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Design of serine proteinase inhibitors by combinatorial chemistry using trypsin inhibitor SFTI-1 as a starting structure

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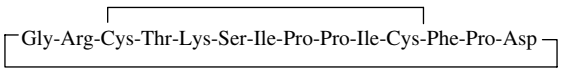
Abstract: A small peptide library of monocyclic SFTI-1 trypsin inhibitor from sunflower seeds modified in positions P_1 and P_4' was synthesized using a portioning-mixing method. The peptide library was deconvoluted by the iterative approach in solution. Two trypsin ([Met^9]-SFTI-1 and [Arg^5, Abu^9]-SFTI-1), one chymotrypsin ([Phe^5]-SFTI-1) and one human elastase ([Leu^5, Trp^9]-SFTI-1) inhibitors were selected and resynthesized. The values of their association equilibrium constants (K_a) with target enzymes indicate that they are potent inhibitors. In addition, the last two analoges belong to the most active inhibitors of this size. The results obtained show that the conserved Pro^9 residue in the Bowman–Birk inhibitor (BBI)s is not essential for inhibitory activity.

Keywords: serine proteinases; trypsin; chymotrypsin; elastase; inhibitors; combinatorial chemistry

INTRODUCTION

Inhibitors of serine proteinases are among the most extensively studied proteins. They are widely distributed in animals and microorganisms and are responsible for the control of physiologically important endogenous proteinases. Plants, especially their seeds, are also rich in serine proteinase inhibitors, which constitute a part of the plant defense system against pests and pathogens. Scientific interest in these compounds is therefore connected with their potential application as therapeutic and agrochemical agents. Many of serine proteinase inhibitors interact with their target enzymes in a substrate-like manner. They are called standard mechanism inhibitors [1] or canonical inhibitors [2]. Extensive studies on the isolation and characterization of these compounds have revealed at least 18 families of canonical inhibitors [2]. They are small proteins (or even miniproteins) consisting of 30–200 amino acid residues. Trypsin inhibitor SFTI-1 isolated a few years ago from sunflower seeds [3] occupies a special position among canonical inhibitors obtained so far. It consists of 14 amino acid residues with its structure cross-linked by two cycles. SFTI-1 exhibits high sequential and structural homology with the trypsin binding loop of the Bowman–Birk inhibitor (BBI) family. Lys^5-Ser^6, being the inhibitor's reactive site P_1–P_1', therefore SFTI-1 forms complexes with the target enzymes in stoichiometric ratio 1 : 1. SFTI-1 displays a well-defined structure both in solution [4] and in crystal, when forming a complex with trypsin [3]. A fragment of the inhibitor comprising Cys^3 – Ile^7 (corresponds to positions P_3–P_2' of the inhibitor) is responsible for most contacts with the enzyme [5]. Its primary structure is shown below:



Considering the small size and high inhibitory activity of SFTI-1, several research groups focused their interest on this inhibitor as a starting structure for structure-activity relationship studies. Recent results have been summarized in three review papers [5,6,7]. In our previous work [8] we have shown that also monocyclic analoges of SFTI-1 displayed strong inhibitory activity. The value of the association equilibrium constant (K_a) determined for the analogue containing disulfide bridge only with bovine β-trypsin was the same, within the experimental error, as that obtained for wild SFTI-1. Trypsin inhibitory activity of the analogue containing head-to-tail cyclization only (Cys residues were replaced by Abu) was approximately 2.5-fold lower, which corresponded with its lower proteolytic resistance [8]. On the basis of these results we have chosen the first analogue as the lead structure to design SFTI-1 analoges [9,10] modified in substrate specificity P_1 position. We have proved that by the introduction of noncoded amino acids in this position it is possible to obtain inhibitors of bovine β-trypsin, α-chymotrypsin and human leukocyte elastase. As stressed by several authors [5,6], Thr^4, Ser^6 and Ile^7 present in the binding loop of SFTI-1 are optimal for its interaction with trypsin. In addition, Pro^8 conserved in position P_3' of BBI formed a *cis* peptide bond with the preceding amino acid residue, essential to retain a β-hairpin structure of the inhibitor binding loop and,

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as a consequence, crucial to its biological activity. The next position is very often also occupied by the Pro residue, but its role is less obvious. Descours *et al.* [11] postulated that the Pro residue in position P_4' is not important for trypsin inhibitory activity. This is in good agreement with results published by Brauer *et al.* [12] who have shown that the presence of the Pro residue in position P_4' of the BBI is not essential for the activity but facilitates stabilization of the *cis* peptide bond formed by the preceding Pro residue. Both groups drew these conclusions from the fact that the BBI analoges modified by Ala in the position discussed, retained trypsin inhibitory activity. More recently, Hilpert *et al.* [13] have shown that also other proteinogenic amino acids are acceptable in this position. We decided to carry out a more detailed analysis, applying previously published combinatorial chemistry procedure [14]. In position P_4' of monocyclic SFTI-1 we introduced all proteinogenic amino acids (excluding Cys), as well as Abu and Nle. In the substrate specificity P_1 position, amino acids suitable for the interaction with S_1 cavities of trypsin, chymotrypsin and elastase were present. The general sequence of the small library synthesized is as follows:

$$Gly^{1}\text{-}Arg\text{-}\overbrace{Cys^{3}\text{-}Thr\text{-}X_1{}^{5}\text{-}Ser^{6}\text{-}Ile\text{-}Pro\text{-}X_2}\text{-}Ile\text{-}Cys^{11}}\text{-}Phe\text{-}Pro\text{-}Asp$$

Where X_1 = Ala, Abu, Val, Leu, Arg, Har, Lys, His, Phe, l-citruline, l-homocitruline, Tyr;

X_2 = all proteinogenic amino acids (excluding Cys), Nle and Abu.

MATERIALS AND METHODS

Peptide Synthesis

All peptides were synthesized by the solid-phase method using Fmoc chemistry. The *C*-terminal amino acid residue (Fmoc-Asp(OBu^t)) was attached to 2-chlorotritylchloride resin (substitution of Cl 1.46 mequv/g) (Calbiochem–Novabiochem AG, Switzerland) in the presence of an equimolar amount of DIPEA in relation to the amino acid in anhydrous condition in DCM solutions. Peptide chains were elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with 30% piperidine in the mixture of DMF/NMP (1 : 1, v/v) with addition of 1% Triton X-100. After completing the synthesis, the peptides were cleaved from the resin simultaneously with the side-chain deprotection in a one-step procedure, using a mixture of TFA/phenol/triisopropylsilane (88 : 5 : 2.5, v/v) [15]. In the last step, disulfide bridge formation was performed by I_2 in MeOH/$H_2$0 (1 : 1, v/v) solution applying the procedure described elsewhere [16]. The progress of the reaction was monitored by HPLC. The crude resynthesized peptides were purified by HPLC on a Beckman Gold System (Beckman, USA) using an RP Kromasil-100, C_8, 5 μm column (8 × 250 mm) (Knauer, Germany). The solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Linear gradient from 20 to 80% B for 30 min., flow rate 2.0 ml/min, monitored at 226 nm. The purity of the peptides synthesized was checked on an RP Kromasil 100, C_8, 5 μm column (4.6 × 250 mm) (Knauer, Germany). Solvent system was 0.1% TFA (A) and 80% acetonitrile in A (B). Linear gradient from 20 to 80% (B) for 30 min, flow rate 1 ml/min, monitored at 226 nm. The mass spectrometry analysis was carried our on MALDI MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany) using α-CCA matrix.

Preparation of the Peptide Library

The peptide library was synthesized by the portioning-mixing method. Initially, 10 g of the solid support (2-chlorotritylchloride resin) was used. Five-fold amino acid molar excess was used for the coupling. Other synthetic methods employed were as described above.

Screening the Peptide Library for Trypsin, Chymotrypsin and Elastase Inhibitors

Deconvolution of the peptide library synthesized was performed by the iterative method in solution. The experiments were performed in the following manner. To 1.5 ml of experimental buffer (see below), 10 μl of enzyme solution (concentrations of enzymes in the experimental cuvettes are given in Figure 1) and 10 or 50 μl of peptide sublibrary (10 mg of lyophilized samples were dissolved in 0.001 M HCl) were added. After incubation for 15 min, 10 μl of appropriate substrate solution was added. The substrate concentrations never exceed 0.2 K_m in the cuvette. The decreasing initial substrate hydrolysis rate was considered as the measure of the inhibitory activity of the sublibrary investigated. The values of the hydrolysis rates obtained in the absence of the sublibrary were used as controls. In the case of similar results obtained for different sublibraries, the experiments were repeated applying two- or three-fold lower enzyme concentration. The experiments were repeated 3–5 times for each sublibrary. Results differing by more than 10% were rejected.

Determination of Association Equilibrium Constants

Bovine β-trypsin (Sigma Chem. Co. USA) concentration was determined by spectrophotometric titration with 4-nitrophenyl-4′-guanidinobenzoate (NPGB) at an enzyme concentration of the order of 10^{-6} M in curatte. Standardized trypsin solution was used to titrate ovomucoid from turkey egg whites, which in turn served to determine the solution concentrations of bovine α-chymptrypsin (Sigma Chem. Co. USA) and human leukocyte elastase (HLE) (Biocentrum Sp. z o.o. Kraków, Poland). The concentrations of SFTI-1 analoges investigated were determined by titration with the standardized solution of the target enzyme. Association equilibrium constants (K_a) were determined by the Green–Work method modified in the laboratory of M. Laskowski [17,18]. Increasing amounts of the inhibitor were added to the constant amount of the enzyme. After suitable incubation time the residual enzyme activity was measured on a Cary 3E spectrophotometer (Varian, Australia) using the turnover substrate. Enzyme-inhibitor interactions were determined in 0.1 M tris-HCl, pH 8.3 buffer containing 20 mM $CaCl_2$ and 0.005% Triton X-100 at 22 °C. In the case of experiments performed for HLE, 0.1 M tris-HCl buffer pH 7.8 containing 20 mM $CaCl_2$, 0.5 M NaCl and 0.005% Triton X-100 was added. The measurements

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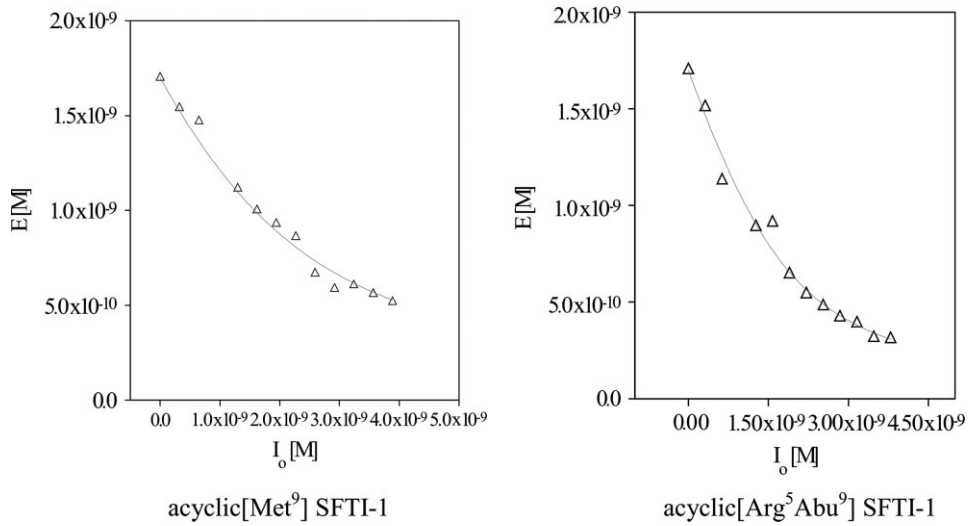

Figure 1 Inhibition curves of bovine β-trypsin by SFTI-1 analoges.

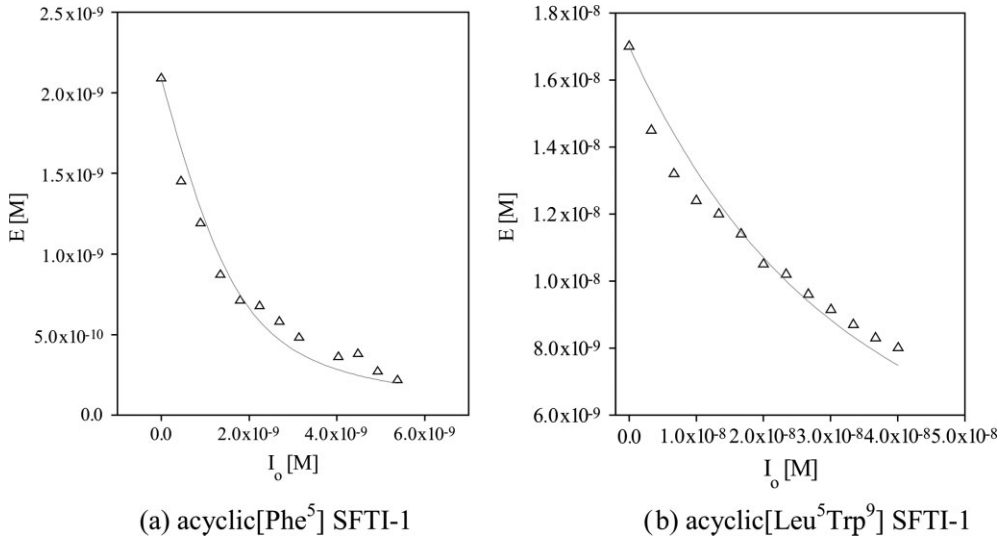

Figure 2 Inhibition curves of (a) bovine α-chymotrypsin and (b) human leukocyte elastase by SFTI-1 analoges.

were carried out at initial enzyme concentrations from the range of 1.7–13 nM for trypsin, 2.0–2.1 nM for chymotrypsin, and 16–23 nM for HLE. After proper incubation time, the residual enzyme activity was measured with Tos-Gly-Pro-Arg-p-nitroanilide (for trypsin), Suc-Ala-Ala-Pro-Leu-p-nitroanilide (for chymotrypsin) and MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide (for elastase). The experimental points were analyzed based on the plot of [E] *versus* [I_o]. The experimental data were fitted to the theoretical values using the program of A. Liwo (University of Gdańsk) utilizing the Marquardt method [19]. The K_a values calculated are given in Table 1, whereas the inhibition curves obtained for selected inhibitors are shown in Figures 1 and 2.

Table 1 Association equilibrium constants of the selected acyclic analogue of SFTI-1 with the experimental enzymes

Analogue	Enzyme	$K_\alpha[M^{-1}]$
SFTI-1 [8]	Bovine β-trypsin	9.9×10^9
[Met^9]-SFTI-1	Bovine β-trypsin	$(9.2 \pm 1.5) \times 10^8$
[Arg^5, Abu^9]-SFTI-1	Bovine β-trypsin	$(1.9 \pm 0.3) \times 10^9$
[Phe^5]-SFTI-1	Bovine α-chymotrypsin	$(2.0 \pm 0.2) \times 10^9$
[Leu^5, Trp^9]-SFTI-1	Human leukocyte elastase	$(3.0 \pm 0.4) \times 10^7$

RESULTS AND DISCUSSION

The library synthesized consists of 252 monocyclic SFTI-1 analoges modified in positions 5 and 9, which correspond to positions P_1 and P_4' of the inhibitor, respectively. Since we decided to check the inhibitory activity of this peptide library against bovine β-trypsin, bovine α-chymotrypsin and human leukocyte elastase, basic, aromatic and hydrophobic amino acids were

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introduced in the substrate specificity P_1 position. Deconvolution of the peptide library in this position is summarized in Figure 3. The presence of Arg and Lys residues appeared to be optimal. In should be pointed out that analoges with L-homoarginine that differ from Arg by the presence of an additional methylene group in the side-chain, are practically inactive. This finding is in good agreement with our previous results obtained for analoges of CMTI-III inhibitor isolated from squash seeds [20]. We have shown that the distance between the positively charged side-chain group in position P_1 of the inhibitor and β-carboxylate of trypsin Asp_{189} is essential for the enzyme-inhibitor interaction. The results presented herein again confirmed, this time by combinatorial chemistry methods, the strictness of this requirement. The Phe residue present in the position discussed yielded analoges with the highest chymotrypsin inhibitory activity. Interestingly, analoges with aromatic Tyr or aliphatic Leu residues appeared significantly less active. As reported by Krowarsh *et al.* [21], all three amino acids mentioned above introduced in position P_1 of BPTI produced chymotrypsin inhibitors with a similar potency. The strongest inhibition of human leukocyte elastase was achieved with Leu residue in the discussed position of the library investigated. Significant inhibition was also observed for Ala, but only the first amino acid was selected for further investigations. The results of the deconvolution of the series of SFTI-1 analoges modified in position P_4' with fixed optimal amino acids in position P_1 are presented in Figures 4 and 5. In the case of trypsin inhibitors, two series of analoges were synthesized,

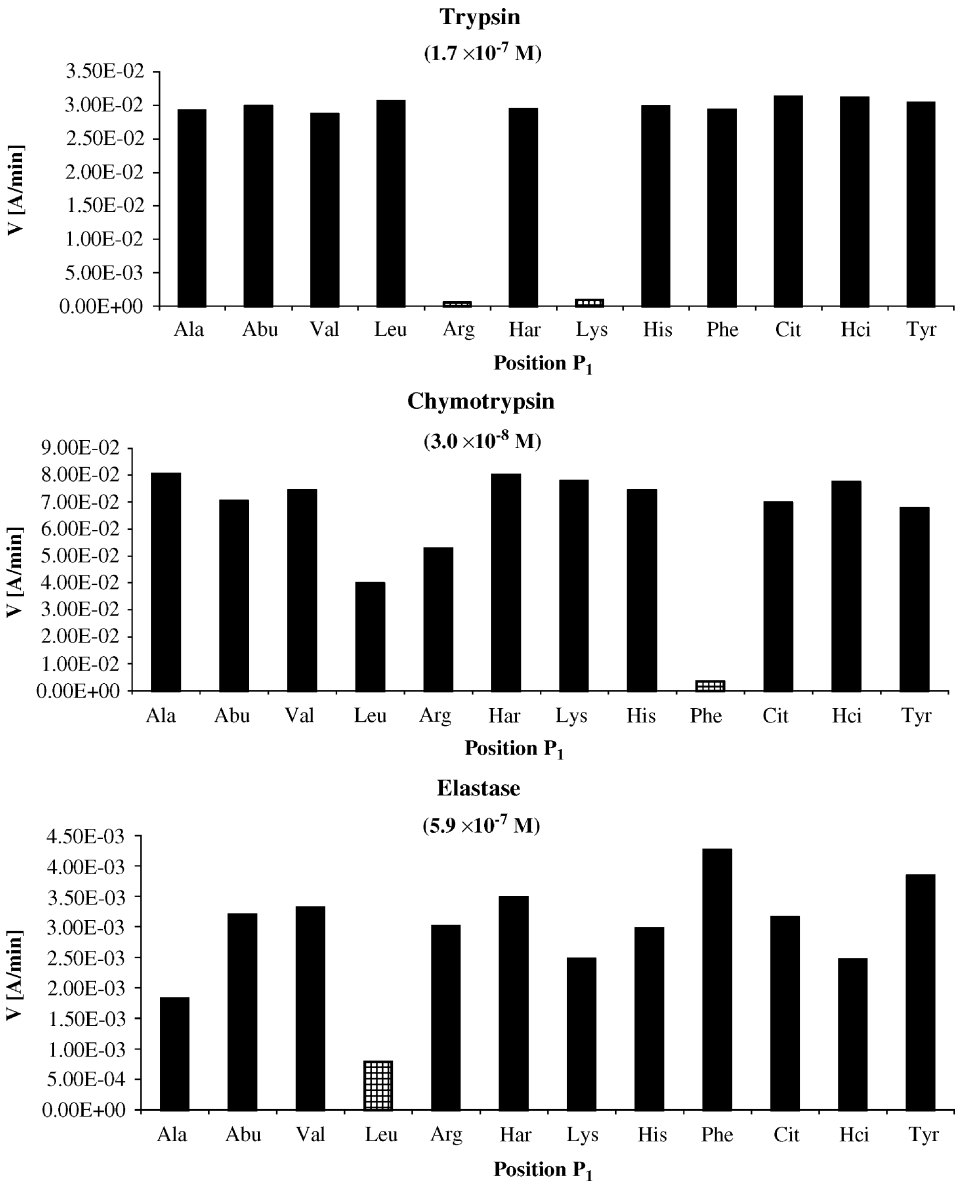

Figure 3 Deconvolution of the peptide library in position P_1 against bovine β-trypsin, bovine α-chymotrypsin and human leukocyte elastase.

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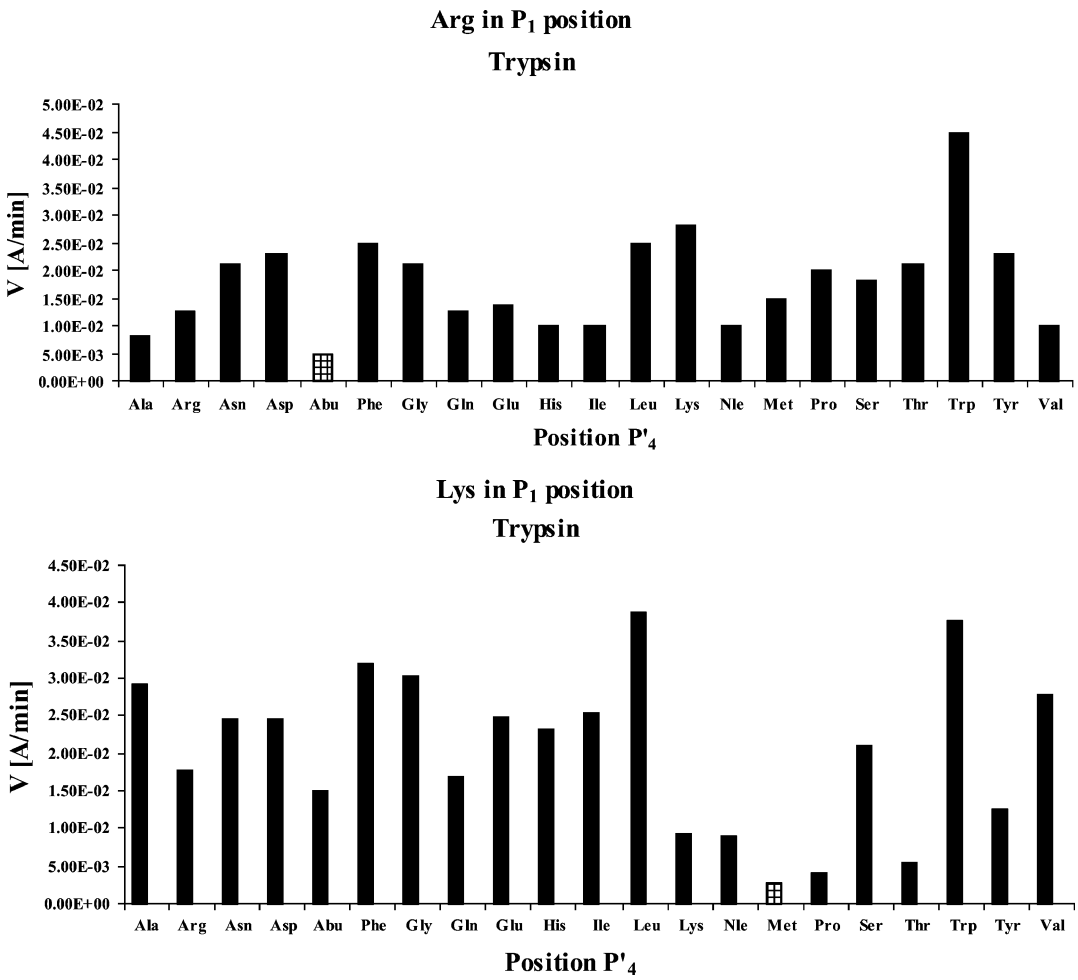

Figure 4 Deconvolution of the peptide library in position P_4' against bovine β-trypsin.

one with Lys and the second with Arg in position P_1. When the Lys residue was present in the position discussed, the lowest substrate hydrolysis rates were determined for analoges with Met and Pro in position P_4'. Surprisingly, a different inhibition profile was obtained in the second series in which noncoded hydrophobic Abu appeared to be optimal. It is worth noticing that other amino acid residues with aliphatic side-chains produced more active SFTI-1 analoges than the conservative Pro residue in this position. On the other hand, among chymotrypsin inhibitors (Phe in substrate specificity P_1 position), the one with the Pro residue in position P_4' displayed the highest activity. Less unambiguous results were obtained for SFTI-1 analoges with fixed Leu residue in position P_1 when screened against human leukocyte elastase. Several amino acids, such as Trp, Ile, and Arg, when introduced in the position discussed, decreased the substrate hydrolysis rates, but the values obtained suggest that these inhibitors display a rather moderate activity.

All analoges with the highest inhibitory activity towards the experimental enzymes were resynthesized by a classical approach and were subjected to kinetic investigations. The results are shown in Table 1. Among three trypsin inhibitors, the highest value of K_a was displayed by monocyclic SFTI-1. The replacement of Pro^9 by Met reduced the trypsin inhibitory activity by one order of magnitude. The analogue with Arg and Abu in positions P_1 and P_4', respectively, appeared to be slightly more potent. The K_a value determined for selected [Phe^5]-SFTI-1 with chymotrypsin was practically the same, but to our knowledge it is the most potent chymotrypsin inhibitor of this size. As reported by the group of Leatherbarrow [6,22], 10- and 11-amino acid peptides were several times less active. Although the last of the selected analoges [Leu^5, Trp^9]-SFTI-1 displayed affinity towards its enzyme (HLE) lower by two orders of magnitude, also this inhibitor can be considered as rather potent and is approximately twice as potent as the strongest HLE inhibitor of a similar size described in the literature [23]. It is worth pointing out that also this inhibitor and the two above mentioned low-molecular trypsin inhibitors were selected by the combinatorial chemistry methods, but in contrast to the results presented herein, deconvolution on solid support was applied.

The results presented above clearly indicate that all selected monocylic analoges of SFTI-1 displayed strong

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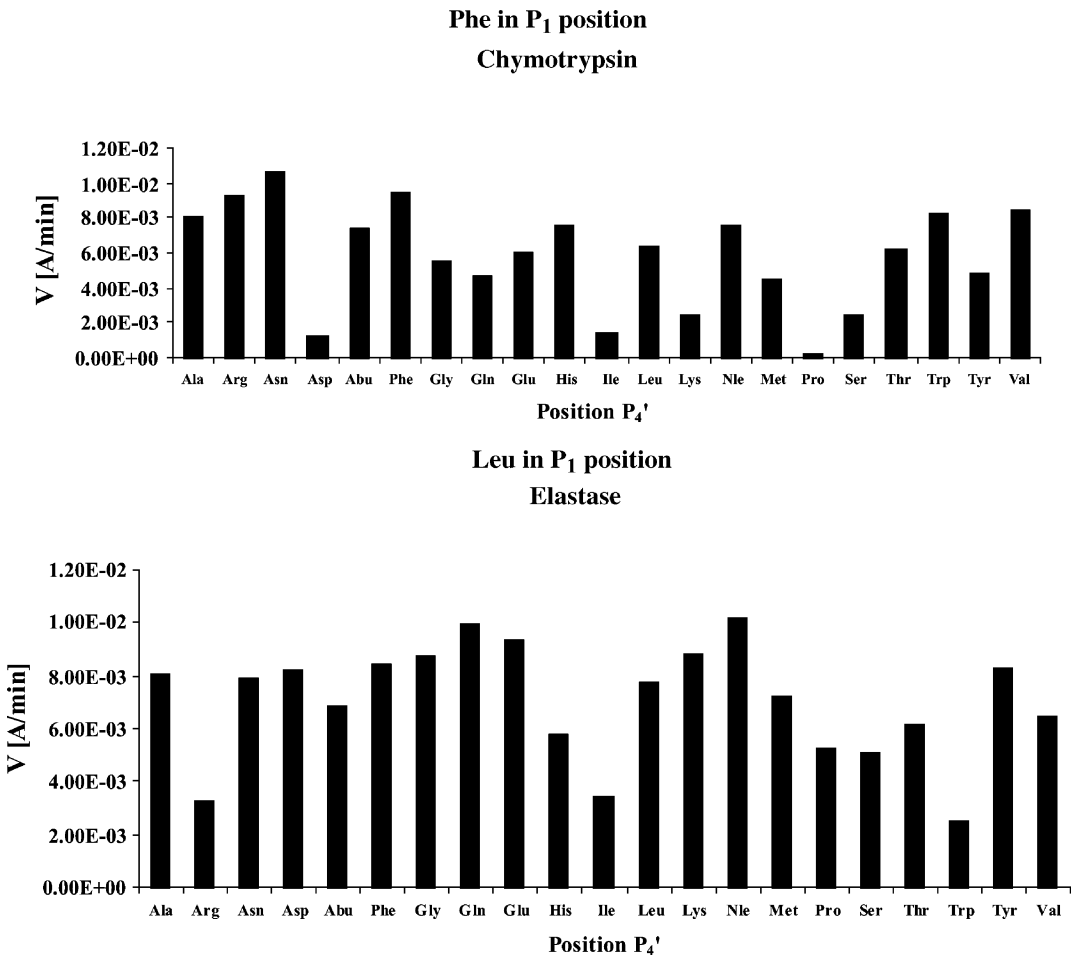

Figure 5 Deconvolution of the peptide library in position P_4' against bovine α-chymotrypsin and human leukocyte elastase.

inhibition of the experimental enzymes. It is worth mentioning that [Phe^5]-SFTI-1 and [Leu^5, Trp^9]-SFTI-1 are probably the most potent peptidic chymotrypsin and HLE inhibitors of this size. One of the main goals of our work was to determine the role of Pro^9 in position P_4' of the inhibitor. The results summarized in Table 1 provide evidence that this amino acid residue can be replaced without significant decrease in the inhibitory activity. In the case of trypsin, the introduction of Abu or Met yielded inhibitors with K_a of one order of magnitude lower as compared with the native sequence. The requirements in this position for chymotrypsin inhibitors are more strict and the Pro residue was optimal. HLE inhibitors can accommodate aliphatic (Ile), aromatic (Trp) and basic (Arg) residues in this position. High inhibitory activity of all selected and resynthesized monocyclic SFTI-1 analoges prove that the straightforward methodology presented herein is a very useful tool for selection of serine proteinase inhibitors.

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