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Report

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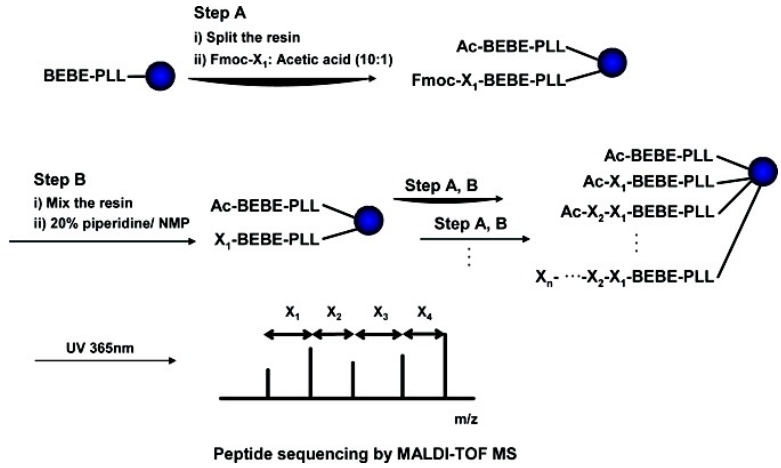

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Reports

Solid-Phase Peptide Library Synthesis on HiCore Resin for Screening Substrate Specificity of Brk Protein Tyrosine Kinase

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Recent developments in the identification of novel drug targets based on the benefits of human genome projects and continued improvements in peptide-delivery technologies have created an increasing demand for highly effective synthetic peptide library systems. Combinatorial chemistry combined with solid-phase peptide synthesis is a technology that allows simultaneous creation of a number of different compounds and enables screening of useful compounds among them. To prepare these peptide libraries, a one-bead one-compound (OBOC) library method was proposed using split and mix synthesis.[1] Split and mix synthesis provides an equimolar mixture of random and large peptide libraries.[2] Such peptide libraries can then be used to screen enzymatic substrates and inhibitors or biomolecule-binding peptides.[3] If a peptide structure in the OBOC library must be identified, the peptide sequence is generally analyzed by Edman degradation.[4] However, this method is time-consuming and expensive. In this regard, the ladder peptide synthesis method can be an efficient alternative.[5] In this method, a small portion of the peptides are N-terminally capped during each coupling cycle in the synthesis of peptides on the polymer beads. For structure identification, the peptide ladders are later released and analyzed by mass spectroscopy.

To prepare an OBOC library, the linker and the spacer on the polymer supports must be designed to allow easy peptide sequence analysis and to ensure sufficient space for an enzyme reaction. For instance, a peptide that contains a methionine linker can be chemoselectively cleaved by cyanogen bromide (CNBr).[6] However, the cleavage of the linker requires a long reaction time (12 h), and a further purification step is needed to yield pure product. Franz et al. reported that photolytic cleavage was not suitable in the analysis of a peptide-encoded combinatorial small molecule library on TentaGel resin because of the poor photocleavage yield.[7] In another report, PEGA (polyethylene glycol polyacrylamide) resin was used in the elution of peptide after the photocleavage step.[3b] However, the diameter of the PEGA resin was 300–800 μm, which restricted the molecular diversities, so that they were at least twenty-five times less than those of the resin generally used for organic synthesis or bioscreening (typical diameter $\approx$ 100 μm).

We previously reported a core–shell-type resin (HiCore resin), the structure of which is segregated into two regions: a rigid core cross-linked by 2,4,6-trichloro-1,3,5-triazine and a flexible shell functionalized by diamino PEG.[8] Because all the amino groups in the HiCore resin are located at the surface, the HiCore resin presumably ensures more effective photocleavage than the gel-type resin, in which functional groups are evenly distributed. In fact, photoelution of peptide on HiCore resin was two times more efficient than that on TentaGel resin when irradiated with UV for 30 min. Moreover, we reported that nonspecific protein binding on HiCore resin was quantifiable with an enzyme-linked on-bead colorimetric assay.[9] In the paper, we found that HiCore resin reduced nonspecific protein adsorption with greater efficiency than TentaGel resin, regardless of the diversities in molecular weights or pI values of several representative proteins. With consideration of these properties, HiCore resin was used as the polymer support for a ladder OBOC library in this study to facilitate the high-throughput screening of unknown active substrates of Brk protein tyrosine kinase (PTK) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

The molecular weights of α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxy-benzoic acid (DHB), which are frequently used as common MALDI matrices, are 189.17 and 154.12, respectively. Since the peptide analyte cleaved from the resin mixes with the matrix compound during MALDI-TOF MS analysis, the low molecular weight peak (<300−400 MW) of a peptide could easily be confused with the matrix signal. Therefore, the lowest molecular weight of peptides in the ladder should exceed 400. In a previous report, the β-alanine-ϵ-aminocaproic acid−β-alanine-ϵ-aminocaproic acid (β-Ala-ϵ-ACA−β-Ala-ϵ-ACA; BEBE) spacer performed well in bioassays on the resin because of the proper accessibility of proteins on the resin.[10] The molecular weight of the BEBE spacer (MW = 427.55) is higher than that of the matrix compounds. Consequently, BEBE is a suitable choice of spacer for solid-phase enzyme reactions and MALDI-TOF MS analyses in this study.

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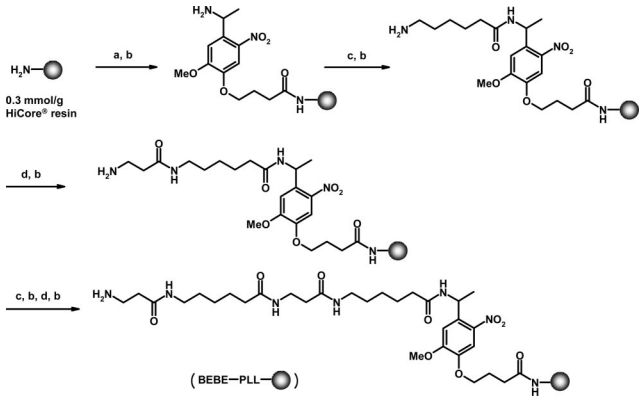
Scheme 1. Introduction of Photolabile Linker and Spacer[a]



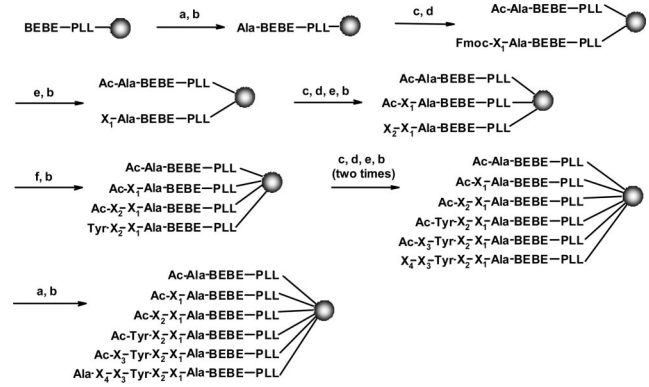
[a] Reagents and conditions: (a) Fmoc-4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy]butyric acid (2 equiv), BOP (2 equiv), HOBt (2 equiv), DIEA (4 equiv), NMP; (b) 20% piperidine in NMP; (c) Fmoc-β-Ala (2 equiv), BOP (2 equiv), HOBt (2 equiv), DIEA (4 equiv), NMP; (d) Fmoc-ϵ-ACA (2 equiv), BOP (2 equiv), HOBt (2 equiv), DIEA (4 equiv), NMP.

To introduce the linker and the spacer on the HiCore resin, Fmoc-4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy]butyric acid (Fmoc-photolabile linker; Fmoc-PLL) was coupled to the resin, and the BEBE spacer was synthesized by Fmoc-chemistry (Scheme 1). After each step, Kaiser's ninhydrin test was performed.[11] To further analyze the peptide coupling, MALDI-TOF MS was performed. A sample of BEBE spacer-coupled resin (10 mg) was acetylated with acetic anhydride. After the acetylated spacer was cleaved from the resin using UV irradiation, the molecular weight of the acetylated spacer on MALDI-TOF MS was found to be 450.59 (calcd $[M+Na^+] = 450.55$). On the basis of the results of Kaiser's ninhydrin test and MALDI-TOF MS, we found that the coupling and Fmoc deprotection were quantitatively performed in each coupling step.

Using the split and mix method, we prepared a ladder peptide library on the linker and spacer-coupled HiCore resin beads (diameter ≈ 100 μm). To prepare the ladder peptide library, the resin beads were equally divided into independent reaction vessels before each coupling reaction. Eighteen Fmoc-amino acids were used in the library synthesis. Cys and Tyr were not included in the peptide library because Cys forms disulfide bonds and Tyr is a reactive site for protein tyrosine kinase (PTK). Instead of the usual Fmoc-chemistry protocol, a mixture of Fmoc-amino acid and 10% (mol/mol) acetic acid was used as the building blocks so that a certain portion of the amino groups was acetylated to generate ladder members on the resin (Scheme 2). After each coupling reaction, the separated resins were pooled into one reaction vessel. After the Fmoc groups were removed, the remaining amino groups were quantified.[12] Upon completion of the peptide coupling, side-chain-protecting groups were removed with reagent K (a mixture of 85% TFA (trifluoroacetic acid), 5% phenol, 5% thioanisol, 2.5% ethandithiol, and 2.5% water).

The peptide library contained four randomized residues. Tyrosine was the center amino acid in the peptide. To elongate the peptide to provide a suitable atmosphere for the enzymatic reaction, an alanine was inserted at both the N-terminus and C-terminus of the peptide sequence. Thus, the resulting peptide library sequence was Ala-X_4-X_3-Tyr-X_2-X_1-Ala-BEBE-PLL-HiCore. The percentages of acetylated/nonacetylated sequences after each coupling step were shown in Table 1 and Figure 1. During the X_1 and X_2 coupling steps, about 49% of all the amino groups were capped by high competitive acetylation against the amino acid coupling reaction. The acetylation yields were about 28% and 30%, respectively. However, after those steps, about 20% of the amino groups were acetylated in each step. Therefore about 24% of all the amino groups were acetylated during Tyr, X_3 and X_4 steps. Consequently, the content of full peptide sequence was $\sim$27%, which was used as substrate for the kinase. This phenomenon was caused by the different accessibility to the resin between acetic acid and Fmoc amino acid. It was because small acetic acid molecule was more efficient to contact the surface of amino group in the early stage of coupling reaction than Fmoc-amino acid.

Scheme 2. Preparation of Ladder Peptide Library on HiCore Resin[a]



[a] Reagents and conditions: (a) Fmoc-Ala-OH (2 equiv), BOP (2 equiv), HOBt (2 equiv), DIEA (4 equiv), NMP; (b) 20% piperidine in NMP; (c) splitting resin into 18 vessels; (d) Fmoc-X_n-OH (2 equiv), acetic acid (0.2 equiv), BOP (2.2 equiv), HOBt (2.2 equiv), DIEA (4.4 equiv), NMP in 18 reaction vessels, respectively; (e) mxing resin into a vessel; (f) Fmoc-Tyr-OH (2 equiv), acetic acid (0.2 equiv), BOP (2.2 equiv), HOBt (2.2 equiv), DIEA (4.4 equiv), NMP.

Phosphorylation of proteins by kinases is a crucial process in the regulation of cell functions.[13] In this study, Brk PTK was selected as a model kinase for substrate screening from an OBOC library. Brk is a human intracellular PTK that is strongly overexpressed in a large subset of breast tumors.[14] In the enzyme reaction test, resin beads that contained the ladder peptide library were incubated with tyrosine kinase in the presence of adenosine triphosphate (ATP). Then, the resin beads were suspended in a solution of alkaline phosphatase-conjugated antiphosphotyrosine antibody and stained by subsequent enzymatic color reaction with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3′-indolyl phosphate p-toluidine salt (BCIP). The binding of antiphosphotyrosine antibody with peptides on the resin was determined by a color change on the resin after treatment with NBT and BCIP.[15] The antibody-bound resin beads were easily sorted by picking out the colored beads. After photoelution of the peptides from the resin through UV irradiation, the peptides were sequenced by mass differences corresponding to each amino acid (Figure 2), except for the isobaric amino acids, Gln/Lys (Q/K) and Leu/Ile (L/I). After

Table 1. Characterization of Acetylated/Nonacetylated Sequences on Each Coupling Step by Fmoc Quantitation

coupling entry	products of each coupling step	relative quantities of amino groups (%)	rates of acetylation (%)/ nonacetylation (%)	populations of acetylated sequences (%)
0	Ala-BEBE	100		
1	Ac-Ala-BEBE/Fmoc-X_1-Ala-BEBE	72.0	28.0:72.0	28.0
2	Ac-X_1-Ala-BEBE/Fmoc-X_2-X_1-Ala-BEBE	50.7	29.6:70.4	21.3
3	Ac-X_2-X_1-Ala-BEBE/Fmoc-Y-X_2-X_1-Ala-BEBE	42.2	16.8:83.2	8.5
4	Ac-Y-X_2-X_1-Ala-BEBE/Fmoc-X_3-Y-X_2-X_1-Ala-BEBE	33.0	21.8:78.2	9.2
5	Ac-X_3-Y-X_2-X_1-Ala-BEBE/Fmoc-X_4-X_3-Y-X_2-X_1-Ala-BEBE	26.7	19.1:80.9	6.3
6	Ala-X_4-X_3-Y-X_2-X_1-Ala-BEBE	26.7		26.7[a]

[a] Population of full length peptide.

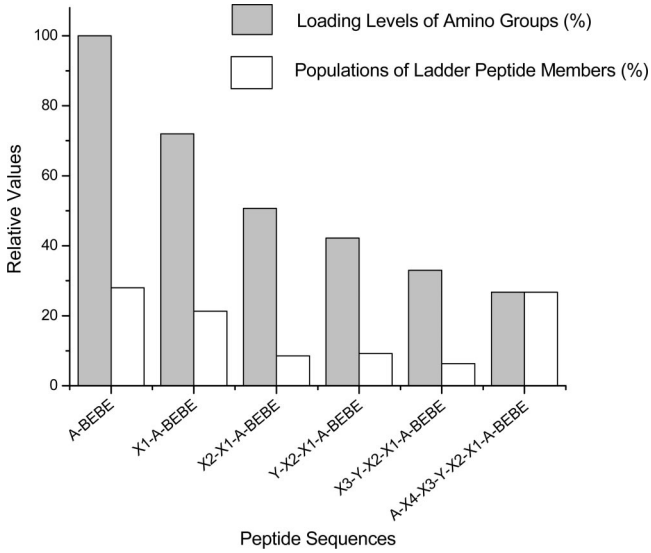


Figure 1. Loading levels and populations of ladder peptide sequences as determined by Fmoc quantitation.

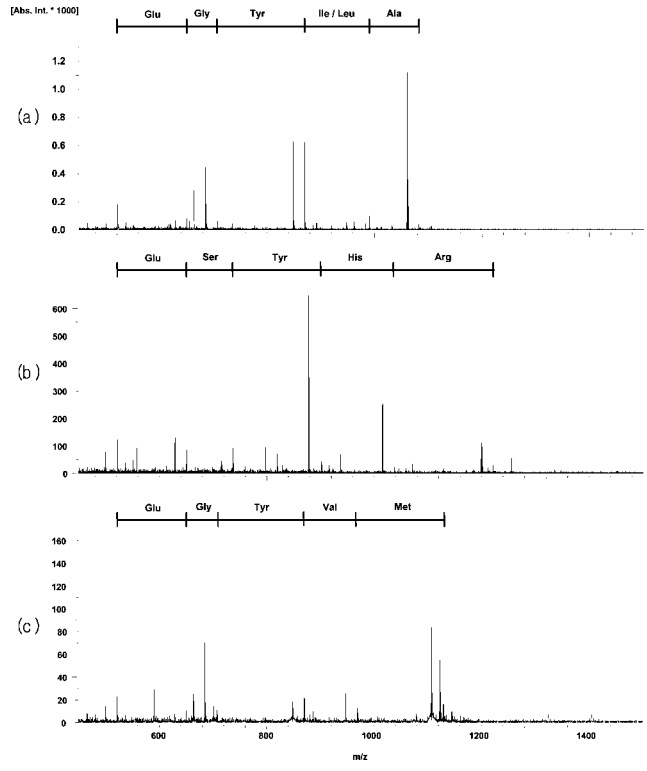


Figure 2. Determination of peptide sequence on one bead by MALDI-TOF MS analysis: (a) AA(I/L)YGEA, (b) ARHYSEA, and (c) AMVYGEA.

the peptide sequences were obtained, three-dimensional modeling of the binding between Brk PTK and optimal

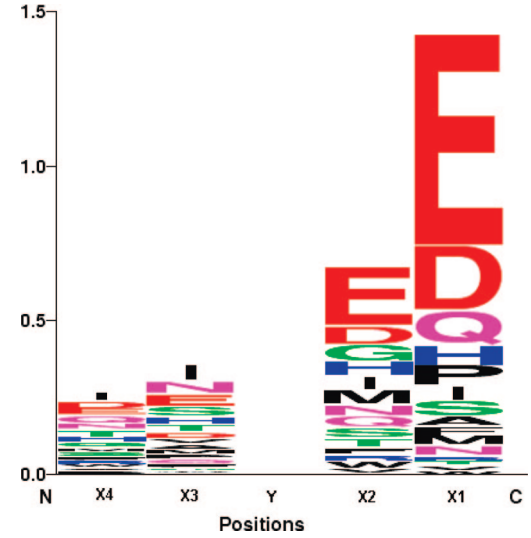


Figure 3. Statistical peptide sequence profile of Brk PTK substrate.[16]

substrate sequences was performed as described in the Supporting Information.

The sequences of 88 beads out of 100 active beads could be analyzed with high-quality MALDI spectra. The identified phosphorylated peptide sequences are summarized in the Supporting Information, and critical residues for the phosphorylation were statistically determined on the basis of the peptide sequencing results. The sequence profile is graphically summarized using WebLogo, which is a web-based program that is used to easily display the frequency of amino acids at each position of a peptide sequence (WebLogo is available at the Web site of http://weblogo.berkeley.edu).[16] The results reveal the key features of Brk substrate specificity. In Figure 3, the size of each letter is proportional to the statistical frequency of that amino acid residue. Brk favors acidic amino acids such as E and D at the C-terminal residue of the substrate sequences. The strongest acidic preferences are found at positions X_1 and X_2. In particular, the X_1 position showed the strongest preference for acidic amino acids (XXYXD or XXYXE; 55 out of 88). Acidic amino acids at the X_2 position were also favored (XXYDX or XXYEX; 33 out of 88). Moreover, the X_3 position was found to be Leu/Ile (X(L/I)YXX, 13 out of 88), Gln (XNYXX, 11 out of 88), and Glu (XEYXX, 10 out of 88). On the other hand, the X_4 position displayed low specificity.

Brk PTK is known to contain SH2 (Src homology 2), SH3 (Src homology 3), and tyrosine kinase catalytic domains in a similar arrangement as Src family kinases.[17] The substrate specificity of Src family kinases has been reported by several groups. Songyang et al. reported the sequence of XXYEX,[18]

and Lou et al. reported the sequence of XIYXX.[19] Kreegipuu et al. summarized the characteristic sequence as XEYXX.[20] On the basis of the consensus of our peptide sequences, in comparison with previous reports, we conclude that our bioassay system could be used for high-throughput screening of tyrosine kinase substrates.

In conclusion, we developed a method to synthesize ladder peptides on a core–shell type solid support (HiCore resin) to efficiently screen the substrate specificity of PTK. Photolabile linker and BEBE spacer were introduced on the resin to allow easy analysis of the peptide sequence and to ensure sufficient space for the enzymatic reaction. Using the split and mix method, ladder peptides of the sequence Ala-X_4-X_3-Tyr-X_2-X_1-Ala-BEBE-NH_2 were synthesized on the polymer beads by partially capping the N-terminus of the growing peptide. The amount of full sequence peptide was ~27%. The Brk PTK-catalyzed phosphorylation reaction was performed on the resin-bound peptide library, and then the resin beads were treated with alkaline phosphatase-conjugated antiphosphotyrosine antibody. After color reaction with BCIP/NBT, the colored beads were selected, and the peptide sequence on each bead was analyzed by MALDI-TOF MS. We found that negatively charged amino acids (Asp and Glu) were in high abundance at the X_1 and X_2 positions, while Leu/Ile, Gln, and Glu were found at the X_3 position in the kinase substrates. From these results, we confirmed that our method was effective and robust in the high-throughput screening of kinase substrate.

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Supporting Information Available. Experimental procedures, phosphorylated peptide sequence details and molecular modeling of the Brk PTK-optimal substrate sequences. This information is available free of charge via the Internet at http://pubs.acs.org.

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