A novel method for screening peptides that bind to proteins by using multiple fluorescent amino acids as fluorescent tags†

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Received (in Cambridge, UK) 30th September 2009, Accepted 16th November 2009
First published as an Advance Article on the web 30th November 2009
DOI: 10.1039/b920426a

We describe a new screening method for simultaneously detecting peptides that bind to a target protein by fluorescence obtained from fluorescent amino acid-modified peptides.

A peptide library is generally used for screening peptides that bind to a target protein. The phage display method and the one-bead–one-compound method are widely used methods for screening with a peptide library.[1–5] These methods essentially need to fix short peptides on very large carriers (sub-micron order) such as phages and beads. This has a drawback because the large carriers often bind to the target protein nonspecifically and specific binding of peptides to the target protein is inhibited. To overcome this drawback, we have developed a new screening method for detecting peptides that bind to a target protein. This new method does not need carriers. Instead of carriers, multiple fluorescent amino acids are connected to peptides. The fluorescent amino acids are utilized as fluorescent tags in this method. Peptides that have bound to a target protein are instantaneously identified and quantified by fluorescence from the tags.

First, to examine whether a peptide that binds to a protein specifically can be detected by our method, we used a combination of a FLAG peptide (DYKDDDDK) and an *anti*-FLAG antibody. It is known that a FLAG peptide specifically binds to an *anti*-FLAG antibody.[6] We prepared a FLAG peptide and its mutant peptides modified with a fluorescent amino acid as a fluorescent tag by conventional solid-phase peptide synthesis (SPPS).[7,8] Fluorescent amino acids (Fmoc-Ala(Acd)-OH, Fmoc-Glu(Edn)-OH, Fmoc-Ala(Bad)-OH, Fmoc-Lys(Cmr)-OH, Fmoc-Ala(Pyr)-OH, and Fmoc-Ala(Ant)-OH) were purchased from Watanabe Chemical (Hiroshima, Japan).[9] Chemical structures of peptides modified with a fluorescent tag and sequences of these peptides are shown in Fig. 1. Fl indicates a fluorescent amino acid. Chemical structures of fluorescent amino acids are also shown in Fig. 1. To improve water solubility, two glutamic acids are connected to the fluorescent amino acid. X_n (n = 1–8) indicates a natural amino acid. The 8-mer peptide consisting of X_n is a library moiety. A long spacer consisting of ethylene glycol units (Sp6) connects the fluorescent amino acid with the library moiety. Ac indicates an acetyl group. These peptides were identified by MALDI-TOF MS (**Acd(KDD)**: calc. $[M + H]^+$ = 1911.80, obs. $[M + H]^+$ = 1912.81; **Cmr(DKD)**: calc. $[M + H]^+$ = 1991.85, obs. $[M + H]^+$ = 1992.89; **Pyr(KEE)**: calc. $[M + H]^+$ = 1946.85, obs. $[M + H]^+$ = 1947.71; **Bad(KAD)**: calc. $[M + H]^+$ = 1917.83, obs. $[M + H]^+$ = 1918.94; **Ant(AAA)**: calc. $[M + H]^+$ = 1749.78, obs. $[M + H]^+$ = 1950.79; **Edn(DDD)**: calc. $[M + H]^+$ = 2011.75, obs. $[M + H]^+$ = 2012.69). A Cy3-labeled *anti*-FLAG M2 antibody and non-labeled *anti*-FLAG M2 antibody used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA).

The peptides modified with a fluorescent tag and the Cy3-labeled *anti*-FLAG M2 antibody were examined by two-dimensional fluorescence (2D-FL) spectroscopy in 10 mM HEPES-NaOH buffer (pH 7.4, 150 mM NaCl) containing 50% MeOH as shown in Fig. 2. The fluorescent tags showed differences in maximum excitation/emission wavelengths (**Acd(KDD)**: 403/420 nm, **Edn(DDD)**: 350/480 nm, **Bad(KAD)**: 485/512 nm, **Cmr(DKD)**: 327/386 nm, **Pyr(KEE)**: 344/378 nm, **Ant(AAA)**: 359/408 nm, Cy3-labeled *anti*-FLAG M2 antibody: 555/570 nm). We also examined the mixture of these six peptides (each 10.0 nM, 30 pmol) and Cy3-labeled *anti*-FLAG M2 antibody (0.2 nM, 0.6 pmol) by 2D-Fl

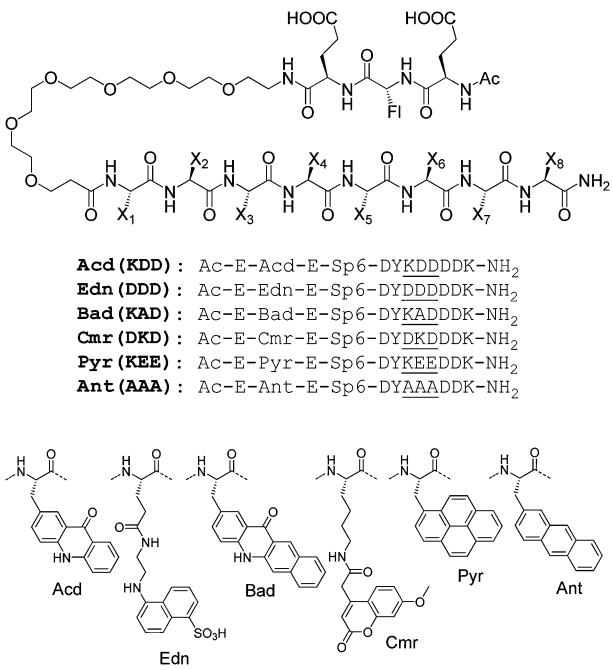

Fig. 1 Chemical structure of the peptide modified with a fluorescent tag and sequences of peptides. Chemical structures of fluorescent amino acids.[9]

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† Electronic supplementary information (ESI) available: Synthesis of new fluorescent amino acids and fluorescent tag-modified peptides. See DOI: 10.1039/b920426a

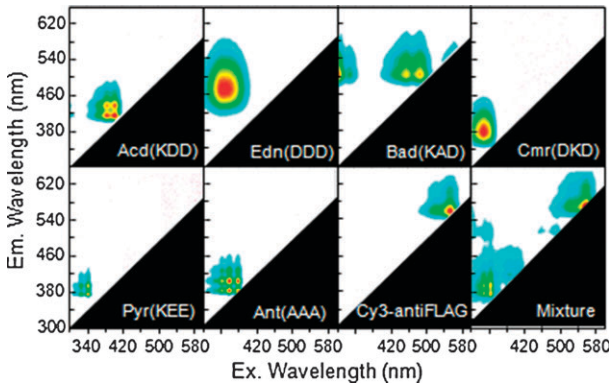

Fig. 2 2D-Fl spectra of fluorescent tag-modified peptides, a Cy3-labeled *anti*-FLAG M2 antibody and the mixture of these peptides (each 10 nM) and the antibody (0.2 nM) over the wavelength ranges of 300–600 nm for excitation and 305–650 nm for emission in 10 mM HEPES-NaOH (pH 7.4, 150 mM NaCl) containing 50% MeOH at room temperature.

spectroscopy (Fig. 2). Then concentrations of fluorescent tag-modified peptides in the mixture were estimated by a least-squares analysis of fluorescent intensity in the 2D-Fl spectrum for the mixture on the basis of component spectra (2D-FL spectra for the fluorescent tag-modified peptides). Consequently, concentrations of these peptides in the mixture were successfully quantified by this method (**Bad(KAD)**: 11.6 nM, **Cmr(DKD)**: 10.8 nM, **Pyr(KEE)**: 10.2 nM, **Edn(DDD)**: 10.4 nM, **Ant(AAA)**: 14.5 nM, **Acd(KDD)**: 8.8 nM and Cy3-labeled *anti*-FLAG M2 antibody: 0.2 nM).[10] The results indicate that fluorescence resonance energy transfer (FRET), quenching and aggregation between peptides did not occur under these conditions. The results also clarified that this method can distinguish and quantify peptides in the mixture.

Next, the Cy3-labeled *anti*-FLAG M2 antibody (50 nM, 5.0 pmol), the non-labeled *anti*-FLAG M2 antibody (1 μM, 100.0 pmol) and the six peptides (each 1 μM, 100.0 pmol) were mixed in 10 mM HEPES-NaOH buffer (pH 7.4, 150 mM NaCl). After overnight incubation at 4 °C, the mixture was fractionated by Superdex 75 pg size exclusion chromatography (GE Healthcare, London, UK). A 2D-FL spectrum for the fraction containing the Cy3-labeled *anti*-FLAG M2 antibody was measured as shown in Fig. 3. Concentrations of all of the components were determined by the protocol described above. **Acd(KDD)** (FLAG peptide modified with Acd: 44.1 pmol) was detected together with a nearly equimolar amount of the *anti*-FLAG M2 antibody (54.0 pmol, estimated from fluorescence intensity of Cy3). We estimated the dissociation constant (K_d) of Acd(KDD)/*anti*-FLAG antibody complex by using least-squares analysis of fluorescent intensity for fractions in which the complex was observed. The K_d value was 17×10^{-9} M, which corresponds to a literature report of K_d (6.5×10^{-9} M).[11] The somewhat higher value may be because of nonspecific adsorption of these peptides and/or the protein to gel by using gel filtration. Other peptides were not detected in the fraction (**Edn(DDD)**: 8.1 pmol, **Bad(KAD)**: 5.7 pmol, **Pyr(KEE)**: 0.6 pmol, **Cmr(DKD)**: <0 pmol, **Ant(AAA)**: <0 pmol). This result indicates that the method can be utilized as a method for detecting a peptide that binds to a protein specifically.

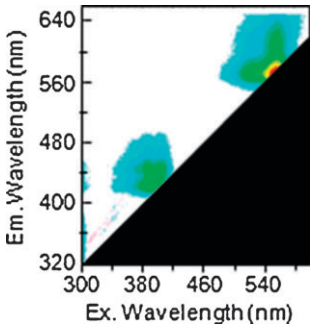

Fig. 3 2D-Fl spectrum of the fraction containing Cy3-labeled *anti*-FLAG M2 antibody obtained after gel filtration chromatography of the mixture of fluorescent tag-modified peptides and the Cy3-labeled *anti*-FLAG M2 antibody.

We preliminarily examined screening for EGFR-binding peptides by this method. An EGFR (epidermal growth factor receptor)[12,13] is a cell-surface receptor, and mutations affecting EGFR expression or activity can result in cancer. Therefore, an EGFR-binding peptide is an attractive medical tool, and some research groups screened for such a peptide by phase display methods.[14–17]

In this study, the extracellular domain of human EGFR fused to the Fc region of human IgG *via* a linker peptide (R & D Systems, Minneapolis, MN, USA) was used as a target protein. Seven fluorescent tag-modified peptides were newly synthesized by SPPS. Fluorescent amino acids (Fmoc-Lys-(Moc)-OH, Fmoc-Lys(Mac)-OH, Fmoc-Lys(Tmr)-OH, Fmoc-Orn(Cm3)-OH, Fmoc-Lys(Fam)-OH and Fmoc-Lys(Hoc)-OH) were used as fluorescent tags on SPPS.[18] Fmoc-Lys(Tmr)-OH and Fmoc-Lys(Fam)-OH were purchased from ABD Bioquest (Sunnyvale, CA, USA) and other fluorescent amino acids were newly synthesized (see ESI†). Sequences of these peptides and chemical structures of fluorescent amino acids are shown in Fig. 4.

These peptides were examined by 2D-FL spectroscopy in 10 mM HEPES-NaOH (pH 7.4, 150 mM NaCl) containing 50% MeOH (maximum excitation/emission wavelengths: 348/400 nm for **Moc(G/A/V)**, 377/464 nm for **Mac(I/L/M)**, 401/421 nm for **Acd(S/T)**, 411/451 nm for **Hoc(D/E)**, 446/491 nm for **Cm3(N/Q/P)**, 501/524 nm for **Fam(H/K/R)**, 542/569 nm for **Tmr(F/W/W)**). Then these peptides were mixed with an EGFR in 10 mM HEPES-NaOH buffer (pH 7.4, 150 mM NaCl), and the mixture was incubated at 4 C for 2 h. Final concentrations of the peptides were 190–280 μM (4.5–14 nmol) and the final concentration of the EGFR was 3.9 μM (190 pmol). The mixture was then fractionated by Superdex

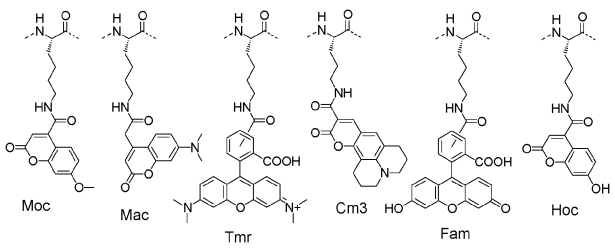

Fig. 4 Sequences of the peptides modified with a fluorescent tag and chemical structures of the fluorescent amino acids.[18]

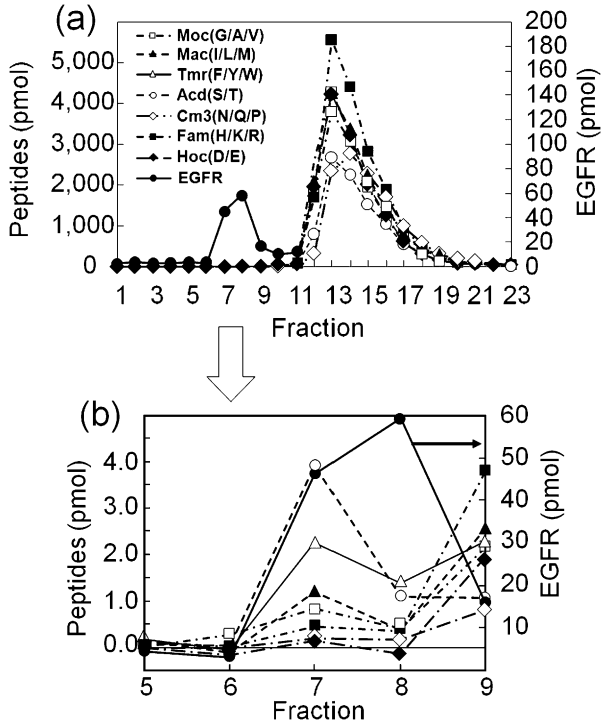

Fig. 5 Quantities of the fluorescent tag-modified peptides and the EGFR estimated from 2D-FL spectra by least-squares analysis.

75 pg size exclusion chromatography. These fractions were measured by 2D-FL spectroscopy. Concentrations of all components in each fraction were quantified by the protocol described above. Quantities of the fluorescent tag-modified peptides and the EGFR in each fraction are shown in Fig. 5. The quantity of the EGFR was estimated from the fluorescence intensity at 333 nm (excitation at 280 nm). EGFR was detected in fraction 7 (46.3 pmol) and fraction 8 (59.5 pmol) as shown in Fig. 5(a). Free peptides were detected in the subsequent fractions. Fluorescent tag-modified peptides were also detected in fraction 7 (Fig. 5(b); magnified fractions 5–9 in Fig. 5(a)). These results indicate that the peptides bind to EGFR and that the EGFR-binding peptides can be successfully detected by this method. **Acd(S/T)** (3.9 pmol), **Tmr(F/Y/W)** (2.3 pmol), **Mac(I/L/M)** (1.2 pmol) and **Moc(G/A/V)** (0.8 pmol) were detected in fraction 7. Other peptides were less than 0.4 pmol. These results indicate that specific peptides that bind to an EGFR can be successfully differentiated by this method.

In summary, we have developed a new method for screening peptides that bind to proteins. We synthesized 8-mer peptides modified with a fluorescent amino acid. The peptides were mixed with an *anti*-FLAG antibody and an EGFR before incubation, and then the protein-binding peptides were recovered by gel filtration chromatography. The binding peptides were able to quantify simultaneously by this method. This method combines fluorescence analysis with gel filtration and it also will be able to combine the analysis with other separation techniques (capillary electrophoresis, ultrafiltration and affinity bead). Determination of an 8-mer peptide sequence that binds to an EGFR by this method is currently underway.

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9 Abbreviations used are as follows: Fmoc, 9-fluorenylmethoxycarbonyl; Acd, 2-[9(10*H*)-acridonyl]; Edn, 5-naphthyl sulfonic acid; Cmr, 7-methoxycoumarin-4-yl; Bad, [benzo[*b*]acridin-12(5*H*)-on-2-yl]; Pyr, 1-pyrenyl; Ant, 2-anthryl.
10 These calculated values were scattered in comparison with the theoretical value (10 nM), especially the value for Ant (14.5 nM). The scattering of the values seemed to be affected by the nature of the fluorescence group. Choice of fluorescence group may be important for analysis correctly by this method.
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