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A simple method for selection of trypsin chromogenic substrates using combinatorial chemistry approach

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Abstract

A tetrapeptide combinatorial library, considered as chromogenic substrates of bovine β-trypsin, was synthesized by the solid phase method. The peptides contain an analog of *p*-nitroanilide, obtained by attaching 5-amino-2-nitrobenzoic acid ($Anb^{5,2}$) to the C-termini. Deconvolution of the peptide library, performed in solution using an iterative method, yielded four efficient trypsin substrates. The most active one, Phe-Val-Pro-Arg-$Anb^{5,2}$-NH_2, appeared to be 125-fold more active than Bz-D,L-Arg-pNA (BAPNA) used as a reference compound. The reported method of designing trypsin chromogenic substrate libraries is straightforward. Such *p*-nitroanilides may be useful for the investigation of any protease substrate specificity.

Keywords: Peptides; Combinatorial chemistry; Chromogenic substrates; Trypsin

Almost one-third of all proteases can be classified as serine proteinases, named for the nucleophilic Ser residue at the active site. They also belong to the group of the most thoroughly examined proteins. The fundamental information for the understanding of the molecular mechanism and the specificity of serine proteinases was obtained from the kinetic data of enzyme–substrate interactions. Although there are many naturally occurring proteins which interact with serine proteinases, their application as substrates in kinetic studies is limited due to their size and the fact that they contain multiple binding sites. Therefore, more conveniently serine proteinases' substrate specificity can be characterized using synthetic chromogenic or fluorogenic substrates. The first efficient chromogenic substrate of bovine β-trypsin, named BAPNA (*p*-nitroanilide of benzoyl-D,L-arginine), was developed by Erlanger et al. [1]. This compound was also used as a starting structure to obtain chromogenic substrates of other serine proteinases. Despite the present availability of a broad spectrum of serine proteinase fluorogenic substrates, *p*-nitroanilides are still very frequently used. Their specificity and sensitivity are sufficient for in vitro experiments and, in addition, the methodology of spectroscopic measurements (as compared with fluorogenic substrates) is much simpler.

In the last decade, a significant progress has been made by the application of combinatorial chemistry methods to characterize the proteinase substrate specificity [2]. These investigations were generally carried out on peptide libraries of fluorogenic substrates. The solid-phase synthesis of peptides with a *p*-nitroanilide moiety at the C-terminus is not a routine work and usually several additional synthetic steps have to be incorporated in the standard procedure [3–6]. Bearing this in mind, the synthesis of such peptide libraries is not an easy task. However, Hojo et al. [7] have recently reported a rather simple modification which considerably facilitated the solid-phase synthesis of peptidic *p*-nitroanilides. They used 5-amino-2-nitrobenzoic acid ($Anb^{2,5}$) instead of *p*-nitroaniline for the preparation of *p*-nitroanilide analogs. Taking such an approach, they attached

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the synthesized peptides to the solid support via the carboxyl group of $Anb^{5,2}$. They have also shown that chromogenic substrates with the amide moiety ($Anb^{5,2}$-NH_2) and carboxyl group ($Anb^{5,2}$-OH) display the same spectroscopic properties as *p*-nitroanilides.

Continuing our studies [3,4] on the influence of modifications in *p*-niroanilide substrates on their interaction with bovine β-trypsin, we decided to apply combinatorial chemistry methods. We synthesized the following library of chromogenic substrates:

$$X_4–X_3–X_2–X_1–Anb^{5,2}–NH_2$$

where X_1, X_2, X_3, X_4-Glu, Arg, Phe, Lys, Ser, Ile, Asp, Val, Tyr, Ala, Pro.

All positions were modified by the same 11 proteinogenic amino acids. Among them basic, acidic, aromatic, and hydrophobic ones are present.

Materials and methods

Peptide synthesis. Peptides for kinetic studies and peptide library were synthesized manually by the solid-phase method using Fmoc chemistry, as described previously [8]. TentaGel S RAM (substitution 0.23 meq/g) (RAPP Polymere, Germany) was used as a support. The amino acid derivatives used for the synthesis were as follows: Fmoc-Ala, Fmoc-Ile, Fmoc-Phe, Fmoc-Pro, Fmoc-Val, Fmoc-Arg(Pbf), Fmoc-Lys(Boc), Fmoc-Ser(tBu), Fmoc-Asp(OtBu), Fmoc-Glu(OtBu), and Fmoc-Tyr(tBu). 5-Amino-2-nitrobenzoic acid was attached to the resin using the TBTU/DMAP method [7]. The C-terminal amino acid residues were incorporated using a method with $POCl_3$ as the coupling reagent [7]. The other amino acid derivatives were coupled by the DIPCDI/HOBt method. After completing the synthesis, the peptides were cleaved from the resin using a mixture of TFA/phenol/triisopropylsilane/H_2O (88:5:2:5, v/v/v/v) [9]. The resynthesized peptides were purified by HPLC by means of a semipreparative RP Kromasil 100 C8 column (250 × 8 mm) (Knauer, Germany). Peptide libraries were used for further experiments without purification.

Preparation of the peptide library. The peptide library was synthesized by the portioning-mixing method [10,11]. Initially, 5 g of the solid support (TentaGel S RAM) was used. Fivefold amino acid molar excess was used for the coupling. Other synthetic methods employed were as described above.

Screening the peptide library for trypsin specificity. Deconvolution of the peptide library synthesized was performed by the iterative method in solution [12]. The stock solution was prepared by dissolving 10 mg of the peptide library in 500 μl DMSO. Ten microliters of stock solution was used in the experiment. The enzymatic hydrolysis of the peptide library was performed in 0.1 M Tris–HCl (pH 8.3) buffer at 25 °C. Measurements were carried out at an enzyme concentration of 10^{-6} M. The increase in absorbency at 410 nm resulting from the release of $Anb^{5,2}$-NH_2 was measured as a function of time. The calculated initial velocities were used as measures of the substrate activity of the peptide library investigated. Each experiment was repeated at least 3 times. Data with a difference up to 10% were rejected.

Kinetic studies. All measurements were performed using a Cary 3E spectrophotometer (Varian, Australia). The concentration of bovine β-trypsin stock solution was determined by titration with NPGB. The stock of the resynthesized substrate solution was prepared by dissolving about 10 mg of the peptide in 250 μl DMSO, and was further diluted 2–20 times, whereas enzyme concentrations ranged from 1.1×10^{-6} to 5.1×10^{-8} M. From three to five measurements were carried out for each compound (systematic error expressed as a standard deviation never exceeded 10%). All details of kinetic studies and the calculation of kinetic parameters, Michaelis constants (K_m), catalytic constants (k_{cat}), and specificity constants (k_{cat}/K_m) were described in our previous papers [3,4].

Results and discussion

The deconvolution of the peptide library (consisting of $11^4 = 14641$ peptides) against bovine β-trypsin is summarized in Fig. 1. The results obtained indicate that the aromatic Phe residue present in position P4 of the

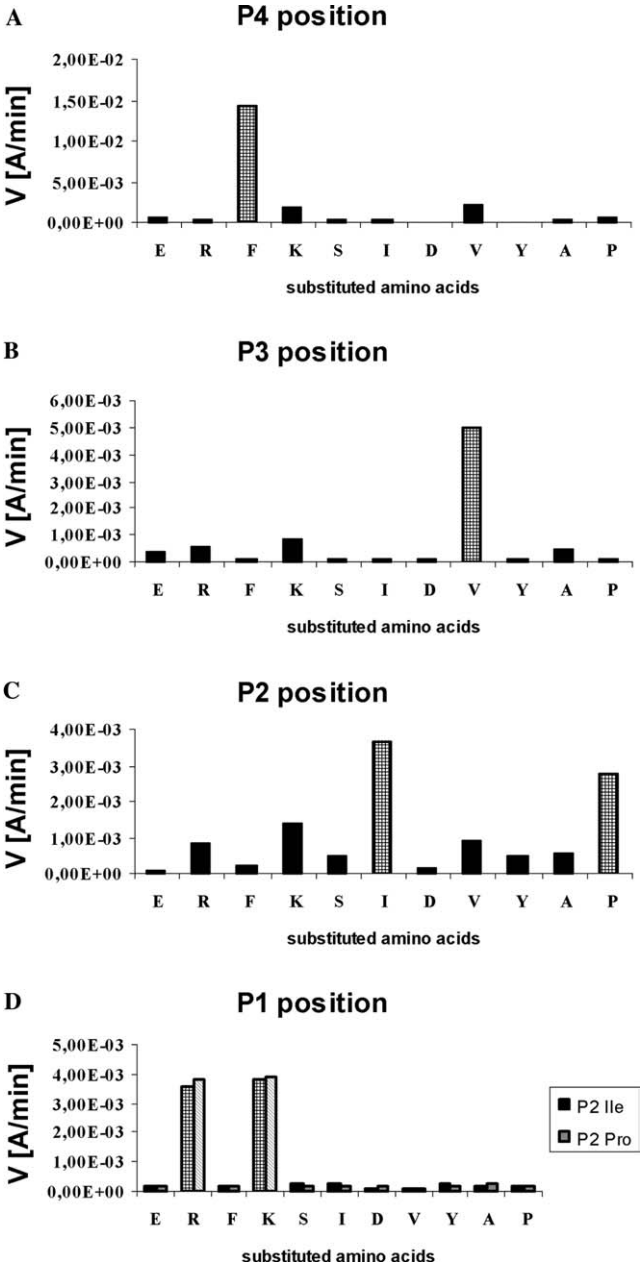

Fig. 1. Deconvolution of the combinatorial library of trypsin chromogenic substrates. Selected amino acid residues are marked.

Table 1
Kinetic parameters with bovine β-trypsin of the selected chromogenic substrates

Substrate	Enzyme concentration [M]	V_{max} [mol/s]	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [s^{-1} M^{-1}]
BAPNA (Bz-D,L-Arg-pNA)	$(11.2 \pm 1.4) \times 10^{-8}$	$(8.9 \pm 0.4) \times 10^{-8}$	0.8 ± 0.1	158.0 ± 9.2	$(0.05 \pm 0.002) \times 10^5$
Phe-Val-Pro-Arg-Anb^{5,2}-NH_2	$(1.1 \pm 0.2) \times 10^{-8}$	$(30.2 \pm 2.7) \times 10^{-8}$	27.4 ± 3.4	43.9 ± 4.8	$(6.24 \pm 0.73) \times 10^5$
Phe-Val-Ile-Arg-Anb^{5,2}-NH_2	$(1.7 \pm 0.2) \times 10^{-8}$	$(7.5 \pm 0.8) \times 10^{-8}$	4.4 ± 0.5	29.5 ± 3.2	$(1.50 \pm 0.12) \times 10^5$
Phe-Val-Pro-Lys-Anb^{5,2}-NH_2	$(5.1 \pm 0.6) \times 10^{-8}$	$(20.4 \pm 3.2) \times 10^{-8}$	4.0 ± 0.3	240.0 ± 27.4	$(0.17 \pm 0.02) \times 10^5$
Phe-Val-Ile-Lys-Anb^{5,2}-NH_2	$(11.2 \pm 1.4) \times 10^{-8}$	$(36.1 \pm 3.5) \times 10^{-8}$	3.2 ± 0.3	96.0 ± 10.5	$(0.33 \pm 0.04) \times 10^5$

synthesized peptide library displays the strongest interaction with the enzyme investigated. Interestingly, the other aromatic residue (Tyr) present in the library had a significantly lower impact on the substrate activity. With the Phe residue fixed in position P4, sublibraries differ in position P3, and with variable positions P2 and P1 they are screened (Fig. 1B). In this case the highest activity was recorded when the Val residue was introduced in position P3. Taking the same approach we were able to show that the Ile residue in position P2 produced the most active trypsin substrates. Considerably high activity (as compared with other amino acid residues) was also achieved when the Pro residue was present in this position. Therefore, both these residues were included for the last step of the screening. This yielded the synthesis of 22 chromogenic substrates. Their relative substrate activity is presented in Fig. 1D. According to the expectations, among the peptides synthesized only those with Arg and Lys in position P1 displayed strong trypsin substrate activity. In this series of peptides, the Ile and Pro residues introduced in position P2 did not change the substrate activity. Therefore, all four active peptides were resynthesized and subjected to kinetic studies with bovine β-trypsin. The experimental data were used for calculations of K_m, k_{cat}, and k_{cat}/K_m values. The results obtained are summarized in Table 1. Compared to the parameters calculated for the standard trypsin substrate, BAPNA, all four selected substrates are considerably faster hydrolyzed and (except one) display higher affinity to the enzyme investigated. The calculated values of the specificity constants indicate that they were also significantly more active. The most active one was the substrate Phe-Val-Pro-Arg-Anb^{5,2}-NH_2. It appeared to be 125-fold more active than the reference compound. The promising results discussed above are a good starting point for further experiments; for example, more specific substrates can be obtained when the free N-terminal amino group is acetylated [4], but other groups might also be worth investigating. Chemical synthesis affords practically a free choice of amino acids to be introduced into the peptide chain, and therefore non-proteinogenic amino acids may also be considered for designing such libraries. In addition, the substrates discussed herein contain an amide moiety coupled to the aromatic ring. By selection of different types of resins, compounds with other constituents can be obtained.

The method described in this paper for selection of trypsin chromogenic substrates by the combinatorial approach is based on the standard solid-phase peptide synthesis. Also the deconvolution method requires no special devices. It can be employed to select chromogenic substrates of any proteinases. Such chromogenic substrate libraries may prove very useful for the determination of proteinase specificity of novel enzymes.

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