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A conformationally purified α-helical peptide library

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Abstract—A conformationally purified peptide library was constructed based on a unique idea combining the combinatorial library concept with de novo peptide design. This peptide library was designed to allow peptides to acquire metal-binding ability when they fold into helix–loop–helix structures. Therefore, a mixture of randomized peptides was purified by immobilized metal-affinity chromatography (IMAC) to provide a library of peptides with the homogenous conformation.

Combinatorial peptide libraries have been broadly recognized as useful sources for screening bioactive ligands that bind to receptors and enzymes.[1] However, the identified peptides generally possess considerable conformational flexibility, so they show poor affinity to target molecules due to the high entropic costs.[2] These facts have accelerated attempts to construct conformationally restricted peptide libraries by grafting them onto a rigid natural protein domain or incorporating them into a stable secondary structural motif.[3] However, some randomized peptide sequences often disorder the parent structural motif. Consequently, in the library screening, unfolded impurities existed in the library inhibit folded peptides from specific binding to target molecules due to the non-specific binding. Here, we propose to purify a mixture of randomized peptides to acquire high quality of conformationally restricted peptide libraries. Thus, a peptide library was constructed based on a unique idea combining the combinatorial library concept.[4] with de novo peptide design for synchronizing metal-binding ability with structural folding.[5] This peptide library was designed to allow peptides to acquire metal-binding ability as they assume proper folding, after which they are purified by immobilized metal-affinity chromatography (IMAC) to provide peptides with a homogenous conformation (Fig. 1).

Helix–loop–helix structures allow polypeptides to form stable α-helices, which often present recognition sequences in biological processes.[6] Therefore, we designed and synthesized an intramolecular antiparallel helix–loop–helix peptide (**1**: Ac-AELAALEAE LAALE-G7-KLAALKAKLAALKAY-NH_2) as a template for peptide libraries[7] The N-terminal and C-terminal segments associate with each other to stabilize the α-helical structures. Since the peptide folds by virtue of the interactions between the amino acid residues positioned inside the helix–loop–helix,[8] the solvent-exposed, outside residues were randomized to give a library of helix–loop–helix peptides. However, in fact, randomization of the outside residues in the C-terminal segment provided a mixture of folded peptides and peptides with multiple random-like conformations. Therefore, we attempted to purify the folded peptides from the crude

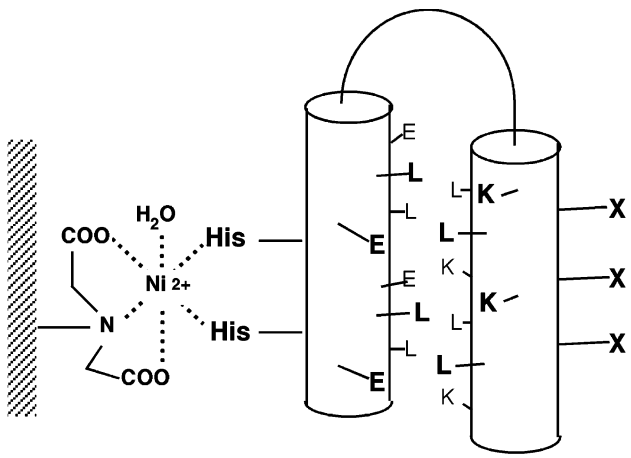

Figure 1. Illustration of the purification of helix–loop–helix libraries by IMAC. The one letter codes, E, L, K, and X, represent glutamic acid, leucine, lysine, and randomized residues, respectively.

Keywords: helix–loop–helix; peptide library; immobilized metal-affinity chromatography.

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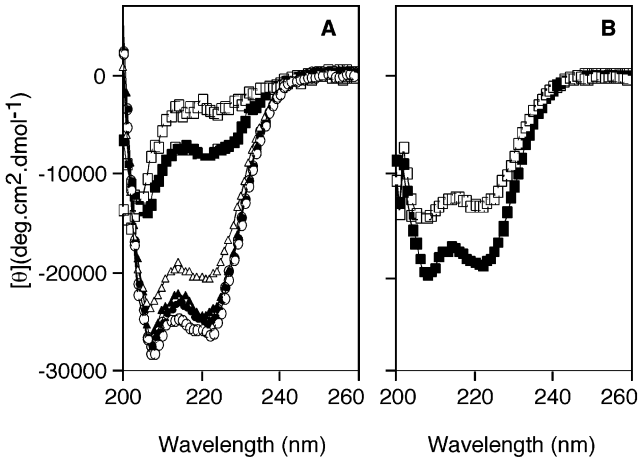

Figure 2. (A) CD spectra of peptides **1** (○,●), **2** (△,▲), and **3** (□,■) in the absence (open symbols) and in the presence (closed symbols) of 100 μM $NiCl_2$. The measurements were performed in 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl at 20°C. (B) CD spectra of the peptide library before (□) and after (■) purification by IMAC. The measurements were performed in 10 mM phosphate buffer (pH 7.0) containing 1 M NaCl in the presence of 100 μM $NiCl_2$ at 20°C. The peptide concentrations were 10 μM.

peptide library by using IMAC,[9] which has successfully been used to purify engineered proteins with metal-binding α-helices and is sensitive to environmental conditions that alter the α-helical structure. For the purification, a metal-binding ability was introduced into the N-terminal α-helix by arranging two histidine residues at the positions, His-X_3-His. Thus, when the randomized peptides have the stable helix–loop–helix folding, the two histidine residues in the N-terminal segments are oriented to chelate transition metals (Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) (Fig. 1). Consequently, on IMAC, the crude randomized library would be purified to provide a mixture of the conformationally defined peptides. In addition, the metal coordination was expected to complement the stability of the α-helical structure.[10]

At first, the ability of IMAC to prove the conformational state of the peptides was examined with using model peptides (**2**: Ac-AEL**H**ALE**H**ELAALE-G_7-KLAALKAKLAAL KAY-NH_2 and **3**: Ac-AEL-**H**ALE**H**ELAALEY-NH_2) possessing a His-X_3-His site in the N-terminal peptide and the well-defined helix–loop–helix peptide (**1**), which has no metal-chelating site, as a reference.[11] To examine the α-helical propensity of the peptides in the IMAC, the CD spectra were measured in the absence and presence of Ni^{2+} ions (Fig. 2A). Peptide **2** (10 μM) exhibited a high α-helical content with large molar ellipticity minimum values at 222 and 208 nm and a maximum ellipticity around 193 nm, which was almost identical with that of **1**. On the other hand, peptide **3** (10 μM) displayed weak α-helicity with a small molar ellipticity at 222 nm.[12] As expected, the α-helical structures of **2** and **3** were induced by the metal chelation. However, the stability of **2** was much higher than that of **3**, due to folding into the helix–loop–helix structure. These peptides were chromatographed on a Ni^{2+}-charged imino diacetate (Ni-IDA) column (Fig. 3A).

On the IMAC,[13] peptide **1** with no metal binding site was eluted in the washing buffer (A: 20 mM phosphate, 1.0 M NaCl, pH 7.2). On the other hand, peptides **2** and **3** were retained in the column with the metal chelation. Peptide **3** was eluted by a buffer including excess NH^{4+} ions (B: 20 mM phosphate, 1.0 M NH_4Cl, pH 7.2), whereas **2** was still retained in the column under the same elution conditions. A lower pH buffer (C: 20 mM phosphate, 1.0 M NaCl, pH 4.0), in which the imidazoles are protonated, was used to elute peptide **2** from the column. These elution profiles were consistent with the α-helical propensity of the peptides. Thus, the helix–loop–helix folding of **2** makes the N-terminal α-helix stable enough to bind strongly to the metal ion, and thus it required more drastic conditions for elution from the IMAC column. The elution profiles paralleled to the metal-binding affinities. The surface plasmon resonance assay of the peptides with a chelated Ni^{2+} ion showed a K_d value of 2.54×10^{-4} M for **2**, but none could be determined for **3**, due to its weak resonance.

Using the column conditions described above, a crude library consisting of 125 peptides was purified by IMAC. In the peptide library (Ac-AELHALEHE-LAALE-G_7-KLA**X**LK**X**KL**X**ALKAF(pNO_2)-NH_2), the amino acid residues at three positions (X_{25}, X_{28}, X_{31}), which are the most exposed in the C-terminal α-helix, were randomized with five amino acids with different properties, in terms of charge and hydropho-

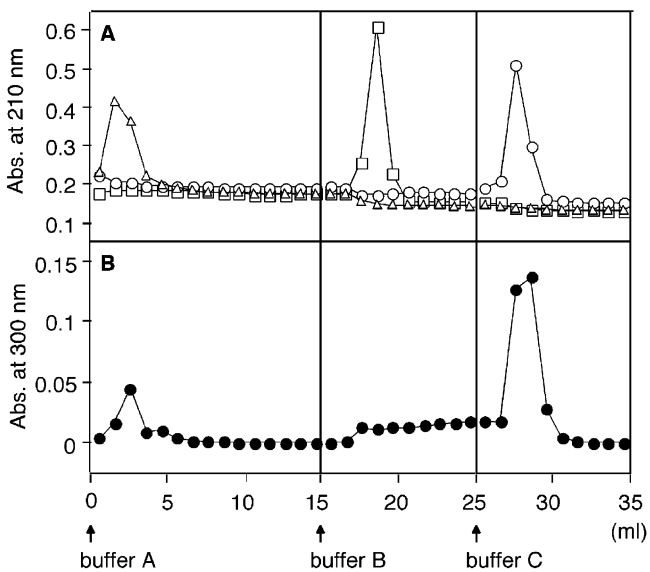

Figure 3. Elution profiles of peptides **1**, **2**, **3**, and the peptide library on the Ni-IDA column. (A) Peptides **1** (△), **2** (○), and **3** (□) (10 nmol/100 μl); (B) peptide library (●) (100 nmol/100 μl). The column was eluted with 15 ml of buffer A (20 mM phosphate, 1.0 M NaCl, pH 7.2), 10 ml of buffer B (20 mM phosphate, 1.0 M NH_4Cl, pH 7.2), and 10 ml of buffer C (20 mM phosphate, 1.0 M NaCl, pH 4.0).

bicity (Ala, Arg, Asp, Thr, and Tyr). The peptide library was prepared by the split-synthetic method.[14] To determine the peptide concentration of the library, *p*-nitrophenylalanine(F(pNO$_2$)) was incorporated into the C-terminus.[15] After cleavage from the resin, the crude peptide library was applied to the Ni-IDA column equilibrated with buffer A. The elution profile is shown in Fig. 3(B). A small amount of the peptides was sluggishly eluted with buffer B, showing a gentle, continuous peak in the chromatogram. When the buffer was changed from B to C, the remaining peptides were completely eluted from the column; approximately 60% of peptides were recovered. Since the peptides with weak α-helicities, such as peptide **3** (see Figs. 2A and 3A), were removed with buffer B, the CD spectrum of the fraction eluted with buffer C showed higher α-helicity as compared with that of the pre-purified library (Fig. 2B). Thus, the IMAC discriminates between the different conformational states of the library peptides to afford a mixture of peptides with a highly stable helix–loop–helix structure. Since the library consists of peptides with varying degrees of the conformational stability, the use of an elongated elution with buffer B on the IMAC should provide a peptide library with more stable and homologous conformations.

In this work, we have demonstrated the first example of a conformationally purified peptide library, based on the unique idea of combining combinatorial chemistry and de novo peptide design. The purified library has the potential to yield bioactive ligands upon selection. Furthermore, we emphasize that this idea opens a way for the de novo design of a new class of stable structural motifs. For example, the purification of a helix–loop–helix peptide library, in which the amino acids inside the helices are randomized, is expected to show a helical packing mode that differ from the hydrophobic interaction based on the heptad rule.

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11. The peptides were synthesized by a solid-phase method using the Fmoc strategy and were purified by reversed-phase C_{18} HPLC. The molecular weights of the peptides were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy (PerSeptive Biosystems Voyager Elite). **1**: $m/z = 3409$ (calcd = 3409); **2**: $m/z = 3541$ (calcd = 3541); **3**: $m/z = 1751$ (calcd = 1750).
12. In the absence of Ni^{2+} ion, peptide **3** (10 μM) indicated the characteristics of an unfolded peptide with a minimum ellipticity below 200 nm.
13. The IMAC was prepared as follows: a HiTrap chelating affinity column (1.0 ml, Pharmacia Biotech AB), in which iminodiacetate (IDA) is immobilized, was charged with 100 mM $NiCl_2$ (1.0 ml) according to the manufacturer's recommendations. After equilibration of the column with 20 mM phosphate, 1 M NaCl, pH 7.2 (equilibration buffer), 10 nmol of the model peptide (10 nmol) or the peptide library (100 nmol) were applied.
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15. The peptide concentration of the library was determined by measuring the *p*-nitrophenylalanine absorbance at 300 nm in 6 M guanidine hydrochloride solutions, using $\varepsilon_{300} = 5895$ (M^{-1} cm^{-1}).