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Conformational and Biochemical Analysis of the Cyclic Peptides which Modulate Serine Protease Activity

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Abstract: Prostate-specific antigen (PSA), a member of the kallikrein sub-group of the trypsin serine protease family, is a widely used marker for prostate cancer. Several sequences with specific binding to PSA have been identified by using phage display peptide libraries. The GST-fusion proteins of the characterized sequences have been shown to increase the enzyme activity of PSA to a synthetic substrate. The corresponding three cyclic synthetic analogues CVFTSNYAFC (A-1), CVFAHNYNYLVC (B-2) and CVAYCIEHHCWTC (C-4) have similar PSA promoting activity. Despite differences in the amino acid sequences, all three peptides bind to the same region of PSA. The conformation of the peptides was investigated by proton NMR spectroscopy. In addition, alanine replacement was used to characterize the prerequisites for binding. It is proposed that interactions with PSA are based on the aromatic and hydrophobic features of the amino acid side chains. Furthermore, it is suggested that peptides form β–turn structures forced by cysteine bridges directing important aromatic side chains to the same side of the turn-structure.

Keywords: prostate-specific antigen; PSA; peptide conformation; amino acid replacement; cyclic peptide; NMR

INTRODUCTION

Prostate-specific antigen (PSA) is a 30 kD serine protease belonging to the kallikrein family. PSA is produced by the epithelial cells of the prostate, from where it is secreted to seminal plasma. PSA participates in the lysis of the seminal coagulum during ejaculation [1]. In prostate tissue and in seminal plasma the protease activity of PSA is inhibited non-competitively by Zn^{2+} ions or competitively by spermide or spermidine. Only minor amounts of PSA appear in human serum and most serum PSA exists as an inactive complex with α_1-antichymotypsin [2,3]. There are contradictory theories of the role of PSA in cancer. It has been shown that PSA activates the urokinase-type plasminogen activator, which is thought to be involved in tumour invasion and metastasis [4,5]. In addition, PSA cleaves insulin-like growth factor binding protein 3 causing the release of IgF-1, which may affect the growth of different tumours [6]. However, PSA has been shown to produce angiostatin-like fragments from plasminogen by partial proteolysis. Angiostatin-like fragments have been shown to inhibit endothelial cell proliferation, migration and invasion [7,8].

The crystal structure of human PSA is not known. However, the 3D-structure of the corresponding equine PSA (HPK) has been determined. The common prostate origin and significant sequence homology between the human and horse PSA

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suggest that HPK may serve as a model for the structure of human PSA. The catalytic site of HPK is similar to the other chymotrypsin-like proteases containing a catalytic triad of His57, Asp102 and Ser195. The common feature for all kallikreins is the kallikrein loop near the active site of the protease, but there are major differences in the kallikrein loop sequences between HPK and other kallikreins. In HPK the kallikrein loop and the loop comprising Trp215-Cys220 form lid-like structures covering the catalytic site. This lid-like structure is suggested to act as a regulator of the protease activity and specificity [9].

An elevated PSA level in serum has been correlated to prostate hyperplasia and cancer [10]. Although the measurement of PSA in serum is widely used as a tumour marker, the major problem in diagnosis is the interference of benign prostatic hyperplasia (BPH). PSA isolated from BPH is clipped by the proteolytic enzyme, whereas in cancer the PSA remains intact [11,12]. This may provide a method of differentiating BPH from prostate cancer [13].

By using phage display libraries, several PSA binding peptides have been identified. These peptides are PSA specific and they do not bind to any other serine protease. Furthermore, the corresponding GST-fusion proteins have been shown to increase the serine protease activity of PSA [13,14]. The same effect can be seen with small cyclic peptides. These peptides are PSA specific and they bind only to the non-clipped form of PSA [15].

The structures of three PSA activating synthetic peptides having one or two disulfide bridges were studied by NMR spectroscopy. Furthermore, the amino acid sequences of the three peptides were modified in order to determine the amino acid residues important for the activity of the peptides. Based on NMR studies and peptide modifications a common structure and the amino acid residues are proposed that are important for the PSA promoting activity of the peptides.

MATERIALS AND METHODS

The Synthesis of Single Peptides for NMR

The peptides were synthesized using a PerSeptive 9050 Plus automated peptide synthesizer, with Fmoc strategy, TBTU/DIPEA as the coupling reagent and NovaSyn TGA with 4-hydroxymethylphenoxyacetic acid linker as the solid phase (Novabiochem, Läufelfingen, Switzerland). The side-chain protecting groups used in synthesis were trityl (Trt) for Asn, Gln and His, O-tert-Butyl (OtBu) for Glu and Asp, t-butyloxycarbonyl (Boc) for Trp and tert-butyl (tBu) for Ser, Thr and Tyr. For Cys both acetamidomethyl (Acm) and Trt protection groups were used. For peptides A-1, B-2 and their modifications Cys(Acm) was used in the Cys position 1: C(Acm)VFTSNYAFC(Trt) and C(Acm)VFAHNYDYLVC(Trt), for peptide C-4 and its modifications Cys(Acm) was used in positions 2 and 3: C(Trt)VAYC(Acm)IEHHC(Acm)WTC(Trt) During the cleavage from the resin with 96% TFA the Acm-protection group remains in the Cys-side chain.

The peptides were purified by HPLC (Shimadzu, Kioto, Japan) with a C_{18} reverse phase column and acetonitrile (ACN) as eluent (0.1% TFA in H_2O/0–60% ACN gradient for 60 min) and verified with a MALDI-TOF mass spectrometer (Bruker) and the purity was determined by analytical HPLC with 240 × 1.4 mm C18 column 0–60% ACN for 30 min.

The Parallel Synthesis of the Modified Peptides

Alanine-replacement sets and other modified peptides were synthesized with an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY). In alanine replacement sets all amino acids except cysteines were replaced one by one. The same synthesis method and amino acids were used as described above.

Cyclization of the Peptides

Peptides with cysteines (Acm) were cyclized using the iodination method. Lyophilized peptide was dissolved in 50% acetic acid (AcOH) at a concentration of 2 mg/ml, 1 M HCl (0.1 ml/mg of peptide) was added followed immediately by 0.1 M iodine solution in 50% AcOH (5 eq./Acm). The solution was stirred vigorously at room temperature for 40 min. The reaction was stopped with 0.1 M sodium thiosulphate. After filtering (0.45 μm) the peptides were purified with HPLC as described above. The formation of the disulphide bonds was verified with a MALDI-TOF mass spectrometer (Bruker Analytic GMBH, Karlsruhe, Germany).

PSA Activity Measurements

The effect of different peptides in the activity of PSA was studied using a chymotrypsin substrate S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA) (Chromogenix,

Mölndal, Sweden). 77 pmoles (2 μg) of PSA substrate and 20 nmoles of B-2 and C-4 series and 9 nmoles with A-1 were incubated in Tris-buffer (10 mM Tris and 150 mM NaCl at pH 8.0) with 1.6 mM concentration of the substrate at room temperature. PSA reaction without any peptide was used as the basic level control. The reaction was measured at 405 mm at 10 min intervals for 60 min using a Multiscan RC photometer (ThermoLabsystems, Helsinki, Finland). The effect of the peptides was calculated as a ratio of the optical density (OD) value of the PSA-peptide complex to the PSA alone after 60 min incubation.

Cross-inhibition Test

The relative binding sites of the peptides on PSA were mapped by competition experiments with chemically synthesized peptide and GST-peptide fusion protein [14]. Briefly, PSA specific monoclonal antibody (Mab5E4) was coated in microtitration plate wells (ThermoLabsystems, Helsinki, Finland) and PSA was bound to the antibody. The GST fusion peptide C-4 (0.17 μM) and each of the synthetic peptides (A-1, B-2 or C4) at 10–100 fold molar excess were incubated with PSA. After 1 h, the wells were washed and Eu^{3+}-labelled anti-GST antibody was added. After a further incubation for 1 h and washing, the bound fluorescence was measured with a 1234 Delfia Research fluorometer (Perkin Elmer Wallac, Turku, Finland).

NMR Spectroscopy

Because of the low solubility in water (especially C-4) all spectra were recorded in DMSO-d_6. NMR samples were prepared by dissolving purified and lyophilized peptides in 600 μl DMSO-d_6 to 5–10 mM. The pHs of the samples were not tested. All spectra were recorded at 300–320 K on a Bruker Avance 500 NMR spectrometer (Bruker) operating at a frequency of 500 MHz for ^{1}H. All one-dimensional experiments were recorded at five different temperatures over the range 300 K to 320 K. The temperature coefficients ($d\delta/dT$) of the amide protons were calculated by analysing the chemical shifts at these five temperatures. All two-dimensional experiments were recorded at either 305 K or 310 K depending on the quality and clarity of the spectra. All the chemical shifts are reported with respect to the DMSO peak at 2.50 ppm (Table 1).

For all 2D experiments, standard pulse programs from the Bruker software library were used. TOCSY spectra were recorded with mixing times of 80 ms by means of a MLEVTP mixing sequence with TPPI phase cycling [16]. NOESY spectra were mainly recorded with mixing times of 400 ms for A-1, 420 ms for B-2 and 300 ms for C-4 using TPPI phase cycling [17]. Various mixing times were tested and the best were determined based on the quality and clarity of the spectra. NOE-build up curves were not determined. Some COSY spectra were recorded with mixing times of 30 ms. Data sets were processed with phase-shifted sine bell functions. Typically the data were recorded with a resolution of 1024 points for both t_1 and t_2.

Structural Analysis

Starting structures for structural analysis were constructed by DYANA software [18]. NOE-intensities were calibrated approximately relative to the β-protons of Tyr7 and Cys10 and the aromatic ring protons of Tyr7 in A-1, the aromatic ring protons of Tyr7 in B-2 and the aromatic ring protons of Trp11 in C-4. NOE correlations were classified either as strong (1.8–2.5 Å), medium (1.8–3.5 Å) or weak (1.8–5.0Å). Pseudo-atom corrections of 1.5 Å for methyl, 1.0 Å for methylene protons and 2.0 Å for tyrosine ring protons were added when needed. Dihedral angles were not restricted mainly because of fluctuation of 3-D structures [19]. The final DYANA structures were modelled and visualized with the software package QUANTA with help of the CHARMM program on a Silicon Graphics workstation [20].

RESULTS

Conformation of Peptides

The most important data derived from NMR measurements were sequential and long-range nuclear Overhauser effects (NOEs), $C^{\alpha}H$ conformational shifts, temperature coefficients of amide protons and appropriate coupling constants, $^3J_{NHC\alpha H}$ [19,21]. The solvent exposures of NH groups were detected by determining the temperature coefficients of the NH groups. Every peptide investigated has NH group or groups showing very low $\Delta\delta/\Delta T$ values (<3 ppb/K) characteristic of strong solvent shielding [22]. Low temperature coefficients of amide protons were found for Tyr7 and especially for Ala8 in peptide A-1, for Tyr7 in peptide B-2 and for His9 in peptide C-4 (Table 1). The upfield chemical shifts

Table 1 ^{1}H Chemical Shifts and Amide Proton Temperature Coefficients of PSA-binding Peptides

Peptide residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	Other	$-\Delta\delta/\Delta T$(ppb/K)
A-1							
Cys1		4.20	2.98				
Val2	8.52	4.23	1.88	0.81			6.1
Phe3	8.36	4.83	3.08, 2.88		7.18	7.13–7.23 ($C^{\varepsilon}H$, $C^{\zeta}H$)	6.0
Thr4	8.02	4.38	4.18	1.04			7.4
Ser5	8.06	4.14	3.72, 3.65				4.7
Asn6	7.99	4.46	2.46, 2.39			6.93, 7.38 ($N^{\delta}H$)	7.2
Tyr7	7.81	4.36	2.86, 2.67		6.94	6.62 ($C^{\varepsilon}H$)	3.7
Ala8	7.89	4.37	1.09				1.8
Phe9	8.00	4.60	3.06, 2.82			7.13–7.23 ($C^{\varepsilon}H$, $C^{\zeta}H$)	7.0
Cys10	8.48	4.58	3.17, 2.96				7.5
B-2							
Cys1	—	4.39	3.03, 2.82				
Val2	8.62	4.42	2.07	0.87, 0.81			4.7
Phe3	8.40	4.77	3.07, 2.73		7.25	7.21–7.24 ($C^{\varepsilon}H$, $C^{\zeta}H$)	9.3
Ala4	8.02	4.25	1.16				7.6
His5	8.42	4.27	3.13, 3.02		7.27 (4H)	8.81 ($C^{\varepsilon}H$, 2H)	8.7
Asn6	7.99	4.26	2.59, 2.59			6.87, 7.44 ($N^{\delta}H$)	5.0
Tyr7	7.85	4.13	2.86, 2.75		6.84	6.58 ($C^{\varepsilon}H$)	0.7
Asp8	8.16	4.53	2.68, 2.50				7.3
Tyr9	7.53	4.58	2.94, 2.72		6.94	6.57 ($C^{\varepsilon}H$)	4.0
Leu10	8.30	4.50	1.35, 1.35	1.33	0.55, 0.53		13.1
Val11	8.21	4.29	1.92	0.88, 0.87			10.8
Cys12	8.75	4.67	3.21, 2.89				10.3
C-4							
Cys1	—	4.22	3.23				
Val2	8.61	4.28	1.98	0.89, 0.88			6.7
Ala3	8.15	4.65	1.20				6.4
Tyr4	7.96	4.71	2.87, 2.66		6.89	6.53 ($C^{\varepsilon}H$)	4.5
Cys5	8.67	5.38	2.83, 2.72				9.9
Ile6	8.55	4.20	1.81	1.14, 1.55 0.92 ($C^{\gamma}H_3$)	0.86		6.5
Glu7	8.98	3.57	2.17, 1.95	2.28			8.5
His8	8.41	3.93	3.27		7.24 (4H)	8.74 ($C^{\varepsilon}H$, 2H)	5.9
His9	7.86	4.72	3.04, 2.87		7.18 (4H)	8.63 ($C^{\varepsilon}H$, 2H)	1.0
Cys10	8.60	5.27	2.74, 2.65				9.1
Trp11	8.58	4.76	3.09, 2.89		7.14 (2H)	7.65 (4H), 6.95 (5H), 6.98 (6H), 7.26 (7H), 10.62 (NH)	4.3
Thr12	8.20	4.40	3.94	1.10			10.3
Cys13	7.97	4.50	3.17				4.0

of C^{α}Hs suggest that there exist α-conformations between residues Ser5-Tyr7 in peptide A-1, between His5-Asp8 in peptide B-2 and between Glu7-His8 in peptide C-4 (Figure 1) [21,23]. Very strong sequential NH(i)-NH(i + 1) NOEs were found for Asn6-Tyr7 in peptide A-1, for His5-Asn6, Tyr7-Asp8 and Asp8-Tyr9 in peptide B-2 and for Val2-Ala3 and His8-His9 in peptide C-4 indicating α-conformations and possible turn structure. Strong sequential C^{α}H(i)-NH(i + 1) NOEs were found between residues Val2-Asn5 in peptide A-1, between Val2-Ala4 and Tyr9-Cys12 in peptide B-2 and between Cys1-Glu7 and His9-Cys13 in peptide C-4 indicating the existence of β-conformations. Long-range inter-residual

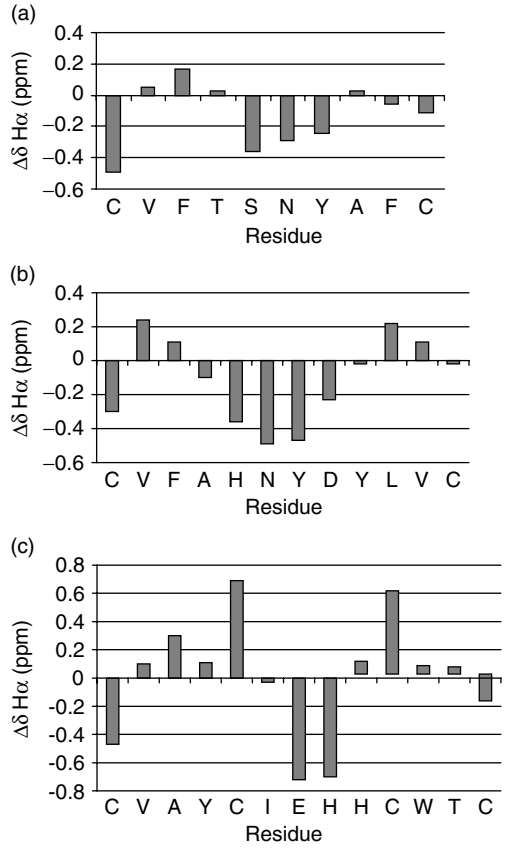

Figure 1 Differences between the $C^{\alpha}H$ chemical shift values in the random coil and values determined experimentally in DMSO-d_6 for A-1 (a), B-2 (b) and C-4 (c).

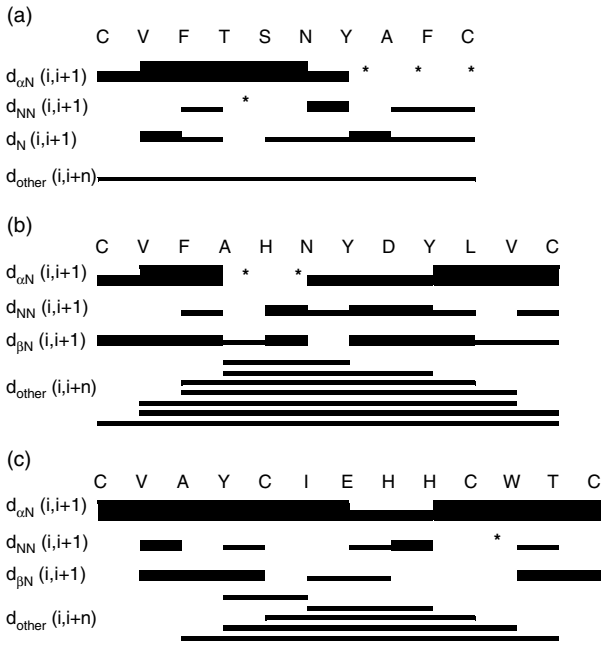

Figure 2 Summary of significant NOE-correlations for peptides A-1 (a), B-2 (b) and C-4 (c) in DMSO-d_6. Relative cross-peak intensities were estimated from volume integration and depicted as strong, medium and weak. * Disturbed NOE-correlation by some other NOE. NOEs indicated as 'other' interaction include long range main chain–main chain, side chain–main chain or side chain–side chain interactions.

NOEs were found for peptides B-2 and C-4. No significant inter-residual NOEs were observed for peptide A-1 (Figure 2). A part of the NH peaks was broadened indicating solvent-exposed NH groups or conformational fluctuation and dynamic behaviour [19,22]. Because of broadening all $^3J_{NHC\alpha H}$ could not be detected and the detected coupling constants were usually between 6.0 and 8.0 Hz.

The common turn structures in the peptides investigated were identified as a type I β-turn between residues Ser5-Ala8 for peptide A-1, a type I β-turn between residues Ala4-Tyr7 for peptide B-2 and a type II β-turn between residues Ile6-His9 for peptide C-4. However, peptide C-4 has a disulphide bond between residues 5 and 10, which causes a tight turn in the sequence between those residues. Whenever a loop structure was formed in the peptides studied the aromatic side chains of phenylalanine, tyrosine or tryptophan were found to locate on the same side of the peptide loop and near each other. In peptide A-1, a relatively strong NH(i)-NH(i + 1) correlation between Asn6 and Tyr7 was detected (Figure 2) supporting the existence of an α-conformation for the backbone of Asn6 and thereby proposing the existence of a type I β-turn between residues Ser5-Ala8 [19]. A moderately low temperature coefficient for the amide proton of Tyr7 (3.4 ppb) was detected (Table 1), which is likely due to an interaction between the NH-proton of Tyr7 and the side chain oxygen of Ser5. This is in line with serine's potential to be at position i of the type I turn [24,25]. However, strong $C^{\alpha}H$(i)-NH(i + 1) correlation for Asn6 and Tyr7 provides evidence for the existence of a significant population of type II turns in the same area (Figure 2). Thus, these mutually exclusive NOE-correlations indicate high flexibility or conformational interconversion in the turn [26].

In peptide B-2, a strong NH(i)-NH(i + 1) correlation between residues His5 and Asn6 and a weak correlation between Asn6 and Tyr7 was found (Figure 2), indicating that there is a type I β-turn at residues 4–7 [19]. Furthermore, weak NOE-correlations between Ala4 $C^{\beta}H_3$-protons and

both Tyr7 and Tyr9 amide protons indicate that a tight turn exists. In peptide C-4, both strong NH(i)-NH(i + 1) correlation between His8 and His9 and strong intraresidual $C^{\alpha}H(i)$-NH(i) correlation for His8 indicates a type II turn (Figure 2). A very strong downfield shift for Glu7 NH evidences its solvent-exposed conformation, which is most probably intrinsic for a type II turn. A NOE was observed between Ile6 NH and Tyr9 NH indicating the existence of a tight turn.

The PSA-promoting Activity of A-1, B-2, C-4 and their Modifications

The activities of the three PSA-promoting peptides and corresponding alanine replacement sets were compared with the activity of PSA alone (Figure 3). Most of the changes inactivated or reduced the activity of peptides A-1 and B-2. The exception was the replacement of His5 by Ala in B-2, which improved the activity. In addition two replacements (F → A and D → A) in the A-1 sequence slightly decreased the PSA reactivity. In the B-2 sequence the carboxy terminal Leu and Val, which are not present in the A-1 sequence, could not be replaced by alanine without losing the activity.

The C-4 peptide contains 13 amino acids and two disulphide bridges. None of the alanine replacements totally inhibit the activity. Reduced activities were observed in Y → A, I → A and W → A modifications. In contrast to this, slightly increased activities were seen when Val2, Glu7, His8, His9 and Thr13 were mutated to Ala.

On the basis of the results from alanine replacements, three sequences of 10 amino acid residues were synthesized and their activities were compared with the A-1 sequence. Reduced activities resulted in the peptides having alanines at positions 2, 4 and 5 (54%) or 2, 4, 5 and 6 and Asp at position 8 (37%). When Tyr7 was replaced by alanine the activity was completely lost (Table 2).

Cross-inhibition of the Peptides

The binding of GST-peptide C-4 to PSA was blocked in a dose dependent fashion by each of the synthetic peptides A-1, B-2 and C-4. The inhibition was up to 60%–70% when using 100-fold molar excess of the peptides showing that these three peptides bind to the same site or to sites which are very close to each other on PSA (Figure 4).

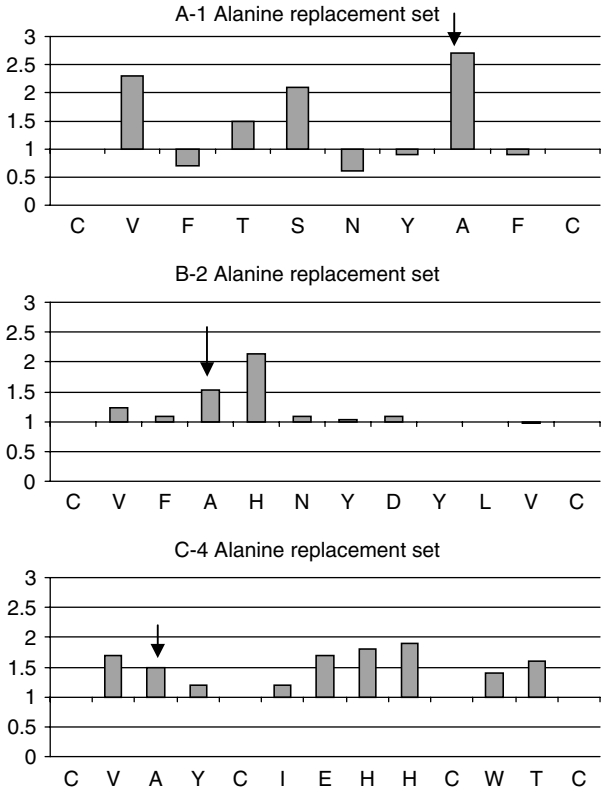

Figure 3 Alanine replacement sets of A-1, B-2 and C-4 peptides. Every column represents amino acid, which is changed with alanine and the height is calculated from the equation: absorbance of peptide PSA/PSA alone. Value 1 illustrates PSA activity alone. In peptide A-1 some of the modifications have values below 1 suggesting PSA inhibitory activity. The parent sequences are shown by the arrow.

Table 2 Sequences with More than One Residue Replacement with Alanine. The Activities of the Modified Peptides Reported are Relative to the Activity of the Parent Sequence

	S/C	%
CVFTSDYAFC	2.52	
CAFAADYAFC	1.83	54%
CAFASAADYC	1.08	5%
CAFAAAYDYC	1.57	37%

DISCUSSION

The PSA-promoting activities of the three cyclic peptides are comparable in spite of the differences in the amino acid sequences and the number of disulphide

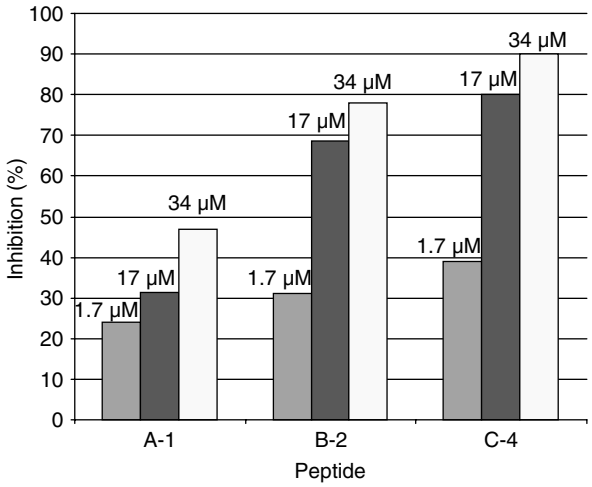

Figure 4 Effect of synthetic peptides on the binding of GST-peptide fusion protein C-4 to immobilized PSA. 1.7, 17 and 34 µM concentrations of synthetic peptides together with 0.17 µM GST-peptide C-4 were added to wells containing PSA bound by MAb 5E4 resulting in concentration dependent inhibition of GST-peptide binding.

bonds. Originally these sequences were determined by using phage display technology. This technology allows the synthesis of millions of different combinations of short peptides, which can be screened with a binding assay to the target molecule. During the selection several sequences with similar binding properties were characterized [27–29]. These sequences serve as valuable lead structures to study the mechanism of the biological interactions. Modern synthesis technology provides several methods to produce directed peptide libraries for interaction studies [30].

In our studies alanine replacement studies showed that the biological activities of all three peptides arise mainly from the aromatic and hydrophobic interactions of Tyr and Phe with the PSA. However, also small hydrophobic residues, Ile, Leu and Val, have a role in the activities of the peptides. This was further studied with consensus peptides, in which the replacement of Tyr totally inactivated the peptide, whereas alanines in other positions reduced the activity. It can be assumed that the aromatic rings or hydrophobic side chains are the contact residues and their spatial arrangement is important for activity while the rest of the peptide acts as a backbone for the active side chains, influencing the conformation of the peptides. In B-2 peptide the replacement of Leu10 or Val11 with alanine inactivates the peptide. On the other hand, A1-peptide, which is similar to B-2 but lacks carboxyterminal Leu and Val, is active.

Aspartic acid stabilizes the turn structures by hydrogen bonding via its side chain Asp, replacement with Ala both in A-1 and B-2 sequences inactivated the peptides. Histidine is not as favoured in position $i+1$ in type I β-turns [24]. We assume that removing this relatively large and basic side chain near the contact residues increases the biological activity. The same effect can be seen in peptide C-4, where replacement of His8 or His9 increases the activity.

The cyclization of the peptides is suggested to force the peptides in turned conformations essential for the PSA-promoting activity. Long-range NOE analyses indicate loop structures and common β-turn structures for each peptide. In A-1 Tyr7 is located at position $i+2$ (turn 5–8) and in B-2 at position $i+3$ (turn 4–7) of a β-turn. Due to the fluctuation of the A-1 backbone, Tyr7 may also locate at position $i+3$. It is suggested that this fluctuation is more favoured in water solutions, because DMSO-d_6 may act only as a hydrogen bond donor, whereas water has hydrogen bond donor and acceptor properties. Therefore, distances between the aromatic residues and the conformations of A1 and B2 are likely to be similar. In peptide C-4, a disulphide bond close to Tyr and Trp is the main factor determining the overall structure of the peptide. In addition, hydrophobic interactions may exist between Tyr, Ile and Trp forming a hydrophobic cluster on one side of the peptide. NOE-correlation peaks were found between the side chains of Tyr4 and Ile6.

The distance between a pair of protons has to be less than 3.5 Å in flexible structures for a NOE to be observed The sequential NH(i)-NH($i+1$) NOE cross peaks are evidence of folded or turned conformations [31]. In the case of short peptides, the NH(i)-NH($i+1$) NOE is seen if the population of a turn conformation is at least 10% [19,32]. Both α- and β-conformations were found for Phe3, Asn6, Ala8 and Phe9 in peptide A-1, for Phe3, Ans6, Tyr7, Asp8, Tyr9 and Val11 in peptideB-2 and for Val2, Tyr4, Glu7, His8 and Trp11 in peptide C-4. Thus, in spite of the fact that these peptides are relatively short cyclic structures, several conformations were observed.

It is suggested that the biological activity of the three peptides is based on the interaction of the aromatic and hydrophobic side chains with PSA. The turn structures of the peptides place the important contact residues in an arrangement crucial for the

PSA recognition (Figure 5). Since it has been shown that B-2 and C-4 peptides bind only to the intact and active form of PSA, the interaction of the peptides is dependent on the native structure of PSA [15].

Although the three peptides contain tyrosine, which has the aromatic side chain of the substrates recognized by PSA, the peptides do not bind to the active site of the protease. The cross-inhibition studies indicate that, despite significant differences in the sequences of A-1 and B-2 compared with C-4, all three peptides bind to the same area of the PSA. Furthermore, in the presence of Zn^{2+}, the affinities of the three peptides increase three- to sevenfold, suggesting the involvement of Zn^{2+} in the binding of the peptides to PSA [14]. It is reasonable to suggest that the PSA-promoting peptides do not directly interact with Zn^{2+} but upon binding of Zn^{2+} the PSA structure is altered so that the peptides' affinities are increased.

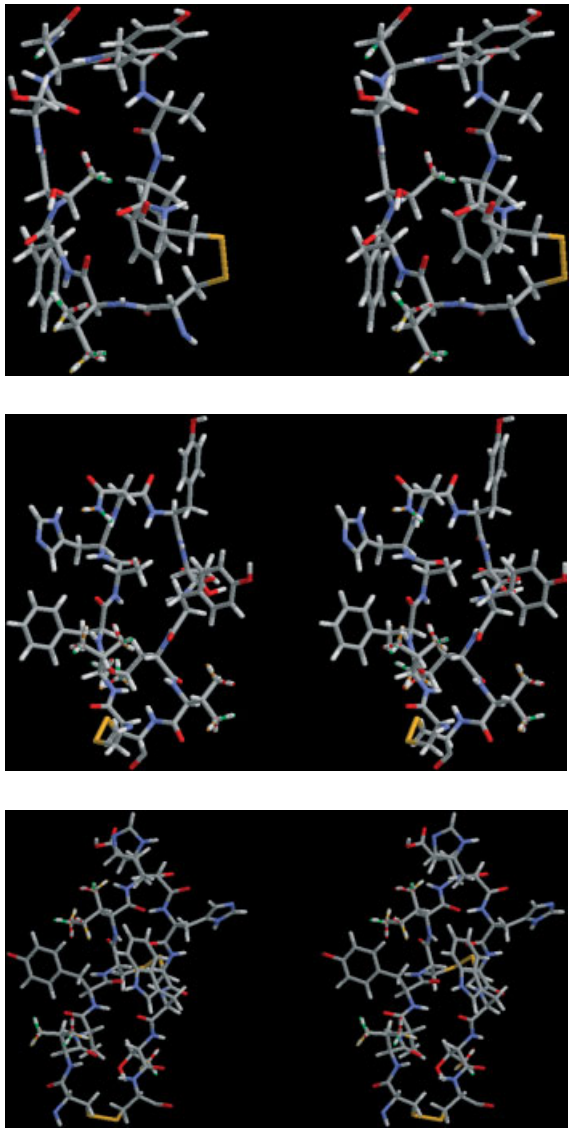

Figure 5 One of the possible theoretical stereotypic molecular models of (A) A-1, (B) B-2 and (C) C-4 peptides. The loop structure forces at least two important aromatic side chains to the same side of the turn structure.

The kallikrein loop of HPK, the corresponding equine PSA, is proposed to be a regulator of protease activity. In human PSA the regulatory mechanism may be similar. Zn^{2+} is a non-competitive inhibitor of PSA and is coordinated by Asp91, His101 and His234 close to the residues of the catalytic triad. The exact binding site and mechanism of the peptides is not known but it is proposed that the peptides bind, like Zn^{2+}, near the active site of PSA and they interfere with the possible regulatory function of the kallikrein loop. The recognition of PSA takes probably place via hydrophobic interactions and aromatic rings of phenylalanine or tyrosine. The specific binding of the peptides may provide a diagnostic tool for selective recognition of an active form of PSA.

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