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Determination of protease subsite preference on SPOT peptide array by fluorescence quenching-based assay

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A peptide SPOT array was synthesized on a glass chip and used to determine protease subsite preference. To synthesize a peptide array for positional scanning, the ratio of the isokinetic concentration was determined for every Fmoc-amino acid except Cys. Based on this ratio, a peptide array consisting of Dabcyl-X-X-P_2-Arg-X-X-X-Lys(FITC) (X: equimolar mixture of 19 amino acids, P_2: one of 19 amino acids) was synthesized on a chitosan-grafted glass chip. Subsequently, the peptide substrates on the array were hydrolyzed by thrombin to screen for subsite specificity using a fluorescence quenching-based assay. The P_2 subsite specificity of thrombin was screened by the fluorescence images obtained after hydrolysis. Pro at the P_2 subsite showed the highest specificity for thrombin based on both the fluorescence quenching-based assay and the solution phase assay. From these results, we confirmed that our mixture-based peptide SPOT array format on the chitosan-grafted glass chips could be used to determine protease subsite preference. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: SPOT synthesis; chitosan-grafted glass chip; isokinetic concentration; FRET assay; protease subsite specificity; fluorescence analysis

Introduction

Peptide microarrays have been developed because of improvements in micro-machining technology [1] and have been used to screen biomolecule ligands, enzyme substrates, and inhibitors in a high-throughput manner [2–4]. The SPOT synthesis method was developed by Ronald Frank as a method for *in situ* peptide microarray synthesis [5]. As the SPOT method is based on conventional solid-phase synthesis with Fmoc-/*t*-Bu-chemistry, it is easily applicable to an automated synthesis system [6].

Combinatorial peptide libraries have been synthesized on polymer beads and on planar surfaces. Since the one-bead one-compound (OBOC) combinatorial library method was developed in 1991, many researchers have used this method to screen small bioactive molecules including peptide substrates [7,8]. The OBOC library can be synthesized rapidly by split and mix synthesis on polymer beads and screened by picking out the active beads [9,10]. However, as the length of the peptide substrates become longer, the number of beads required increases exponentially. Therefore, the OBOC library may not cover the entire compounds.

The reagent mixture synthesis method is an alternative way instead of the OBOC approach from the statistical point of view to screen bioactive molecules. Once the relative reaction rates of the protected amino acids are determined by analyzing the competitive coupling efficiencies, the combinatorial equimolar peptide libraries can be synthesized. Therefore, the method of mixture-based combinatorial library synthesis is useful for positional scanning [11].

Proteases play essential roles in numerous biological processes, such as hormone activation, proteasomal degradation, and apoptosis. Furthermore, proteases are involved in diverse diseases, including many viral and parasitic infections, cardiovascular disease, cancer, and Alzheimer's disease. One of the most important aspects of proteolytic pathways is the ability of proteases to selectively cleave target substrates in the presence of other proteins [12]. Therefore, identifying substrate specificities for a protease may not only elicit valuable information on their biological function but also create the basis for designing potent substrates and selective inhibitors that can be used as novel drug targets [13].

The fluorescence-based method of protease substrate screening provides useful information on protease substrate specificities so they can be readily applied to microarray formats. Because of the ability to screen thousands of compounds on a miniaturized scale [14], protease substrate screening on a microarray has been studied in a high-throughput manner. For example, the use of coumarin-containing enzyme substrate derivatives immobilized on microarrays has enabled screening of the proteolytic activities of different proteases by detecting the differences in resulting fluorescence of the fluorogenic coumarins after enzymatic cleavage. As a result, substrate-dependent enzyme activity profiles were obtained using analysis of the fluorescence patterns [15,16]. Additionally, protease inhibitory activities can be screened by printing mixtures of inhibitor candidates and fluorogenic substrates on the microarray platform [17].

We previously developed a simple method to pre-pattern SPOT arrays on a slide glass, which allowed for more convenient SPOT synthesis of peptides and their assays. The SPOT synthesis platform on a glass surface was developed with a perfluorinated background. Peptide coupling reactivities were assessed on

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various surfaces, and the protein binding assay was performed on a synthesized peptide SPOT array [18]. In the present study, to further apply peptide SPOT arrays in bioassays, the isokinetic concentration was determined for Fmoc-amino acids except Cys to synthesize an equimolar peptide mixture array. Using the reagent mixture synthesis method, we prepared peptide SPOT arrays of internally quenched fluorogenic substrates on a chitosan-grafted chip, which produced a high peptide synthesis coupling yield with reduced non-specific binding of proteins [19–21]. Finally, the SPOT arrays were used to determine thrombin subsite preference as a model study.

Materials and Methods

3-Glycidoxypropyltrimethoxysilane, 3-aminopropyltriethoxysilane (γ-APTS), chitosan (75–85% deacetylated chitin, molecular weight = 50 000–190 000), *N*,*N*-diisopropylethylamine (DIPEA), fluorescein isothiocyanate (FITC), thioanisole, 1,2-ethanedithiol, ninhydrin, phenol, pyridine, piperidine, dichloromethane (DCM), and *N*-methyl-2-pyrrolidone (NMP) were purchased from Aldrich (St. Louis, MO, USA). *N*-{4-[4′-(dimethylamino)phenylazo]benzoyloxy}succinimide (Dabcyl-OSu) and Fmoc-ε-aminocaproic acid (Fmoc-ε-ACA) were purchased from Novabiochem (Darmstadt, Germany). Fmoc-β-alanine (Fmoc-β-Ala) was purchased from Bachem AG (Bubendorf, Switzerland). Benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP), and 1-hydroxybenzotriazole (HOBt) were purchased from GL Biochem (Shanghai, China). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Geel, Belgium). 1*H*,1*H*,2*H*,2*H*-Perfluorodecylmethyldichlorosilane was purchased from Lancaster Synthesis (Ward Hill, MA, USA). Rink amide-AM SURE resin (0.60 mmol NH_2/g) and other Fmoc-amino acids were purchased from BeadTech (Seoul, Korea). Trityl was used for the side chain protection of Asn, Gln, and His; Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl chloride) for Arg; *t*-Boc (*t*-butoxycarbonyl) for Lys and Trp; and *t*-Bu (*t*-butyl) for Asp, Glu, Ser, Thr, and Tyr. Glass slides (75 × 25 mm, Micro Slides no. 2948) were purchased from Corning Glass Works (Corning, NY, USA). Photoresist AZ 4330 was purchased from Clariant Corporation (Muttenz, Switzerland). Amino group-modified SPOT arrayed glass slides (4 × 10 SPOT arrays) were prepared according to procedures reported previously [18]. A microarray scanner GenePix 4000B (Axon Instruments, Foster City, CA, USA) was used to evaluate fluorescence by excitation at 532 nm. High performance liquid chromatography (HPLC) was carried out using a YoungLin Autochro 2000 system (YoungLin Instrument, Seoul, Korea). The peptide samples were analyzed on a Waters μBondapak C18 analytical column (pore size: 12.5 nm, particle size: 10 μm, 3.9 × 150 mm spherical packing) employing gradients composed of 0.1% aqueous TFA (eluent A) and 0.1% TFA in acetonitrile (eluent B). Elution occurred at a flow rate of 1.0 ml min^{-1}, and peaks were detected at 260 nm. Matrix-assisted laser desorption and ionization–time of flight mass spectroscopy (MALDI-TOF MS) analysis was performed using a Bruker Datonics Biflex IV TOF MS (Bremen, Germany), and the spectra in positive mode were obtained using α-cyano-4-hydroxycinnamic acid as a matrix.

Measuring the Isokinetic Concentration for Each Fmoc-Amino Acid on the Spot Arrayed Glass Surface

Solutions of each Fmoc-amino acid, BOP, HOBt, and DIPEA in NMP (10 mM) were prepared individually. The stock solutions were then diluted to various concentrations (4~40 mM) to produce the experimental sets. The same molar solutions of Fmoc-amino acid, BOP, HOBt, and DIPEA in NMP were mixed and vortexed for 5 min for pre-activation. Then, these pre-activated solutions (200 nl) of the various Fmoc-amino acids (with 1 equivalent of BOP, HOBt, and DIPEA) in NMP were spotted onto γ-APTS treated glass chips at various concentrations ranging from 1 to 10 mM, and the chips were incubated at 25 °C for 1 h. After capping the remaining amino groups by acetylation and subsequent Fmoc deprotection, the liberated free amino groups were labeled with FITC to quantify the amount of coupled amino acids. The concentration of each amino acid required to complete coupling was determined by reading the concentration at which fluorescence intensity reached a maximum.

Synthesis of an Equimolar Peptide Mixture Array on a Spot Arrayed Glass Chip

A 6.7-mM solution of Fmoc-Lys(Boc), BOP, HOBt, and DIPEA in NMP was added to the $(\beta$-Ala-ε-ACA$)_4$ spacer on a chitosan-modified SPOT array at 25 °C for 2 h. After the Boc group was removed by treating the slide with 50% TFA in DCM for 1 h, it was treated with a 5-mM solution of FITC and DIPEA in NMP at 25 °C for 2 h, and then thoroughly washed with NMP (×5) and DCM (×5). Fmoc-amino acids were freely mixed at their isokinetic concentrations and dissolved in NMP to yield a 6.7-mM solution (with 1 equivalent of BOP, HOBt, and DIPEA). The pre-activated solution was spotted onto its proper spots (200 nl/spot) and incubated at 25 °C for 1 h. After building up the final sequence, a pre-activated solution of Dabcyl-OSu in NMP (10.0 mM with the same equivalent of HOBt and DIPEA) was added to each spot (200 nl) and incubated at 25 °C for 15 h. Finally, the glass slides were treated with reagent K (82.5% TFA : 5% phenol : 5% water : 5% thioanisole : 2.5% ethanedithiol) at 25 °C for 1 h [22], thoroughly washed with NMP (×5) and DCM (×5), and air dried.

Thrombin Treatment on Internally Quenched Fluorescent Peptide Array

A 250-nM solution of thrombin in 100 mM Tris–HCl buffer (pH = 7.0, with 150 mM NaCl) was spotted on the peptide mixture array and incubated at 37 °C for 2 h in a closed chamber under humid conditions. Then, the glass slide was sonicated successively with 0.1% (v/v) Tween 20 in 50 mM PBS (pH 7.5) (×3) and water (×3) for 5 min, and the fluorescence profile was measured with a microarray scanner.

Solution Phase Thrombin Subsite Screening of Model Peptides

The peptide sequences of Fmoc-Ile-Thr-P_2-Arg-Gly-Phe-Ala-NH_2 (P_2: Pro, Ala, Asp, Asn, and Lys) were synthesized using Fmoc-Rink amide AM SURE resin (0.6 mmol/g, confirmed by Fmoc titration) by solid phase Fmoc chemistry [23]. After building up the final sequence, the samples were Fmoc deprotected and reacted with 5 equivalents of Dabcyl-OSu, HOBt, and DIPEA at 25 °C for 24 h. Then, each resin was suspended in 3 ml of reagent K, stirred at 25 °C for 1 h and filtered. The filtrates were evaporated with a vacuum evaporator, and the remaining product mixture was cooled in a refrigerator for 2 h. The peptide products precipitated after slowly adding cold ether (30 ml) to the mixtures at 0 °C. The peptide samples were washed with cold ether (5 × 20 ml) and then dried under reduced pressure.

A solution of 0.6 μM thrombin (2 μl) in 100 mM Tris–HCl buffer (pH = 7.5, 150 mM NaCl) was added to each 200-μl aliquot of 6 μM dabcyl-heptapeptide amides in Tris–HCl buffer. The reaction mixtures were then incubated in a rotary shaker (200 rpm) at 37 °C for 15 min. Absolute ethanol (1 ml) was added, and the samples were centrifuged for 10 min to finish the enzymatic reaction. The supernatants from the reaction mixture were air dried and analyzed using MALDI-TOF MS.

Results and Discussion

To prepare an equimolar peptide mixture array by SPOT synthesis, the isokinetic concentration of each amino acid was calculated to compensate for their reaction rate differences. Each stock solution of BOP-activated Fmoc-amino acid was diluted to yield various concentrations and then spotted onto γ-APTS treated glass surfaces according to their concentrations and incubated for 1 h (Figure 1). Unreacted amino groups were capped by acetylation. Fmoc groups attached on the surface were removed by treating with piperidine solution followed by FITC coupling. The fluorescence intensities of each spot, which corresponded to the coupling yield of Fmoc-amino acids, were measured to obtain the concentrations of complete reaction for 1 h and to determine the isokinetic concentrations (Table 1). Amino acids such as Arg, Asp, and Asn with bulky side chain protecting groups showed lower reactivity than other small-sized Fmoc-amino acids. Ostresh *et al.* reported on the coupling efficiency of Boc-amino acids on a polystyrene support [24]. They found that the coupling rates of Ile, Val, and Thr were slower than those of other less hindered amino acids. Comparing their results with ours, we found no significant correlation between the coupling efficiencies of Fmoc-amino acids and Boc-amino acids because of the differences in the side chain protecting groups.

We designed Dabcyl-P4-P3-P2-P1-P1′-P2′-P3′-Lys(FITC) as a model peptide sequence to screen the thrombin substrates. Peptide couplings were carried out using Fmoc chemistry with BOP/HOBt. The detailed scheme is summarized in Figure 2. The peptide synthesis was performed on chitosan-grafted SPOT arrayed glass chips, which reduce non-specific protein binding and result in a high coupling yield from peptide synthesis [19–21]. As we previously reported that the $(\beta\text{-Ala-}\varepsilon\text{-ACA})_4$ spacer produces high affinity between the target protein and the peptides on the glass surface, a spacer was introduced on the chitosan-grafted glass surface before the peptide sequence was synthesized to improve accessibility of the protease to the peptides on the surface [18].

Dabcyl and FITC were coupled on the peptide as a quencher and a reporter, respectively, to apply the peptide array to the fluorescence quenching-based screening [25]. In particular, the P_1 site was fixed with Arg and the P_2 site was diversified with 19 amino acids. The randomized amino acids were incorporated with equimolar proportions at the P_1', P_2', P_3', P_3, and P_4 positions, respectively, based on the isokinetic concentration of each amino acid (Table 1). The final form of peptide sequence synthesized on the surface was Dabcyl-X-X-P_2-Arg-X-X-X-Lys(FITC).

After all of the peptide sequences were synthesized on the SPOT arrayed glass chip, the chip was incubated with thrombin solution to obtain fluorescence intensities corresponding to the degrees of hydrolysis of the internally quenched fluorescent peptide substrates caused by thrombin. As a result, Pro at the P_2 subsite showed the strongest fluorescence and Asn and hydrophobic residues such as Val and Leu demonstrated significant hydrolysis, which was consistent with that in the literature (Figure 3) [26,27]. Additionally, we found that Gln, Arg, Thr, and Glu gave higher preferences. These differences in preferences are allowable because the enzymatic reaction in the literature occurs on polymer beads [26] or in the solution phase [27], respectively.

Five representative peptide sequences of Dabcyl-heptapeptide amides (Dabcyl-Ile-Thr-P_2-Arg-Gly-Phe-Ala-NH_2) were synthesized and reacted with thrombin in the solution phase. Pro and Asn at the P_2 position were picked as highly preferred sequences; Ala as a hydrophobic sequence, Asp as a negative sequence, and Lys as a positive sequence. The overall yields of the peptide synthesis were 80–90%. The purities of the resulting Dabcyl-heptapeptide amides were verified using HPLC and summarized in Table S1. Each Dabcyl-heptapeptide amide was treated with thrombin and then the reaction mixtures of the hydrolyzed peptides were analyzed using MALDI-TOF MS (Table S2). Measuring the degree of hydrolysis caused by thrombin, Pro at the P_2 subsite showed that the proteolytic reaction completed, whereas other peptide sequences showed only partial hydrolysis (Figure S1). Although some differences in screening methods were observed, Pro at

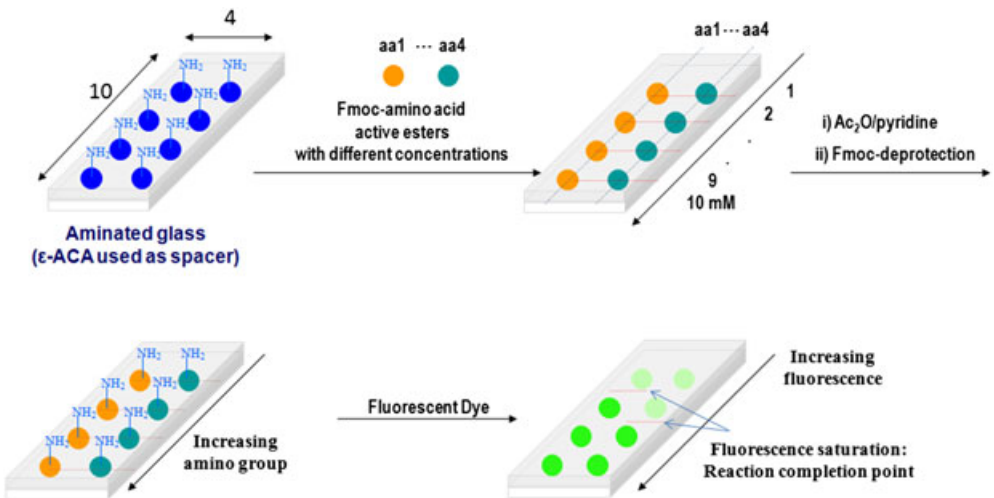

Figure 1. Determination of the isokinetic Fmoc-amino acid concentrations for the coupling reaction onto a SPOT arrayed glass chip.

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Table 1. Coupling rates of Fmoc-amino acids on SPOT arrayed glass chip and the ratio of isokinetic concentration[a]

Amino acid[b]	Concentration for complete reaction (mM)	Ratio of isokinetic concentration[c] (%)	Amino acid	Concentration for complete reaction (mM)	Ratio of isokinetic concentration (%)
G	2.5	3.6	H	3.5	5.0
A	3.0	4.3	K	4.0	5.8
V	2.5	3.6	R	5.0	7.2
L	3.0	4.3	W	4.5	6.5
I	3.5	5.0	D	5.0	7.2
P	4.5	6.5	E	2.0	2.9
F	3.0	4.3	S	3.5	5.0
M	3.5	5.0	T	4.0	5.8
N	5.0	7.2	Y	4.0	5.8
Q	3.5	5.0	—	—	—

[a]Molar ratio to build a randomized sequence without the Cys residue.
[b]All Fmoc-amino acids are abbreviated with single letters.
[c]Ratio of isokinetic concentration was obtained from the concentration data for the complete reaction.

the P_2 subsite showed the highest thrombin specificity for both fluorescence quenching-based chip assay and solution phase assay. From these results, we confirmed that the mixture-based and internally quenched fluorescence peptide SPOT array can be used for subsite screening of proteases.

Conclusion

The ratio of isokinetic concentration was determined for every Fmoc-amino acid except Cys to synthesize a peptide array for positional scanning. Then, an internally quenched fluorescent peptide array, Dabcyl-X-X-P_2-Arg-X-X-X-Lys(FITC), was built on a chitosan-grafted SPOT array chip. P_2 subsite thrombin activities were screened using fluorescence imaging, and the result was compared with one from solution phase hydrolysis results obtained using MALDI-TOF MS. Despite the difference in the activities between the fluorescence quenching-based assay and the solution phase assay, Pro at the P_2 subsite showed the highest specificity for thrombin in both assays. We conclude that the fluorescence quenching-based assay can be applied to determine protease subsite preferences.

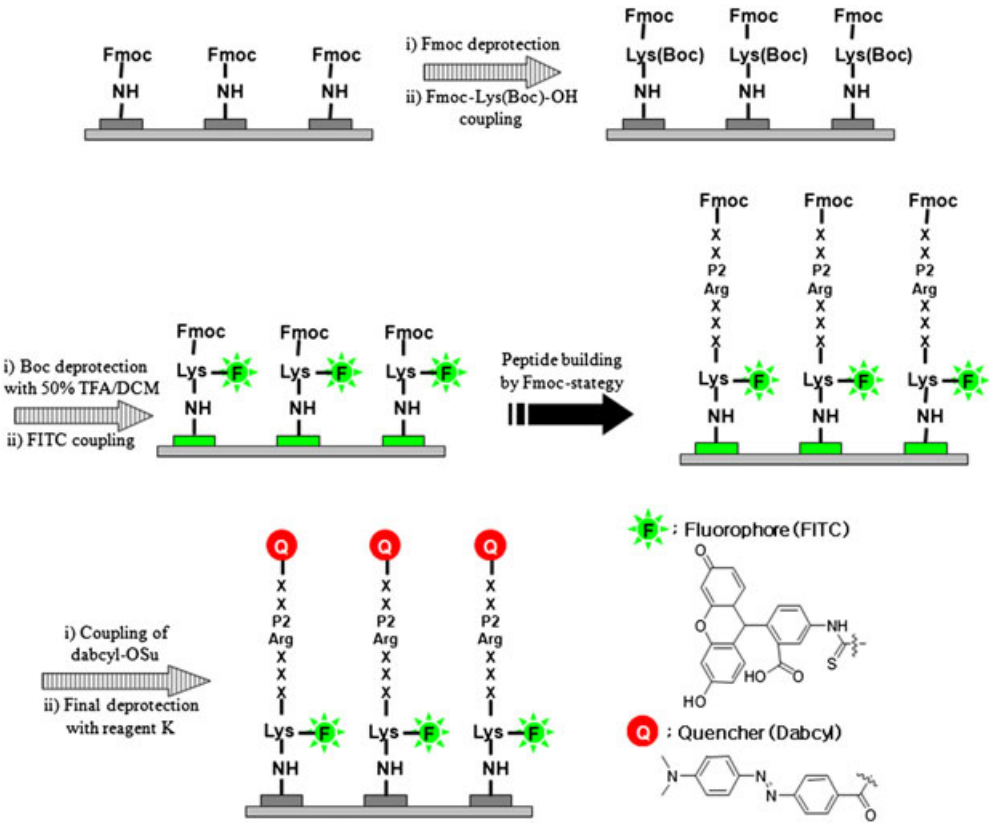

Figure 2. Synthesis of internally quenched fluorescent peptide substrates for protease subsite screening on a SPOT arrayed glass chip.

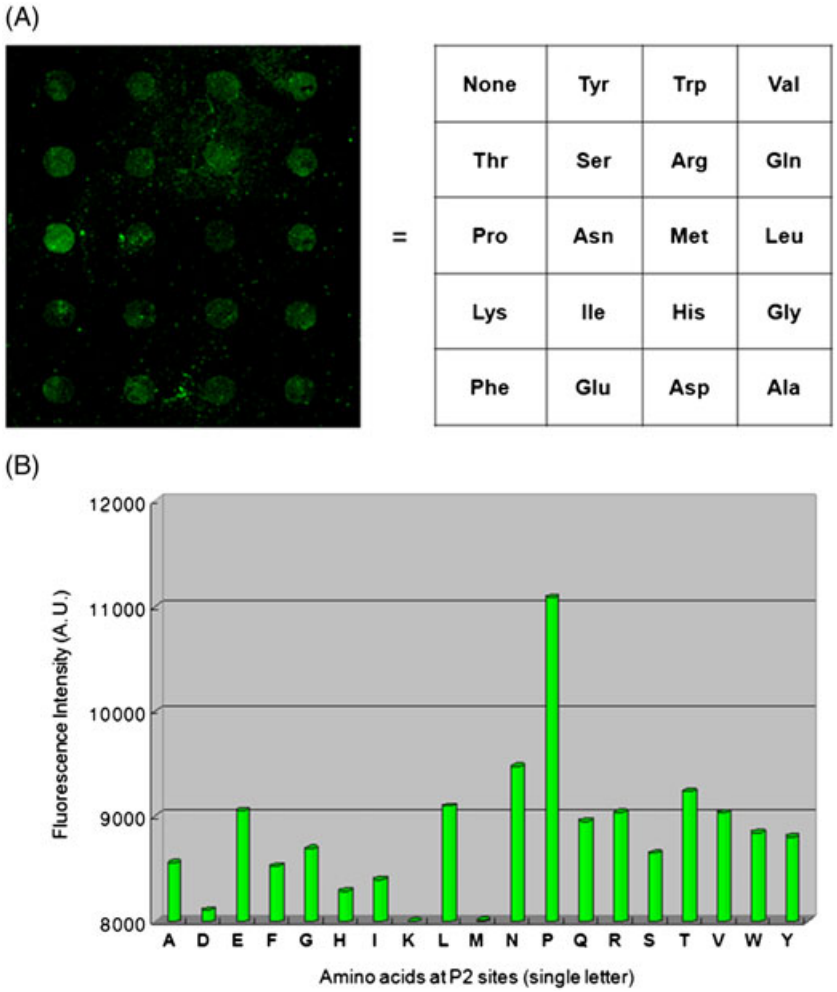

Figure 3. Screening for thrombin subsite preference on a SPOT arrayed glass chip via fluorescence quenching-based assay: (A) fluorescence scanning images for FITC and (B) the degree of thrombin-catalyzed hydrolysis of heptapeptides (X-X-P_2-Arg-X-X-X) according to the P_2 position.

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