α-amino acids as metal tags on chips in chelating ability.

According to our previous report, a unique peptide sequence of HGGHHG showed a chelating ability with a transitional metal ion on a chip better than hexa-His peptide.[20] It indicated that amino acids in the peptide sequence play an important role in affinity binding. Moreover, it is well known that free α-amino acids have good chelating ability to coordinate with transitional metal ions.[1,2] Therefore, we focused on investigating the α-amino acids that have the strongly affinity binding with the His-Tagged protein. We also examined the affinity binding of His-Tagged protein on chips to coordinate with transitional metal ions in aqueous solution. Briefly, an appropriate α-amino acid was introduced onto a commercial amine-glass chip (SuperAmine, TeleChem International Inc.) using a peptide synthesizer (Rainin) by two different ways for coupling of amino acids to amino-glass chip: (i) coupling with side-chain carboxy groups of Glu and Asp (Boc-Glu-*O*-*t*-Bu and Boc-Asp-*O*-*t*-Bu, 0.1 mmol), and (ii) derivatization of amino-glass with succinic anhydride followed by coupling with the side-chain amino and hydroxy groups of Orn and Ser (Boc-Orn-*O*-*t*-Bu and Boc-Ser-*O*-*t*-Bu, 0.1 mmol). The protecting groups of the amino acid

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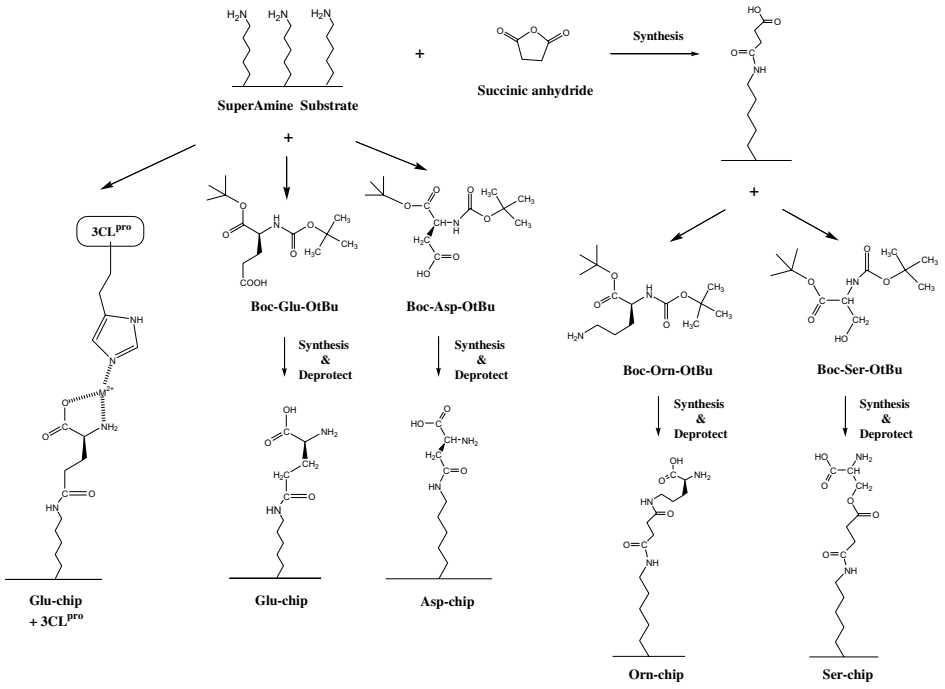

Figure 1. Coupling of the α-amino acid derivatives (0.01 mM) onto a SuperAmine chip. The recombinant His-Tagged $3CL^{pro}$ protein is conjugated on chips via transitional metal ions (M^{2+}).

derivatives on the chips were cleaved by 95% TFA, 2.5% TIS, and 2.5% water (Fig. 1). Each chip was compared the affinity binding with a recombinant SARS CoV main protease ($3CL^{pro}$, 0.15 mg/ml), which is essential for life cycle of SARS coronavirus and expressed in *E. coli* containing a His-Tagged at C-terminus[21–24], in a mild aqueous solution (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, and 10 mM metal ions, such as Cu^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+}) for 30 min. Using the first antibody rabbit anti-$3CL^{pro}$ (1/1000 dilution) against the $3CL^{pro}$ protein and its secondary antibody labeled with FITC (1/50 dilution, FITC Goat Anti-Rabbit IgG conjugate, Zymed Laboratories Inc.), there were clearly monitored the fluorescent spots on chips by a fluorescence scanner (Typhoon 9200 image scanner, Amersham Biosciences). The specificity and affinity binding of bound His-Tagged $3CL^{pro}$ on chips were examined by chelating with different metal ions and by eluting with imidazole (150 mM) as a chelating reagent for 30, 90, and 120 min, as shown a model of His-Tagged array in Figure 2. The positive controls without metal ions and normal proteins without His-Tagged were shown to have no binding to the chips.

After each chip was against antibody and washed in washing buffer containing 50 mM NaH_2PO_4 pH 8 and 300 mM NaCl for 30 min, we found that the recombinant His-Tagged $3CL^{pro}$ protein was found to have the different specific binding on each immobilized chip (Fig. 3). The binding order based on their binding

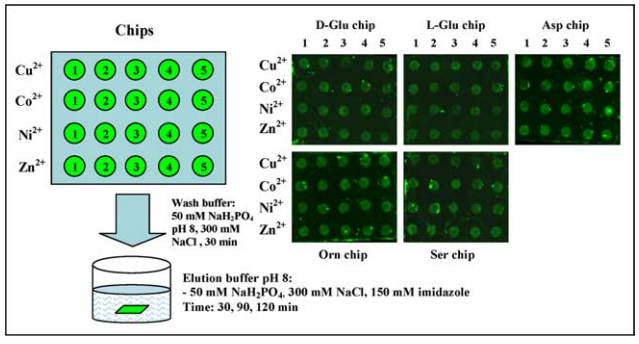

Figure 2. Immunofluorescent binding test of recombinant His-Tagged $3CL^{pro}$ protein on different immobilized chips of D-Glu, L-Glu, Asp, Orn or Ser, after chelating with different metal ions (10 mM) and eluting by 150 mM imidazole for 30, 90, and 120 min. Each chelating metal ions were repeated in five spots (no. 1–5).

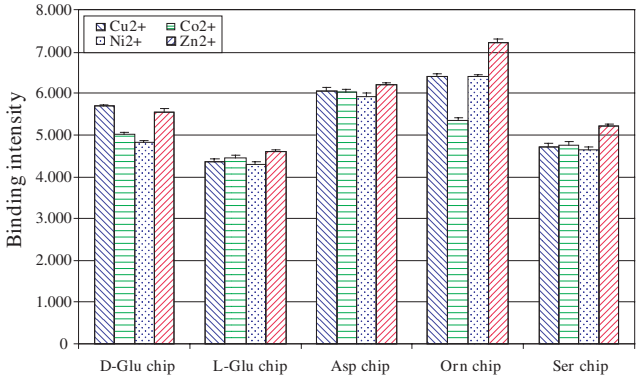

Figure 3. Binding intensity of recombinant His-Tagged $3CL^{pro}$ protein on different immobilized chips of D-Glu, L-Glu, Asp, Orn, and Ser after conjugation with FITC-labeled antibody and detection under fluorescent scanner. Each chip was conjugated with a His-Tagged $3CL^{pro}$ protein by using different chelating metal ions of Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}, without elution of imidazole.

fluorescent intensity was Orn > Asp > D-Glu > Ser > L-Glu chip, respectively. Orn chip was found to have higher specific binding with $3CL^{pro}$ protein than other chips, especially in the presence of Zn^{2+}. By comparison of transitional metal ions, all transitional metal ions had the different coordination ability in binding with the recombinant His-Tagged $3CL^{pro}$ protein on different immobilized chips. Zn^{2+} as a chelating metal ion formed the complex with protein on chips better than other metal ions, except on D-Glu chip. Depending on the functional side chain and polarity of amino acid, Orn chip having a basic side chain and polar group of NH_2 was found to have the specific binding with $3CL^{pro}$ protein higher than other chips having acidic (D-/L-Glu and Asp) or neutral (Ser) side chains and polar group. It indicated that the amino acid side chain is also directly affected to the specific binding of protein on chips. According to our previous report, a unique peptide sequence of HGGHHG on chip which contained Gly having a neutral side chain and nonpolar group showed to have the specific binding with His-Tagged protein lower than a homologue peptide sequence of 6 × His having polar group.[20] Hence, D-Glu, L-Glu and Asp chips having acidic side chain and polar group showed a few difference of specificity with $3CL^{pro}$ protein, in which Asp chip was found to have the specific binding with $3CL^{pro}$ protein rather than D-Glu and L-Glu chips. In addition, D-Glu chip showed the specific binding with $3CL^{pro}$ protein than L-Glu chip. It indicated that the orientation of conformation of amino acid derivative plays an important role for the specific binding of protein interaction.

Furthermore, the binding ability and stability of His-Tagged $3CL^{pro}$ protein conjugated on chips were examined by eluting with imidazole (150 mM, pH 8.0) in different times of 30, 60, and 120 min (Fig. 4). We found that all transitional metal ions had the different coordination ability in binding with His-Tagged $3CL^{pro}$ protein on chips along 120 min elution. The fluorescent intensities of bound His-Tagged $3CL^{pro}$ on each chip via different transitional metal ions decreased when the elution time was longer. Otherwise, the affinity binding orders of $3CL^{pro}$ protein on chips at different times were L-Glu > Ser > Orn > D-Glu > Asp for 30 min elution and Ser > L-Glu > D-Glu > Orn > Asp for 90 and 120 min elution. After 120 min elution, the Ser chip showed the highest affinity binding while the Asp chip showed the lowest affinity binding. The comparison of metal-binding order of His-Tagged $3CL^{pro}$ protein on chips at different times is summarized in Table 1. All of transitional metal ions have the coordination ability

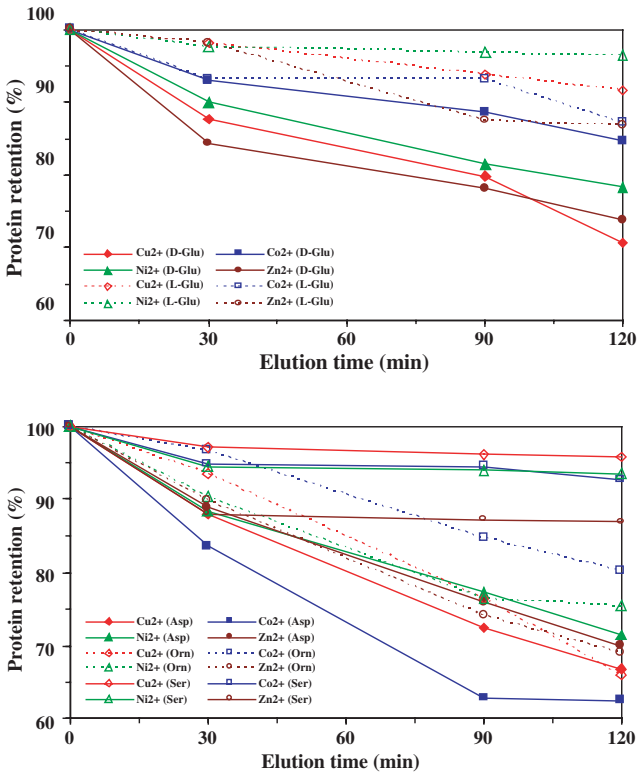

Figure 4. Protein retention of His-Tagged $3CL^{pro}$ protein bound on different chips of D-Glu, L-Glu, Asp, Orn, and Ser after chelating with different metal ions and eluting with imidazole (150 mM) at different times. The protein intensities in five experimental spots are averaged and the initial protein intensity at 0 min is defined as 100% protein retention.

with different selectivity and binding affinity to protein on each immobilized chip. Herein, the metal-binding order on chips is dependent on the site-specificity on α-amino acids, orientation of conformation, functional structure, and transitional metal ions. In the same manner, the specificity and affinity binding of His-Tagged protein on chips via transitional metal ions also had the different coordination ability in sequence order of polypeptides.[20,25] Based on amino acid side chain and charged group, L-Glu and Asp chips having the same acidic side chain and negatively charged group showed the highest specific binding in the presence of Zn^{2+} and the highest affinity binding in the presence of Ni^{2+}. D-Glu chip showed the different specific and affinity binding of His-Tagged $3CL^{pro}$ protein via transitional metal ions from L-Glu and Asp chips because of the different orientation of conformation. Orn chip

Table 1. Specific metal-binding order of recombinant His-Tagged $3CL^{pro}$ protein conjugated on different immobilized chips via the complexation of different transitional metal ions after elution with imidazole for 30, 90, and 120 min

Chips	Side chain/charged	Specific metal-binding orders			
		0 min	30 min	90 min	120 min
D-Glu	Acidic/negative	Cu > Zn > Co > Ni	Co > Ni > Cu > Zn	Co > Ni > Cu > Zn	Co > Ni > Zn > Cu
L-Glu	Acidic/negative	Zn > Co > Cu > Ni	Zn > Cu > Ni > Co	Ni > Cu > Co > Zn	Ni > Cu > Co > Zn
Asp	Acidic/negative	Zn > Cu > Co > Ni	Zn > Ni > Cu > Co	Ni > Zn > Cu > Co	Ni > Zn > Cu > Co
Orn	Basic/positive	Zn > Cu > Ni > Co	Co > Cu > Ni > Zn	Co > Ni > Cu > Zn	Co > Ni > Zn > Cu
Ser	Neutral/uncharged	Zn > Co > Cu > Ni	Cu > Co > Ni > Zn	Cu > Co > Ni > Zn	Cu > Ni > Co > Zn

having basic side chain and positively charged group showed the highest specific binding in the presence of Zn^{2+} and the highest affinity binding or stability in the presence of Co^{2+}. Ser chip having neutral side chain and uncharged group showed the highest specific binding in the presence of Zn^{2+} and the highest affinity binding in the presence of Cu^{2+}. Thus, Zn^{2+} was the best transition-metal ion for affinity binding with His-Tagged $3CL^{pro}$ protein on all chips except D-Glu, while the stability of proteins on chips along 120 min elution is dependent on the amino acid structure and transitional metal ions. Likewise to our previous result of a unique peptide sequences on chip,[20] the His-Tagged protein formed the coordination on chips via transitional metal ions with the different specific and affinity bindings. Therefore, it indicated that the binding ability and stability of His-Tagged protein on chips is dependent on some important factors such as structure of amino acid including polarity, functional side chain and charged group, orientation of amino acid conformation, and transitional metal ions. Although this microarray used only one protein of SARS CoV $3CL^{pro}$ obtained from crude extract for testing, the results indicated that it can be used for detection of this protein that is very important for diagnostics. Otherwise, our results also indicated that one amino acid derivative on chip could conjugate the His-Tagged protein via the transitional metal ion with the high specificity and affinity binding. In addition, this technique using α-amino acids as metal Tags on chips can be used as an alternatively microarrays for further detection and purification of His-Tagged proteins and/or their binding analysis. Moreover, we believe that this technique on chip can also be used as a high throughput technique for studying protein–protein or protein–drug interaction, drug screening, and design against SARS that are very important for therapeutic applications.

In conclusion, we have successfully developed an effective method to prepare a protein microarray by utilizing different chelating properties of α-amino acids. The recombinant His-Tagged $3CL^{pro}$ protein was conjugated on different immobilized chips, containing only one amino acid derivative, via transitional metal ions as protein microarray with the high specificity and affinity binding. Orn chip was shown to be the best ligand for specific binding while Ser chip was the best ligand for affinity binding or stability. Thus, it is believed that this technique can be alternatively used to prepare the protein microarrays for rapid detection and efficient purification of other Tagged proteins. Moreover, it can be further used for protein interaction and drug screening which are very important for therapeutic applications.

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