J. Biochem. **138**, 383–390 (2005)
doi:10.1093/jb/mvi140

Complete Substitutional Analysis of a Sunflower Trypsin Inhibitor with Different Serine Proteases

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Received April 19, 2005; accepted July 10, 2005

Here we present a method to simultaneously characterize and/or optimize both the binding loop towards the protease and a cysteine-stabilized scaffold. The small peptidic sunflower trypsin inhibitor (SFTI-1) was chosen as a model system for these experiments. The inhibitor was investigated for positional specificity against trypsin, elastase and proteinase K using complete substitutional analyses based on cellulose-bound peptide spot synthesis. Inhibitor variants optimized for elastase or proteinase K inhibition by several rounds of substitutional analyses exhibit K_i values in the micromolar range and high specificity for the corresponding protease. The results of this easy-to-perform assay can be used to design an improved peptide library using classical methods.

Key words: cellulose bound peptides, elastase, protease inhibitor, proteinase K, serine protease, substitutional analysis, sunflower inhibitor, trypsin.

A recently discovered naturally occurring protease inhibitor (SFTI-1, see Fig. 1A) from the seeds of sunflower (*Helianthus annuus*) contains only 14 amino acids with a molecular mass of 1,513 Da and is, to date, the smallest known naturally occurring serine protease inhibitor (*1*). SFTI-1 belongs to the Bowmann-Birk inhibitor family, a class of serine protease inhibitors found in legumes and other plants. Members of this family typically have a molecular mass in the range of 6,000–8,000 Da and contain seven disulphide bonds to stabilize the conformation (*2*). They are usually able to inhibit two different proteinases simultaneously and independently, in most cases trypsin and chymotrypsin (*3*). It has been shown that a disulfide-stabilized active site loop fragment of these inhibitors can also act as a strong protease inhibitor (*4*, *5*). Similarly, the SFTI-1 is monofunctional and also exhibits strong inhibitory activity. In a subnanomolar range, SFTI-1 can inhibit bovine pancreatic β-trypsin [$K_i = 1 \times 10^{-10}$ M (*1*) or 1.06×10^{-9} M (*6*)], cathepsin G [$K_i = 1.5 \times 10^{-10}$ M (*1*)] and human matriptase [$K_i = 9.2 \times 10^{-10}$ M (*6*)] Weak inhibition of bovine pancreatic chymotrypsin [$K_i = 7.4 \times 10^{-6}$ M (*1*)], bovine thrombin [$K_i = 1.4 \times 10^{-4}$ M (*1*), or 5×10^{-6} M (*6*)], porcine pancreatic elastase [$K_i = 1 \times 10^{-4}$ M (*1*)] and human urokinase type plasminogen activator [$K_i = 5 \times 10^{-4}$ M (*6*)] has also been reported.

SFTI-1 is stabilized by two cyclizations: one disulfide bridge and a connection of the N- with the C-terminus. A backbone cyclization was also observed for the serine protease inhibitor MCoTI I and II from *Momordica cochinchinensis*, both of which belong to the squash inhibitor family (*7*). A comparison of the crystal structure of SFTI-1 in complex with bovine pancreatic trypsin (*1*) with the solution NMR structure (*8*) revealed an almost identical conformation stabilized by these cyclizations. The influence of both cyclizations was investigated in detail. Two variants were synthesized, one [SFTI-1 (V2)] with a break in the cyclic backbone between residues 1 and 14, and one with an isosteric substitution of both cysteines (residue 3 and 11) by α-aminobutyric acid [SFTI-1 (V1), see Fig. 1A]. Both variants showed similar inhibitory activity against trypsin compared to the native inhibitor ($K_i = 1 \times 10^{-10}$ M and 2.2×10^{-10} M, respectively) (*9*). NMR measurements demonstrated that SFTI-1 (V2) has a very similar structure to the wild-type SFTI-1 (*8*). SFTI-1 and SFTI-1 (V2) displayed a very high stability against proteolytic attack by trypsin, in contrast to SFTI-1 (V1), which was hydrolyzed remarkably faster. Furthermore, a backbone acyclic variant with a break between the scissile (Lys5 and Ser6) peptide bond also acted as a trypsin inhibitor, with the protease resynthesizing the cleaved bond (*10*).

Due to their small size and the fact that they do not contain any non-proteinogenic amino acids, SFTI-1 and the monocyclic (disulfide bridged) variant SFTI-1 (V2) are easily accessible to peptide synthesis on resin or cellulose. Their strong binding affinity together with a high proteolytic stability make them ideal candidates to investigate protease/inhibitor interaction in general and as a lead structure for developing new therapeutic agents.

We have applied a powerful strategy to investigate the interaction of small linear peptitic inhibitors with serine proteases using parallel peptide synthesis on cellulose supports (*11*). In the present study, we demonstrated that this technique can be used to investigate the interaction of an inhibitor stabilized by a disulfide bond with different serine proteases, measuring the importance of each position for these interactions. In consequence, information on the binding loop and scaffold features can be obtained in a single experiment. This opens up a new dimension of applications to investigate and/or optimize inhibitors against a given protease.

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A

GRCTKSIPPICFPD	backbone cyclic	SFTI-1
GRαTKSIPPIαFPD	backbone cyclic	SFTI-1 (V1)
GRCTKSIPPICFPD	backbone open	SFTI-1 (V2)
GRCTLSIPPICFPD	backbone open	OSFEI I
GVCTLSIPPICFPD	backbone open	OSFEI II
GRCTISIPPICFPD	backbone open	OSFPKI I
GRCTISILPICFFD	backbone open	OSFPKI II
GRCTASILPICFFD	backbone open	OSFPKI III
GPCTLEYPPICFPD	backbone open	HEI-TOS

α = α-aminobutyric acid

B

GRCTKSIPPICFPD	SFTI-1	*Helianthus annuus* (sunflower)
…TTCTKSIPPICSCG…	BBI(put.)	*Oryza sativa* (rice)
…AVCTRSIPPICRCM…	BBI	*Triticum aestivum* (bread wheat)
…AMCTRSIPPICRCV…	BBI	*Coix lacryma-jobi* (Job's tears)
…AVCTRSIPPICTCM…	BBI	*Hordeum vulgare* (barley)
…CRCTKSIPPQCHCA…	BBI	*Vigna radiata* (mung bean)
…CFCTKSNPPICQCR…	BBI	*Erythrina variegata* (coral tree)
…CACTKSIPPKCRCS…	BBI	*Vigna mungo* (black gram)
…CACTKSIPPQCRCS…	BBI	*Phaseolus vulgaris* (kidney bean)
…CLCTKSIPPQCQCA…	BBI	*Vigna angularis* (adzuki bean)
…CTCTKSIPPQCHCN…	BBI	*Macrotyloma axillare* (horsegram)
…CPCTKSIPPQCRCS…	BBI	*Medicago sativa* (alfalfa)
…CLCTRSFPPQCHCT…	BBI	*Pisum sativum* (pea)
…CACTRSIPAKCRCF…	BBI	*Amburana acreana* (amburana)
…CACTKSIPPQCRCS…	BBI	*Phaseolus vulgaris* (kidney bean)
…AICTKSFPPMCRCM…	BBI II	*Triticum aestivum* (bread wheat)
…CICTRSLPPQCRCI…	BBI	*Pisum sativum* (pea)
…QTCTKSIPAFCRCR…	BBI	*Setaria italica* (foxtail millet)
…CRCTKSEPPQCQCQ…	BBI	*Dioclea glabra* (bean)
…CLCTKSNPPTCRCV…	BBI II	*Pisum sativum* (pea)
…AFCNKMNPPTCRCM…	BBI	*Oryza sativa* (rice)
…CICSKSIPPQCHHT…	BBI	*Vigna unguiculata* (cowpea)
…CLCTRSQPPTCRCV…	BBI	*Vicia sativa* ssp.nigra (garden vetch)
…EKCSRSIPPRCRCM…	BBI III	*Triticum aestivum* (bread wheat)

Fig. 1. **Sequence comparison for Bowman-Birk type protease inhibitors.** A: Sunflower trypsin inhibitor (SFTI) derived inhibitor peptides. B: Comparison of sunflower trypsin inhibitor (SFTI-1) with the binding loop sequences of Bowman-Birk type inhibitors from different plants.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Cellulose-bound peptides for binding experiments were prepared using a pipetting robot (Abimed, Langenfeld, Germany) and Whatman 50 cellulose membranes (Whatman, Maidstone, United Kingdom) as described previously (*12*, *13*). The cyclization of the peptides bound on the membrane was performed by incubation with 0.1 M Tris-buffer, pH 8.5 at 4°C overnight, followed by incubation with 10% DMSO in the same buffer for 8 h at 4°C and with 20% DMSO overnight at room temperature. The peptides used for K_i determination and stability investigations were prepared by Interactiva (Ulm, Germany), or in the lab of R. Volkmer-Engert according to standard Fmoc synthesis chemistry using a multiple peptide synthesizer (Abimed, Langenfeld, Germany). The cyclization protocols for these free peptides were as described previoulsy (*14*). The degree of cyclization was characterized by determining the amount of free SH groups with 5,5′-dithiobis (2-nitrobenzoic acid). The cyclization procedure was applied until no free SH groups were detectable.

Binding Experiments with Cellulose Bound Peptides—All proteases were purchased from Serva, Heidelberg, Germany. To detect bound proteases the enzymes were labeled with horseradish peroxidase (HRP, Boehringer, Mannheim, Germany) using the method of Wilson and Nakane (*15*). The activities of the labeled enzymes (indicated with *) were determined to be 78% residual activity for porcine pancreatic elastase, 49% for bovine trypsin, and 77% for proteinase K.

After the membrane-bound peptides were oxidized, the membranes were washed with 96% ethanol for 5 min, equilibrated with 0.1 M Tris (Applichem GmbH, Darmstadt, Germany) buffer, pH 8.5, then blocked by incubation with blocking reagent (Genosys, Cambridge, UK) in 0.1 M Tris buffer, pH 8.5, for 2 h at room temperature, washed three times with the same buffer for 3 min each and incubated for 30 min at room temperature with the corresponding proteases (22 nM PPE*, 30 nM trypsin*, and 57 nM proteinase K*) in 0.1 M Tris buffer, pH 8.5. The membranes were finally washed with 0.1 M Tris buffer, pH 8.5, once for 5 min and five times for 10 min, then incubated for 3 min with an HRP substrate to yield a chemiluminescent product (chemiluminescence kit from Pierce, Illinois, USA). The light arising from spots with bound protease* was measured with a Lumi-Imager™ (Boehringer, Mannheim, Germany).

K_i and Proteolytic Stability Determination—All protease substrates were purchased from Serva, Heidelberg, Germany. The activity of the enzymes was measured by monitoring hydrolysis of the corresponding substrates at 400 nm on a recording spectrophotometer model UV-160A (Shimadzu, Duisburg, Germany). By varying the substrate (0.125 mM to 1 mM) and inhibitor concentrations (0.1 μM to 1 mM) the K_i values were determined at 25°C. A 0.1 M Tris buffer was used with pH 8.5 for PPE and proteinase K, and

pH 8.0 for trypsin. The activities of PPE and proteinase K were determined with Suc-Ala-Ala-Ala-pNA as a substrate, while that of trypsin was determined with *N*α-benzoyl-DL-Arg-pNA. All substrates were solubilized in DMSO.

Proteolytic stability of the inhibitor peptide OSFEI II with PPE was measured (determined *via* residual protease activity) by preincubation of OSFEI II at a concentration of 2.4×10^{-4} M with 1 mg/ml PPE at 22°C in 0.1 M Tris buffer, pH 8.5. At varying time intervals over a period of up to 6 h, aliquots were withdrawn and the PPE activity measured in a standard activity assay at 25°C. Relative increase of that activity means decrease in inhibitor concentration, which can be calculated from the corresponding velocity equation.

Structure Modeling—The structure of the elastase/SFTI-1 (V2) complex was modeled by hand-docking the inhibitor in the elastase active site with the trypsin/SFTI-1 complex (PDB code 1SFI) as a reference structure for proper orientation, and subsequent energy minimization using the CHARMm force field as implemented in QUANTA97 (Accelrys Inc., San Diego).

RESULTS AND DISCUSSION

Substitutional Analysis of SFTI-1 to Investigate Trypsin Interaction—Generally, with cellulose-based peptide spot synthesis, the peptides are bound to the cellulose *via* a C-terminal linker, but they can also be cleaved from the solid support. Earlier comparative experiments demonstrated that the proteases were able to bind to the cellulose-bound peptide inhibitors. An inhibition experiment using a protease activity assay with the soluble peptides cleaved from the cellulose support gave similar results (*11*). Thus, in the present study, we used binding experiments only. This study was undertaken solely to show that this approach could, in principle, be used to develop inhibitors using membrane-bound peptide libraries, and consequently, the optimization was stopped once inhibitors with micromolar activity were found. A recent review discusses a comparison of this method with others (*16*).

A substitutional analysis of SFTI-1 (V2) was performed on cellulose, in which all positions of the inhibitor sequence were substituted by the remaining 19 proteinogenic L-amino acids. The peptides were cyclized and then incubated with HRP-labelled trypsin. The result is given in Fig. 2A. The main features of this analysis were as expected. Both cysteines are important for the interaction, and the positive results with the cysteine-containing peptides demonstrate that the cyclization was successfully performed on the cellulose support. The lysine at position 5 determines the trypsin specificity of the inhibitor [P1 according to the nomenclature of Schechter and Berger (*17*)] and can be substituted only by arginine. Of the other positions only Thr-4 (P2), Ser-6 (P1′) and Asp-14 were comparably sensitive to substitution: Thr-4 can be substituted by serine only and Ser-6 by several other amino acids like glycine, asparagine, or alanine but with significant reduction of binding affinity. Other positions show medium specificity (Pro-8 in P3′ can be substituted by Asp and Glu, Pro-9 by Ala, Glu, Gln and Ser) or low specificity (Gly-1, Arg-2, Ile-7, Ile10, Phe-12, Pro-13). This observation is supported by the observation that shortening the sunflower trypsin inhibitor by the residues Gly-1, Pro-13 and Asp-14 did not diminish inhibitory activity (*18*). In several

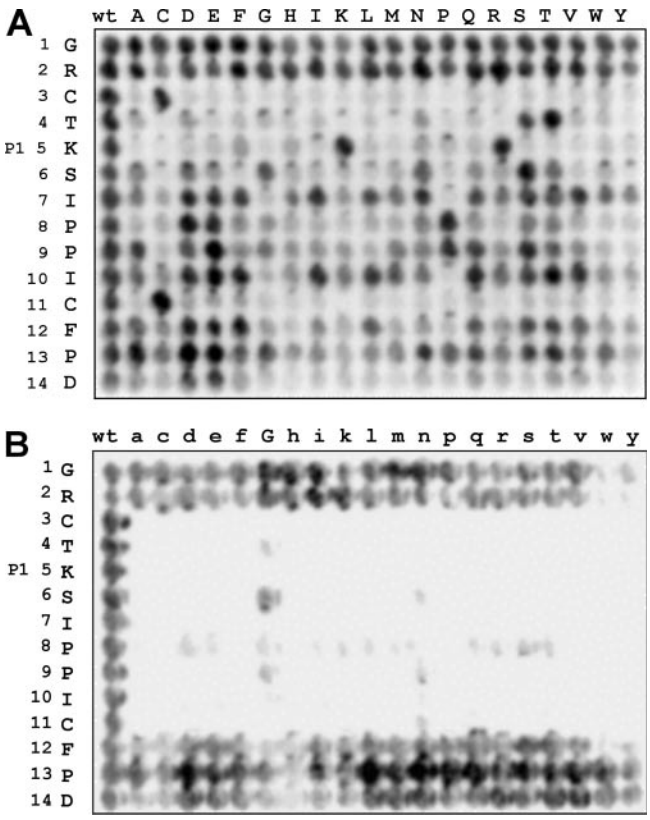

Fig. 2. **Substitutional analysis for the sunflower trypsin inhibitor (SFTI-1) with bovine trypsin.** Positions P1 and P1′ indicated. A: All positions substituted by all proteinogenic amino acids, B: All positions substituted by D-amino acids. wt = wild-type peptide (*e.g.* first line: wt and G are identical peptides, second line: wt and R are identical peptides *etc.*). Trypsin was coupled with HRP for detection of peptide-bound enzyme.

positions, a negative charge seems to compensate to some extent for loss in affinity upon substitution, but this may be an artifact of the cellulose-bound peptides.

An inspection of the published structure of the complex of SFTI-1 with trypsin (*1*) may explain most of the features described above. The H of Gly-1, and the side chains of Arg-2, Ile-7, Ile-10, Phe-12 and Pro-13 are more or less exposed to the solvent and thus can be exchanged with many other amino acid side chains. An H-bond between Arg-2(I) and Asn-97 of trypsin does not contribute much to the affinity of the inhibitor. This arginine and the other positions mentioned above are not conserved in the family of Bowmann-Birk inhibitors or are occupied by cysteines for additional disulfide bridges (see Fig. 1B). The Thr-4(I) is highly conserved in the Bowmann-Birk trypsin inhibitor family (see Fig. 1B) and its importance is reflected in the substitutional analysis. This may be explained by a well-defined environment surrounding this residue (van der Waals contact to His-57, Leu-99, H-bonds to Ser-10(I) Oγ and Ile-10(I) NH which may critically stabilize the loop conformation). The same observation holds true for Ser-6(I) with contacts to His-57 and Ile-10(I) and H-bonds to Thr-4(I) and Pro-8(I) C=O. Pro-8(I) and Pro-9(I) are also strongly conserved, with the exception of Pro-9 exchanged for Ala in two cases. This is also

reflected by the substitutional analysis. Both prolines may be critical in stabilization of a proper turn conformation of the inhibitor. It has been shown for the BBI reactive site loop that one of the two prolines, that in position P3′, adopts a *cis*-peptide bond configuration (*19*). Asp-14(I) may also contribute to a favourable overall conformation by H-bonds to Gln-175 and the peptide NH of Gly-1(I) and Arg-2(I), but this residue is not at all conserved in this inhibitor family (Fig. 1B). Substitution experiments at special subsites of the active site loop fragment from Bowman-Birk family trypsin inhibitors reveal a mixed picture: substitutions at the P1′ position show that exchange of serine for alanine lowers the affinity towards trypsin about fourfold (*20*), which correlated with our binding experiment. A complete substitutional analysis at the P2′ site with the corresponding K_i values for trypsin inhibition is given by Gariani *et al*. (*21*). Here Asp and Glu show low affinity, supporting the hypothesis that these spots are artefacts in our experiment caused by charge compensation effects. The observation that Gly and Pro are extremely unfavourable in this position also agrees with our results. However, in our study Ile does not appear to be better in P2′ than, *e.g.*, Leu, Val or Asn, whereas in the inhibition experiment it appears to be (*21*). Certainly, in our experiment, binding is also low if there is rapid hydrolysis of the corresponding peptide. But as we find Arg and Lys in P1 as the most important residues with trypsin binding, this effect should be negligible. It should also be mentioned that our peptide is three residues longer than the BBI reactive site loop (SCTKSXPPQCY) and further differs by the residues in position P4, P5′ and P7′.

An alanine walk performed with a shortened peptidomimetic of the sunflower trypsin inhibitor (*18*) showed almost no change in inhibitory activity with Ala in the position P4′. In addition, substitution of alanine in P2, P1′, P2′ and P5′ resulted in diminished inhibition, but no measurable inhibition with Ala in P1 and P3′. These effects are clearly reflected in Fig. 2A.

The substitutional analysis of the SFTI-1 (V2) against D-amino acids underlines the high stereospecificity of trypsin (Fig. 2B). Inspecting the trypsin/SFTI complex structure and taking into consideration that the side chain of a D-amino acid occupies the hydrogen position of the L-amino acids, at positions Gly-1, Arg-2, Phe-12 , Pro-13 and Asp-14 there is enough free space or solvent exposition for substitution by D-amino acid side chains, and as mentioned above, it appears that Gly-1, Pro-13 and Asp-14 could be omitted without loss in inhibition (*18*). If we ignore the glycine row, which corresponds to that in Fig. 2A, there are only weak signals in the line corresponding to the Pro-8 position, where several substitutions to D-amino acid side chains are additionally allowed but with comparatively low affinity. An exchange for D-amino acids at positions Thr-4 , Lys-5, Ser-6 and Pro-9 may lead to a direct clash with the enzyme, but there are two exceptions: Ile-7 and Ile-10. The Cα hydrogen atoms of these residues are directed outwards, and therefore side chains at these positions should not cause steric hindrance. The result of this substitutional analysis suggests that the backbone conformation of the inhibitor is changed significantly by substitutions against D-amino acids within the binding loop, thus dramatically decreasing the affinity of the inhibitor for the protease.

Substitutional Analyses of SFTI-1 with Porcine Pancreatic Elastase—A weak inhibitory activity of SFTI-1 towards porcine pancreatic elastase (PPE) was reported ($K_i = 1 \times 10^{-4}$ M) (*1*). To investigate this interaction and to demonstrate that it is possible to increase the affinity systematically, a second substitutional analysis of SFTI-1 (V2) was performed and the binding towards PPE was measured (Fig. 3A). A binding signal for the wild-type peptide was detectable on a low level. Most of the inhibitor sequence positions are substitutable. As expected, the P1 position Lys-5 showed a markedly higher signal if substituted by amino acids more suitable for elastase specificity, especially alanine, leucine or threonine. Surprisingly, the Arg-2 at position P4 also seems to be unfavourable for the interaction with elastase. A substitutional analysis of an SFTI-1 variant (OSFEI I, see Fig. 1A) with leucine in position 5 was performed. The name OSFEI resulted from the nomenclature of the sunflower inhibitor (**O**ptimised **S**un **F**lower **E**lastase **I**nhibitor). The result of the substitutional analysis is given in Fig. 3B. As reflected in position P1 of the substitutional analysis, pancreatic elastase has a broader specificity than trypsin, which accepts almost exclusively arginine and lysine at P1. For elastase, alanine and threonine seem to be more favorable at that position than leucine. Also, the positions P2 and P1′ allow more substitutions with elastase than with trypsin. The positions Ile-7 and Ile-10 are similarly substitutable as in the case of trypsin, and the prolines at positions 8 and 9 are comparatively restricted. Again, the arginine at position 2 is unfavourable for the interaction with elastase. An exchange with valine showed the highest binding signal. The disulfide bridge obviously does not contribute much to the affinity. A new substitutional analysis was performed with valine in position 2 (Fig. 3C). The specificity of the positions 2 to 12 is somewhat more pronounced now and especially the disulfide bridge becomes more important again. For affinity determinations in solution, two peptides were synthesized: GRCTLSIPPICFPD (OSFEI I) and GVCTLSIPPICFPD (OSFEI II). The K_i values were determined for OSFEI I and II to be $3.5 \pm 0.2 \times 10^{-5}$ M and $3.0 \pm 0.5 \times 10^{-7}$ M, respectively. Thus the exchange of the unfavourable side chain at position P1 to a side chain found in natural elastase inhibitors resulted only in 3-fold increase, but the additional exchange at position 2 (=P4) increased the affinity towards elastase by 330-fold in comparison with SFTI-1 (V2).

A structure model for the complex of elastase with OSFEI II (Fig. 4A) reveals that Val-2 is positioned within a well-defined hydrophobic surrounding (methyl group of Thr-175, side chains of Val-99, Ala-99A, Trp-172, and Phe-215 of elastase) in comparison to the corresponding Arg-2 of SFTI in a more polar and less defined pocket in trypsin (Asn-97, Gln-175, Trp-215, see Fig. 4B). In a paper from McBride *et al*. (*22*), a synthetic library randomized at positions P1, P4, and P2′ of the Bowman-Birk inhibitor active site loop was screened for the inhibition of human leucocyte elastase. In this case, only Ala or Thr was preferentially selected at P1, but not Leu. At P4 they selected the aromatic side chains and, less frequently, Ile, Met, Val and even Arg (with again Leu missing); and at P2′, Ile and Leu, and less frequently the aromatic residues. These results agree only partly with those of our substitutional analysis, but the different specificities of these elastases

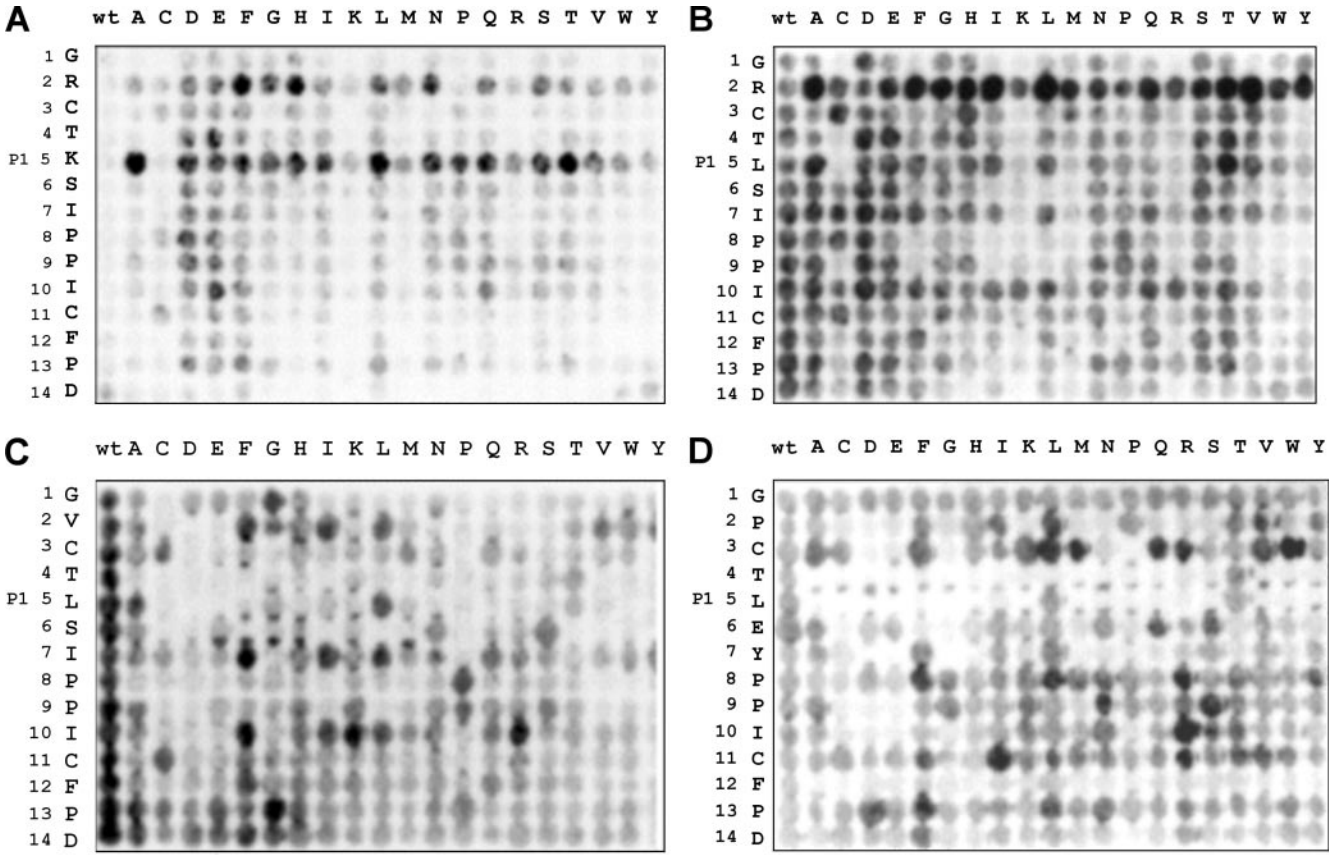

Fig. 3. **Substitutional analysis for sunflower trypsin inhibitor and derived inhibitors with porcine elastase, all positions substituted by all proteinogenic amino acids.** A: SFTI-1; B: OSFEI I with leucine at position 5; C: OSFEI II with leucine at position 5 and valine at position 2; D: HEI-TOS with an ovomucoid inhibitor derived sequence (PXTLEY) inserted. Elastase was coupled with HRP for detection of peptide-bound enzyme.

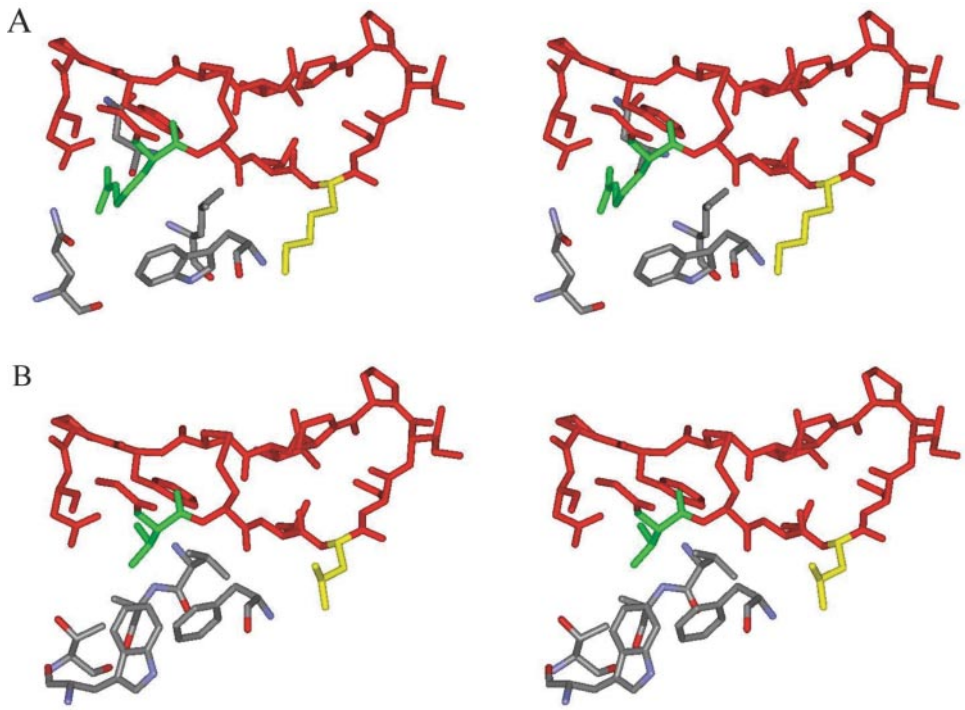

Fig. 4. **Model structure for OSFEI II bound to porcine elastase in comparison with SFTI-1 bound to bovine trypsin.** A: elastase/OSFEI II; inhibitor in red, position 2 (Val) in green, elastase residues in contact with Val-2 in gray (Val-99, Ala-99A, Trp-172, Phe-215), P1 (Leu) in yellow. B: trypsin/SFTI-1 derived from PDB code 1SFI; inhibitor in red, position 2 (Arg) in green, trypsin residues in contact with Arg-2 in gray (Asn-97, Gln-175, Trp-215), P1 (Lys) in yellow.

(*11*) may explain these findings, underlining the importance of subsites other than P1 for the affinity and specificity of peptidic protease inhibitors.

Substitutional Analysis of HEI-TOS, a Hybrid Optimized Inhibitory Peptide/SFTI-1 Peptide—In a previous work we optimized the binding loop of the third domain of turkey ovomucoid inhibitor (OMTKY3) for PPE inhibition (*11*). This optimized inhibitory peptide was introduced into the binding loop of a trypsin-specific inhibitor from the squash family (*23*). The resulting hybrid inhibitor had a K_i of 3.8×10^{-7} M, showing a similar affinity and specificity as the free optimized peptide. Next, we performed a substitutional analysis of the sunflower inhibitor in which the optimized inhibitory peptide (PMTLEYR) was introduced into the binding loop of SFTI-1 (GPCTLEYPPICFPD: HEI-TOS, **H**ybrid **E**lastase **I**nhibitor **T**urkey **O**vomucoid **S**unflower; see Fig. 1A). The result is shown in Fig. 3D. There is a binding signal for the wild type, which shows that the trypsin specificity is changed to inhibit elastase. Again, the disulfide bridge is not very favourable, suggesting that conformational flexibility is important for the interaction of the optimized inhibitory peptide fragment with PPE. The K_i value for the soluble peptide with PPE was determined to be $7.3 \pm 1.1 \times 10^{-6}$ M. The positions Thr-4 and Leu-5 show a markedly different pattern than those of OSFEI I (see Fig. 3B). In the case of HEI-TOS both positions are very sensitive to any substitution. Thr-4 cannot be substituted at all and Leu-5 only by threonine, whereas in OSFEI I both positions can be substituted by many other amino acids without a marked loss of binding signal. The amino acids at position 4 and 5 in OSFEI I and HEI-TOS are the same and therefore the difference between the interaction of these peptides with PPE must be caused by an influence of Pro-2, Glu-6 and/or Tyr-7 positions. Several positions of HEI-TOS, especially Pro-2 and Ile-10 together with the disulfide bridge, are not the optimal ones for the interaction with PPE. This is reflected by the more than one order of magnitude lower affinity of HEI-TOS in comparison to the squash-family hybrid inhibitor. Thus further exchanges would be necessary to adapt HEI-TOS for optimal PPE inhibition.

From this data it can be concluded that both the sequence directly interacting with the subsites of the enzyme's substrate-binding region and the framework where this sequence is integrated are of importance for an optimal inhibitor affinity.

Substitutional Analyses of SFTI-1 with Proteinase K—Proteinase K, which belongs to the subtilisin-type serine proteinases (and thus to a different structural class than trypsin and elastase) was also investigated with respect to the interaction with SFTI-1 (V2). Proteinase K has broader substrate specificity than other subtilisin proteinases, the S1 site being lined by hydrophobic residues and large enough to accomodate almost all amino acid side chains (*24*). There is a weak inhibition of the proteinase K activity at high concentrations of SFTI-1 (V2), but a K_i value cannot be properly determined. A substitutional analysis of SFTI-1 (V2) was performed on cellulose and the binding of proteinase K was measured (Fig. 5A). As was expected, the wild type inhibitor shows a very low binding signal. The Lys-5 at the P1 site is unfavourable for this interaction. Several other side chains give a higher binding signal, especially threonine and the hydrophobic residues other than the large tryptophan. Similar to the interaction of SFTI-1 with PPE there are two other positions also unfavourable for the protease/inhibitor interaction. The substitution of proline 13 or aspartic acid 14 by phenylalanine resulted in a higher binding signal. Also both prolines in position 8 and 9 seem to be problematic. There is also no indication that the disulfide bridge is advantageous for the inhibitor binding.

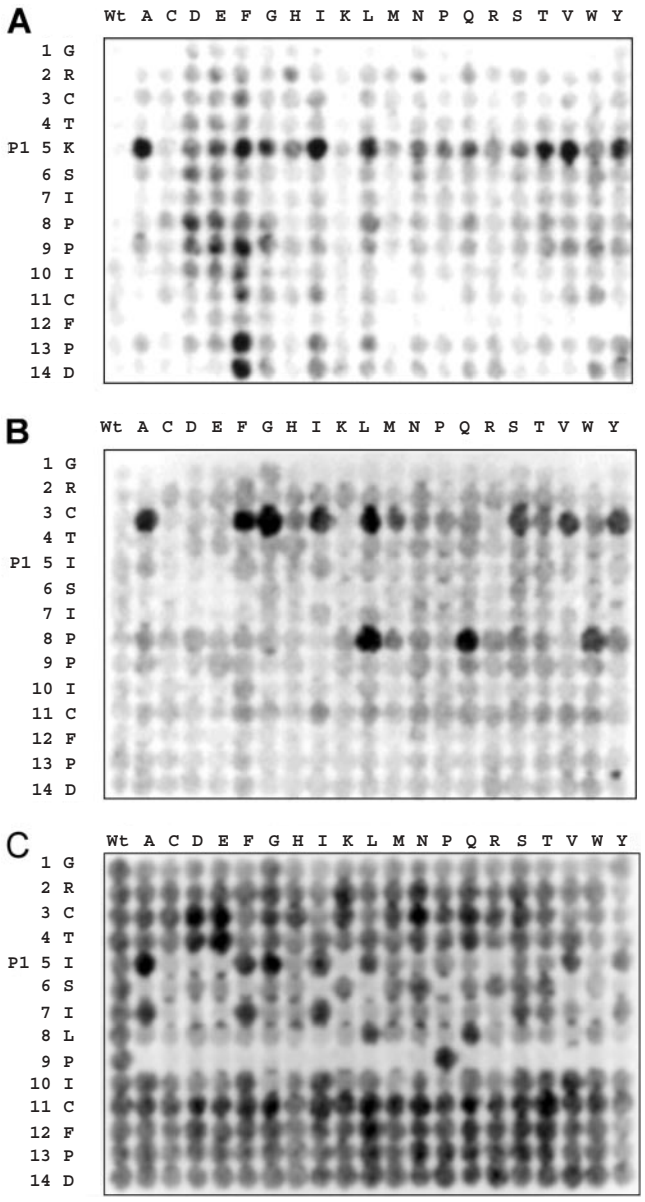

Fig. 5. **Substitutional analysis for sunflower trypsin inhibitor and derived inhibitors with proteinase K from *Tritirachium album limber*, all positions substituted by all proteinogenic amino acids.** A: SFTI-1; B: OSFPKI I with isoleucine at position 5; C: OSFPKI II with isoleucine at position 5 and leucine at position 8. Proteinase K was coupled with HRP for detection of peptide-bound enzyme.

An additional substitutional analysis was performed with substitution of Lys-5 by isoleucine (OSFPKI I, **O**ptimized **S**un **F**lower **P**roteinase **K** **I**nhibitor). The result of the binding experiment is given in Fig. 5B. The low

Table 1. **Summary of K_i values of different inhibitor variants against porcine pancreatic elastase (PPE) and proteinase K.**

Sequence	Name	Porcine pancreatic elastase	Proteinase K
GRCTKSIPPICFPD	SFTI-1	1×10^{-4} M*	n.d.
GRCTKSIPPICFPD	SFTI-1 (V2)	n.d.	w.i.
GRCTLSIPPICFPD	OSFEI I	$3.5 \pm 0.2 \times 10^{-5}$ M	n.d.
GVCTLSIPPICFPD	OSFEI II	$3.0 \pm 0.5 \times 10^{-7}$ M	$1.1 \pm 0.17 \times 10^{-4}$ M
GRCTISIPPICFPD	OSFPKI I	n.d.	$3.9 \pm 0.8 \times 10^{-4}$ M
GRCTISILPICFFD	OSFPKI II	n.d.	$2.1 \pm 0.8 \times 10^{-4}$ M
GRCTASILPICFFD	OSFPKI III	$4.8 \pm 0.84 \times 10^{-5}$ M	$7.4 \pm 2.4 \times 10^{-6}$ M
GPCTLEYPPICFPD	HEI-TOS	$7.3 \pm 1.1 \times 10^{-6}$ M	n.d.

*Value taken from Ref. *1*. w.i.: There is a weak inhibition of the proteinase K activity at high concentrations of SFTI-1 (V2) but a K_i value cannot be properly determined. n.d.: not determined.

specificity of proteinase K at P1 remains obvious. Interestingly, the substitution of lysine by isoleucine greatly influences the interaction mode at other positions. Substitution of proline-13 and aspartic acid-14 by phenylalanine did not show the markedly higher binding signal seen with SFTI-1. In contrast, Cys-3 appears unfavourable for this interaction. Substitutions at this position by alanine, glycine or hydrophobic side chains showed a markedly higher binding signal. There was also a higher binding signal when Pro-8 was substituted by leucine, glutamine, or even tryptophan. The K_i for the inhibition of proteinase K by the free inhibitor OSFPKI I was determined to be $3.9 \pm 0.8 \times 10^{-4}$ M. To increase further the affinity of the inhibitor, another substitutional analysis was performed with Pro-8 of this peptide substituted by leucine (OSFPKI II, Figs. 1A and 5C). In this peptide, position 9 showed a remarkable preference for the proline, which suggests that a proper turn conformation is important. In addition, the binding signal increased when the Ile-5 in P1 was substituted by alanine or glycine. As with previous peptides, the disulfide bridge seems to be of little importance. With the corresponding free peptide the measured affinity for OSFPKI II increased by only a factor of about 2 ($K_i = 2.1 \pm 0.8 \times 10^{-4}$ M). A third variant (OSFPKI III) was synthesized with alanine in position 5. In this peptide, the affinity increased markedly by a factor of 28 in comparison to OSFPKI II ($K_i = 7.4 \pm 2.4 \times 10^{-6}$ M). Obviously, the degree of freedom in accepting different side chains in the proteinase K subsite S1 binding pocket becomes more restricted with tighter binding of the whole inhibitor molecule.

Both optimized inhibitors, OSFEI II for elastase and OSFPKI III for proteinase K, show quite high specificity. The affinity of OSFEI II for elastase had a K_i of 3.05×10^{-7} M about three order of magnitude higher than that for trypsin ($K_i = 2.1 \pm 1.6 \times 10^{-4}$ M) or that for proteinase K ($K_i = 1.13 \pm 0.17 \times 10^{-4}$ M). The specificity of OSFPKI III for proteinase K was somewhat lower with a K_i of 7.4×10^{-6} M for proteinase K as compared to $K_i = 4.8 \pm 0.84 \times 10^{-5}$ M for elastase, but with a not exactly measurable K_i in the millimolar range for trypsin. A direct comparison of the different inhibitor variants is given in Table 1.

The inhibitor peptides are fairly stable against proteolytic degradation by the proteases. When OSFEI II at a concentration of 2.4×10^{-4} M was preincubated with 1 mg/ml elastase at 22°C, the initial residual activity of 15% measured in a corresponding activity assay (see "EXPERIMENTAL PROCEDURES") increased after 6 h to 26% (data not shown). It can be calculated from the equation for the case of competitive inhibition using the known K_i and Km values that the inhibitor concentration drops by about 50% during this time. It becomes more difficult to make a similar estimation for SFTI I (V2) with trypsin or OSFPKI III with proteinase K because of the higher autolysis rate of these proteases during prolongated incubation times at room temperature (data not shown).

Conclusion—Resin-based chemical synthesis of individual peptides with variations at specified positions is costly and time-consuming. Our complete substitutional analysis using cellulose-bound peptide spot synthesis offers an inexpensive, rapid, and highly parallel way to semi-quantitatively investigate the influence of all amino acid positions on the inhibitory activity of an inhibitory peptide or small protein inhibitor. On the basis of the sunflower trypsin inhibitor framework, it is possible to develop and optimize stable and specific peptidic inhibitors against target proteases of quite different substrate specificity.

Certainly, for affinity optimization an iterative procedure is necessary with selected peptides resin-synthesized and purified, for verification of the observed effects and for affinity determination in solution. Alternative methods, including screening of generally or positionally randomized peptide libraries after *in vivo* or *in vitro* display, may be hampered by the limited size of such libraries and restrictions in the synthesis or expression schemes used. The synthesis, measurement and analysis of a complete substitutional analysis of a peptidic inhibitor like SFTI I can be performed in about 4 days. Therefore, this approach is an excellent method to gain the necessary information to design an effective peptide library. In fact, this new method can support the classical methods very efficiently and improve the success rate of finding a specific high-affinity inhibitor.

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