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Identification of novel peptide inhibitors for human trypsins

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Abstract

Human trypsin isoenzymes share extensive sequence similarity, but certain differences in their activity and susceptibility to inhibitors have been observed. Using phage display technology, we identified seven different peptides that bind to and inhibit the activity of trypsin-3, a minor trypsin isoform expressed in pancreas and brain. All of the peptides contain at least two of the amino acids tryptophan, alanine and arginine, whereas proline was found closer to the N-terminus in all but one peptide. All peptides contain two or more cysteines, suggesting a cyclic structure. However, we were able to make synthetic linear variants of these peptides without losing bioactivity. Alanine replacement experiments for one of the peptides suggest that the IPXXWFR motif is important for activity. By molecular modeling the same amino acids were found to interact with trypsin-3. The peptides also inhibit trypsin-1, but only weakly, if at all, trypsin-2 and -C. As trypsin is a highly active enzyme which can activate protease-activated receptors and enzymes that participate in proteolytic cascades involved in tumor invasion and metastasis, these peptides might be useful lead molecules for the development of drugs for diseases associated with increased trypsin activity.

Keywords: computer modeling; inhibitors; peptidases; phage display; serine proteases.

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Introduction

Trypsin is one of the best characterized proteolytic enzymes. It is produced as a proenzyme, trypsinogen, mainly by the pancreas, and it is activated in the gastrointestinal tract by enterokinase (Haverback et al., 1960). Active trypsin participates in the digestion of food by degradation of dietary proteins and by activation of other digestive enzymes in the small intestine. However, trypsinogen is also expressed in various other normal and malignant tissues (Koivunen et al., 1990; Nyberg et al., 2006; Paju and Stenman, 2006). Eight different trypsinogen genes have been found in humans, three of which (*PRSS1*, *-2* and *-3*) have been shown to encode protein, i.e., trypsinogen-1, -2 and -3, also called cationic-, anionic- and mesotrypsinogen, respectively (Paju and Stenman, 2006). The gene encoding trypsinogen-3 gives rise to additional splicing variants expressed in the brain (trypsinogen-4 A and B) (Wiegand et al., 1993), differing only in the sequence preceding the activation peptide, i.e., the active enzymes are identical and, thus, referred hereinafter as trypsin-3. Trypsinogen-C (TRY6) is thought to be a transcribed pseudogene (Chen and Ferec, 2000).

Trypsin-3 is a minor trypsin-isoform in the pancreas and it differs from the major trypsin-isoforms, i.e., trypsin-1 and -2, by its resistance to several protein inhibitors and by substrate specificity (Nyaruhucha et al., 1997; Szmola et al., 2003). The physiological function of trypsin-3 is unknown, but it has been suggested to regulate trypsin activity by degrading trypsin inhibitors (Szmola et al., 2003). It also cleaves several other proteins, such as myelin basic protein (Medveczky et al., 2006) and protease-activated receptors (Cottrell et al., 2004).

Abnormally high trypsin activity as a result of overexpression of trypsin, mutations in trypsin causing increased activity, or reduced trypsin inhibition is thought to play a pathogenic role in pancreatic diseases and cancer (Whitcomb et al., 1996; Nyberg et al., 2006; Paju and Stenman, 2006). Increased trypsin activity could also be associated with neurological diseases (Medveczky et al., 2006; Wang et al., 2008). Therefore, specific inhibitors for different trypsin-isoforms have potential therapeutic utility in disorders characterized by increased trypsin activity. Trypsin-binding reagents could also be useful for diagnosis and imaging of such diseases. However, systemic inhibition of all trypsin-isoforms at the same time can cause unwanted side effects, whereas specific inhibition of individual trypsin-isoforms, such as trypsin-3 might be expected to be better tolerated.

Peptides and peptidomimetic compounds are potential alternatives to antibodies and traditional small molecule drugs, and they can also be used for diagnostic applications (Wu et al., 2004). Although antibodies are specific and have high affinity, their size can limit their therapeutic and diag-

Table 1 Amino acid sequences of trypsin-3 binding peptides identified by phage display.

Code	Phage library[a]	Aminoacidsequence	No. of isolates
TRY3-C-1	$CX_3CX_4CX_2X$	C G C V C W F E R C C P C	3
TRY3-C-2	$CX_3CX_4CX_2X$	C P A R C W A R S C N E C	2
TRY3-C-7	$CX_3CX_4CX_2X$	C P T R C F A R D C G H C	2
TRY3-C-9	$CX_3CX_4CX_2X$	C V P T C W W R S C L L C	1
TRY3-X10-1	X_{10}	I P C S W F R T G C	10
TRY3-A-1	CX_8C	C I P Y Q W A R G C	7
TRY3-A-3	CX_8C	C T L P N T W W A C	1

The amino acids common for most of the identified peptides are highlighted. Cysteines are shown in bold font.

[a]C is cysteine and X is any of the 20 naturally occurring amino acids. Subscript numbers refer to the number of amino acids.

nostic use *in vivo*. Peptides that specifically recognize active proteases can be selected by phage display (Koivunen et al., 1999; Nixon, 2002; Koistinen et al., 2008).

Here, we report development of peptides binding to trypsin-3 and characterization of their reactivity with human trypsin-1, -2, -C and -3. These peptides have potential use as lead molecules for the development of treatment for disorders characterized by increased trypsin activity and for diagnosis of such conditions.

Results

Isolation of trypsin-3 binding phage

Screening of five different phage libraries revealed seven different phage binding to recombinant trypsin-3 that was captured to the solid phase with a monoclonal antibody (Table 1). Phage binding to the capture antibody (not shown) was excluded from further analyses. The trypsin-specific phage recognized active, but not inactive, trypsin-3 (Figure 1). All of the selected phage clones displayed peptides containing two or more Cys-residues. Furthermore, the peptides contained at least two of the amino acids Trp, Ala and Arg (in that order), whereas Pro was found close to N-terminus in all but one peptide.

Characterization of peptides expressed as fusion proteins with GST

Four peptides, TRY3-C-1, -C-7, -A-1 and -X10-1 were expressed as fusion proteins with GST (referred hereafter to as GST-peptides). The inhibitory effect of these GST-peptides was studied with trypsin-1, -2, -C and -3. TRY3-X10-1-GST inhibited efficiently trypsin-1 and -3, and to a lesser degree trypsin-2 and trypsin-C, whereas TRY3-A-1-GST inhibited only trypsin-1 and -3 significantly (results not shown). The other GST-peptides were only weakly active, if at all. The binding of TRY3-X10-1-GST and TRY3-A-1-GST to trypsin-3 was studied by an immunopeptidometric assay (IPMA) (see the materials and methods section). Both bound to trypsin-3 in a dose dependent manner, but TRY3-X10-1-GST bound more strongly.

The inhibitory effects of synthetic peptides

When we studied the ability of synthetic peptide analogs to inhibit different trypsin-isoenzymes (Table 2), peptides derived from TRY3-X10-1 showed significant inhibition of trypsin-1 and -3 at submicromolar concentrations. At higher concentrations some of the peptides also inhibited trypsin-2 and -C (Figure 2). Replacement of each amino acid of peptide #1 to Ala (peptides #2–8) showed that a change of Ile1 (numbering according to original phage associated peptide, Table 1), Pro2, Trp5, Phe6 or Arg7 significantly decreased the activity of the peptide, indicating the importance of these amino acids on the activity of the peptide. However, none

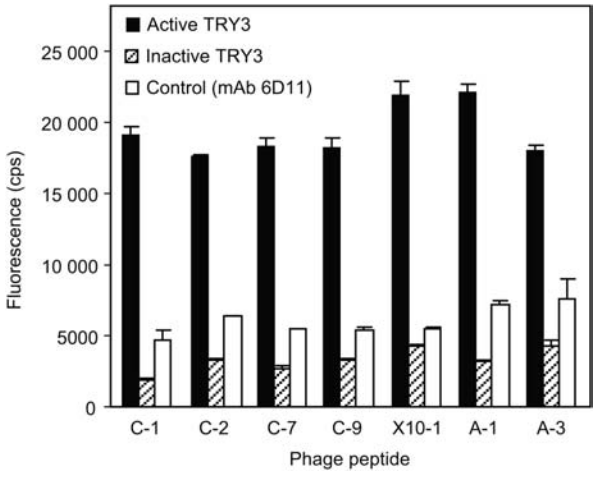

Figure 1 Reactivity of active trypsin-3 (active TRY3) with phage. Each of the phage was incubated in wells containing equal amounts of trypsin-3, which was captured by monoclonal antibody 6D11, and activated by enterokinase. The binding of phage was quantified by phage IFMA. Non-activated trypsinogen-3 (inactive TRY3) and wells containing only antibody (mAb 6D11), but not trypsin, were used as controls. Data represent mean values from duplicate wells±standard error (SE). Amino acid sequences of the phage displayed peptides are given in Table 1.

Table 2 The effect of synthetic variants of peptides identified by phage display on the activity of different trypsin-isoenzymes [trypsin (TRY)-1, -2, -C and -3] and plasma kallikrein (KLK).

Peptide code	Peptide sequence[a]	Inhibition[b,c]				
		TRY1	TRY2	TRYC	TRY3	KLK
Cyclic variants of TRY3-X10-1						
1	**G** I P C S W F R T G C **A**	+++ (0.2)	- (5.1)	+ (7.1)	+++ (0.3)	+++
2	**G A** P C S W F R T G C **A**	-	-	-	-	
3	**G** I **A** C S W F R T G C **A**	+	-	-	+	
4	**G** I P C **A** W F R T G C **A**	++++	-	-	+++	
5	**G** I P C S **A** F R T G C **A**	-	-	-	-	
6	**G** I P C S W **A** R T G C **A**	-	+	+	+	
7	**G** I P C S W F **A** T G C **A**	+	+	+	+	
8	**G** I P C S W F R **A** G C **A**	+++ (0.4)	+ (6.7)	- (6.5)	+++ (0.3)	+++
9	**G** I P C S W F R T **F** C **A**	++	-	-	++	
10	**G** I P C S W F R T **S** C **A**	+++	-	-	++	
11	**G** I P C S W F R T **D** C **A**	+++	-	-	+++	
Linear variants of TRY3-X10-1						
12	**G** I P **A** S W F R T G **A**	+++	+	+	+++	
13	I P **A** S W F R T **NH_2**	+	-	+	+	++++
14	I P **A A** W F R T **NH_2**	+	-	+	+	++++
15	I P **A** S W **A** R T **NH_2**	-	-	+	+	+
16	I P **A A** W **A** R T **NH_2**	-	-	+	+	-
17	**Ac** I P **A** S W F R T **NH_2**	++	+	-	++	++++
18	**Ac** I P **A A** W F R T **NH_2**	++	+	-	++	++++
19	**Ac** I P **A** S W **A** R T **NH_2**	++	-	-	++	-
20	**Ac** I P **A A** W **A** R T **NH_2**	+	-	-	++	-
21	**G** I P **A** S W F R **G** G **A**	+++	+	-	+++	++++
22	**G** I P **A** S W F R **A** G **A**	+++	+	-	+++	++++
23	**Ac** I P A S W F R **G** **NH_2**	+++ (0.3)	+ (2.7)	- (6.7)	++ (0.4)	++++
24	**Ac** I P **A** S W F R **A** **NH_2**	++	+	-	++	++++
25	**Ac A** I P **A** S W F **K** T **A** **NH_2**	+ (2.2)	- (>20)	- (>20)	- (8.7)	++
26	**Ac A** I P **A A** S W F R T **A** **NH_2**	-	-	-	-	+
27	**Ac A** I P **A** S W F **A** R T **A** **NH_2**	-	-	-	-	+
28	**Ac A L** P **A** S W F R T **A** **NH_2**	+	-	-	-	++++
29	**Ac A** I P **A** S W F R T **A** **NH_2**	++ (0.4)	- (5.2)	- (8.2)	++ (0.7)	++++
30	**Ac A** I P **A** S W F R	++	-	+	++	
31	**Ac A** I P **A** S W F R **NH_2**	++	-	-	++	++++
TRY3-C-9						
32	**A** C V P T C W W R S C L L C **A**	++	-	-	+	+
Variants of TRY3-A-1						
33	**A** C I P Y Q W A R G C **A**	- (3.7)	- (>20)	- (>20)	- (15)	-
34	**A A** I P Y Q W A R G **A A**	- (5.2)	- (>20)	- (>20)	- (5.1)	-
35	**A A** I P Y Q W A R **T A A**	-	-	-	-	-
36	**Ac A** I P Y Q W A R **NH_2**	-	-	-	-	+
37	**Ac A A** I P Y Q W A R **P A A** **NH_2**	+ (4.4.)	- (>20)	- (16)	- (N.I.[d])	-
38	**SA A** I P Y Q W A R G **A A** **NH_2**	-	-	-	-	-
39	**Ac A A** I P Y Q W A R G **A A** **NH_2**	+	-	-	-	-

Amino acids or other modifications that differ from the original peptides (Table 1) are shown in bold font.

[a]Amino acid sequence of the peptides. Ac, acetyl; SA, sarcosine; NH_2, C-terminal amide.

[b]Effect of 1 μM peptides on trypsin activity or 10 μM peptides on plasma kallikrein activity, ++++ very strong inhibition (i.e., 80–100% inhibition); +++, strong inhibition (60–80%), ++ average inhibition (30–60%); + weak inhibition (10–30%); - no inhibition or very weak inhibition (<10%).

[c]For selected peptides, IC_{50} values (μM) are shown in parentheses.

[d]No inhibition was observed even at 20 μM concentration.

of the Ala replacements affected the isoform-specificity profile significantly. The replacement of Gly9 with larger amino acids, i.e., Phe, Ser or Asp (peptides #9–11), did not have any major effect on activity.

A linear form of TRY3-X10-1 peptide was synthesized by replacing Cys-residues with Ala (peptide #12). The inhibitory activity of this form was similar to the cyclic form (peptide #1). Shortening of the peptide by removal of the N-terminal Gly, and the C-terminal Gly and Ala, and amidation of C-terminus led to a significant loss of activity (peptide #13). The activity was partially restored by acetylation of the amino terminus (peptide #17). The effect of replacement of Ser or Phe, or both, with Ala in peptides lacking C-terminal Gly was also tested with and without amino-terminal acetylation (peptides #13–16 and #17–20, respectively). Non-acetylated peptides showed only weak inhibition, whereas the acetylated versions were more active. Replacement of Thr by Ala or Gly had only a minor effect on the activity of the peptide (peptides #21–24, as compared to #12). Replacement of Arg with Lys (#25) or Ile with Leu (#28) greatly reduced or abolished the activity. The distance between Ile-Pro and Trp, and between Trp and Arg appears to be critical, as addition of Ala between these amino acids inactivated the peptide (peptides #26 and #27, as compared to peptide #29). Significantly, an N-terminally acetylated peptide with a C-terminal Arg (#30) and its C-terminally amidated version (#31) inhibited trypsin-1 and -3.

Cyclic and linear versions based on TRY3-A-1 and TRY3-C-9 were also synthesized (peptides #32–39). Most were active at high concentrations, but they were less effective than TRY3-X10-1-based peptides. A linear version of TRY3-A-1 (peptide #34) had similar activity as the cyclic version (peptide #33), inhibiting trypsin-1 and -3 at high concentrations. Another linear variant of TRY3-A-1 (peptide #37), in which Gly was changed to Pro, the N-terminus was acetylated and C-terminus amidated, inhibited trypsin-1 at micromolar concentration, whereas it did not show any inhibition of trypsin-3 even at 20 μM concentration (Figure 2).

Synthetic variants of TRY3-X10-1 (#1 and #4) and TRY3-A-1 (#37) were also tested for their ability to inhibit α-casein degradation by trypsin-1 (Figure 3). In keeping with the results obtained with a peptide substrate (Table 2), all these peptides inhibited trypsin-1 activity towards α-casein, peptide #37 being least active.

Studies on enzyme kinetics showed that peptides #1, #23, #25 and #37 all significantly increased the K_m value of trypsin-1, without significantly affecting V_{max} (Table 3). Similar results were obtained with trypsin-3, except for peptide #37, which did not inhibit trypsin-3.

Effect on the enzymatic activity of other proteases

Several of the TRY3-X10-1 variants in which Phe, but not Ala, preceded Arg were found to effectively inhibit plasma kallikrein (Table 2). Peptide #1 also inhibited chymotrypsin with moderate activity (52% inhibition). Plasmin and KLK2 were only weakly, if at all, inhibited (<30% inhibition) by

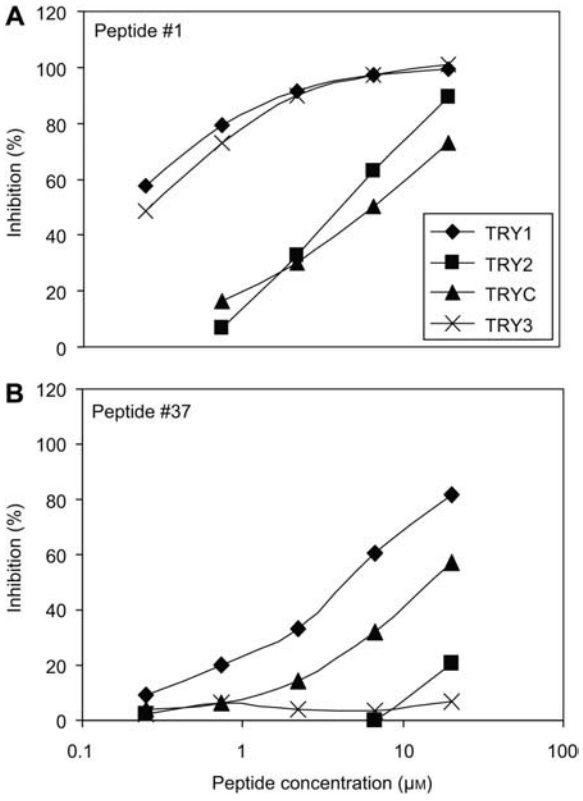

Figure 2 Effect of the peptides #1 (A) and #37 (B) on the activity of trypsins (TRY).
The activity of trypsins with and without peptides was measured using a colorimetric substrate. The absorbance values were converted to inhibition percent using the formula: 100%-100%* (absorbance with inhibitor/absorbance without inhibitor). 100% represents total inhibition of trypsin activity and 0% no inhibition. For peptide numbers, refer to Table 2.

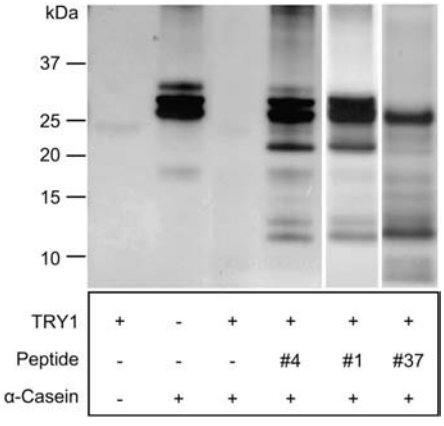

Figure 3 Inhibition of trypsin-1 mediated degradation of α-casein by peptides.
Trypsin-1 was preincubated with different peptides, or with buffer as a control, before the addition of α-casein. The degradation was analyzed by SDS-PAGE and silver staining. For peptide numbers, refer to Table 2.

Table 3 Effect of the selected peptides on V_{max} and K_m values of trypsin-1 and -3 with substrate S-2222.

Peptide	TRY1		TRY3	
	V_{max} (μM/min)	K_m (μM)	V_{max} (μM/min)	K_m (μM)
Without peptide	3.9±0.2	189±20	7.1±0.2	123±2
Peptide #1 (1 μM)	4.3±0.5	1290±23	7.6±0.7	705±78
Peptide #23 (1 μM)	4.0±0.6	1853±428	7.5±0.4	846±68
Peptide #25 (10 μM)	5.1±1.4	1735±245	7.4±0.2	342±4
Peptide #37 (10 μM)	4.7±0.1	880±73	7.0±0.2	138±9

Refer to Table 2 for the numbers of peptides. V_{max} values are for 1 nM trypsin. The data represent average ±SE of two separate experiments.

peptides #1, #28, #31 and #37, whereas prostate-specific antigen (PSA) was not inhibited (results not shown).

Cleavage of the peptides by trypsin-3

The stability of selected peptides (peptides #23, #25, #28, #29 and #31) was determined with trypsin-3. Most of the tested peptides were found to be significantly cleaved by trypsin-3 after Arg or, in case of peptide #25, after Lys. In peptide #31, which has C-terminal amidated Arg, the C-terminal amide was converted to the carboxylic acid. The most stable peptide was #23, of which less than 50% was cleaved during 1 h incubation with trypsin-3. The degradation rate of peptides correlated to their inhibitory efficacy, the most stable peptides being the most effective inhibitors.

Binding of peptides to trypsin-1 and -3

Peptides #1 and #12 were incubated with trypsin-1 and -3, followed by the separation of the free peptides from the trypsin-bound peptides by a centrifugal device and analysis with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Intact peptide #1 had [M+H]+ of 1295.03, whereas the cleaved peptide was 18 Da larger (1313.03), indicating that the fragments are still connected by a disulfide bridge. The intact and cleaved forms of peptide #12 had [M+H]+ of 1117.98 and 932, respectively. Almost all (>85%, n=2 for both trypsins) of the trypsin-1 and -3 bound cyclic peptide #1 was intact, whereas in the flow through the cleaved peptide was 4.8±1.0-fold (mean±SE, n=4) more abundant than in trypsin-containing fraction. Similar results were obtained with linear peptide #12, but the differences were not as dramatic as those with peptide #1 (59±14% and >90% of the peptide bound to trypsin-1 and -3, respectively, was intact, whereas cleaved peptide was 2.4±0.3-fold more abundant in flow through than in trypsin-containing fraction).

Molecular modeling of the binding of peptides to trypsin-3

Molecular dynamics (MD) simulation based binding conformations of trypsin-3 complexed with cyclic peptide #1, and its linear variant peptide #12 is shown in Figure 4. Binding of the first eight residues of both peptides, i.e., from the N-

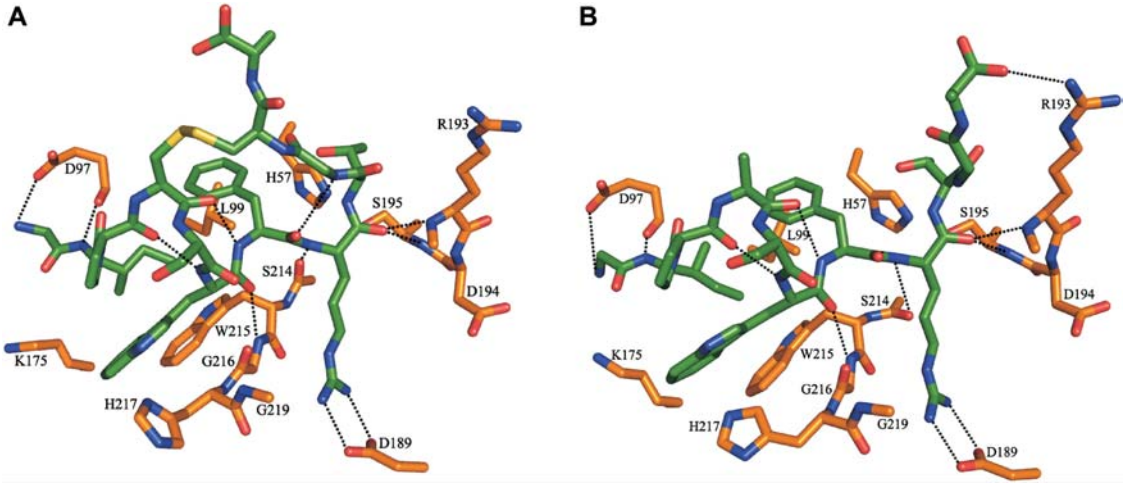

Figure 4 Binding of cyclic peptide #1 (A) and linear peptide #12 (B) to trypsin-3.
Hydrogen bonds are shown by dashed lines. Note the extra hydrogen bond formed between the N-terminal carboxylate group of the linear peptide (B) and Arg193. Structures are averages from the last 0.5 ns of the trypsin-peptide MD simulations. Peptides are shown in green and trypsin-3 in orange. Heteroatom colors: nitrogen=blue, oxygen=red and sulfur=yellow. For peptide numbers, refer to Table 2.

terminus to arginine, is highly similar. The N-terminal part of the peptides, and arginine and two residues preceding it make hydrogen bonds with trypsin-3. Between these two sites of specific contacts there is an extensive hydrophobic patch of interactions, which presumably is important for the stability of the trypsin-peptide complexes. The cyclic peptide #1 contains two type I β-turns. In the linear variant (#12) the first turn is similar to that of the cyclic peptide, but owing to the lack of a disulfide bond, the C-terminus is able to adopt a less strained conformation. In this conformation an ionic bond is formed between the C-terminal carboxylate group of the peptide and the guanidium group of Arg193.

The N-terminus of the peptides is firmly bound to trypsin by an ionic bond between the positively charged amino group and the carboxylate group of Asp97 and a hydrogen bond between the NH group of peptides Ile-residue and the backbone carbonyl group of Asp97. The side chain of Ile is located in the middle of a hydrophobic cluster of side chains and, therefore, its importance for inhibition is obvious. Ile makes hydrophobic interactions with Leu99 and Trp215 of trypsin-3, and Trp and Phe of the peptides. The hydrophobic patch extends to the contacts made by Trp and Phe: Trp makes additional contacts with Trp215 and His217 and is stacked with Pro of the peptides, whereas Phe makes extensive interactions with Leu99.

Close to the catalytic site of trypsin, the contacts formed between trypsin-3 and the peptides are similar to contacts known to exist between trypsin and its canonical protein inhibitors or substrate peptides (Krowarsch et al., 2003). Arg, which was found in all peptides showing notable inhibitory potency, binds to the specificity pocket of trypsin-3 and makes an ionic bond to the carboxylate group of Asp189. The carbonyl group of Arg makes a short hydrogen bond with the NH group of Arg193, but the distance to the other NH group of the oxyanion hole of trypsin is somewhat longer (∼4 Å). In the predicted conformations Trp and Arg of the peptides make backbone-backbone interactions with Ser214 and Trp21 of trypsin-3.

Sequence similarity

Among human proteins found in the Swiss-Prot database, only obscurin-like protein 1 and endoplasmic reticulum-Golgi intermediate compartment protein 1 were found to harbor the sequence found to be important for the activity of TRY3-X10-1 peptide, i.e., Ile, Pro, Xaa, Xaa, Trp, Phe, Arg, in which Xaa denotes any amino acid. None of the original peptides identified by phage display (Table 1) showed identity with known proteins in more than five consecutive amino acids.

Discussion

Increased proteolytic activity is associated with several diseases including cancer, in which proteases are thought to play a major role in invasion and formation of metastases (Koblinski et al., 2000; Overall and Kleifeld, 2006b). Thus, proteases are potential drug targets. Although traditional small molecule drugs have several advantages over peptides and proteins, their specificity for modulation of the activity of highly similar proteases could be a problem, which might cause undesired side effects (Overall and Kleifeld, 2006a; Turk, 2006). To overcome this, we have used phage display technology to develop peptides that bind to trypsin and inhibit its activity.

Seven trypsin-3 binding phage, presenting different peptides on their surface, were found. All the peptide sequences identified are likely to be cyclic as they contained two or more Cys-residues. Four of the identified peptides contained four or six Cys-residues, suggesting that these peptides formed bicyclic or tricyclic structures, respectively. Library X10, which is not 'cysteine engineered', also produced a peptide with two cysteines, suggesting that a cyclic conformation is preferable for trypsin-binding. However, synthetic linear variant of the X10-1 peptide retained bioactivity, indicating that, although a cyclic-conformation might be preferable for screening conditions, it is not necessary for trypsin inhibition. The identified peptides contained characteristic consensus amino acids, i.e., most contained Arg, preceded by Ala and Trp, and Pro close to N-terminus. Trypsin cleaves proteins after Arg and Lys (Halfon et al., 2004; Gosalia et al., 2005) (http://merops.sanger.ac.uk/), and trypsin was found to cleave the linear and cyclic (peptide #1, not shown) synthetic analogs of the peptides after Arg and Lys residues with variable efficiency. In peptide #31, trypsin converted the C-terminal amide of arginine to a carboxylic acid. As it is possible that the cleaved peptides still bind to trypsin, we tested whether intact or cleaved forms of cyclic peptides #1 and #12 are the main trypsin-1 and -3 bound forms. Mostly intact peptides were found to be bound to trypsin. The binding of the intact peptides is also supported by the experiment showing that the most inhibitory peptides are also most stable ones. Furthermore, we purified fragmented peptide #1 and found that it had lost most of its inhibitory activity (not shown). However, it is possible that the conformation of the peptide, in which the fragments are held together with disulfide bridges, is different after purification from the peptide that is cleaved and retained in active site of the protease. Peptides #29 and #31, which trypsin converts identical to peptide #30, inhibit trypsins with similar activity as peptide #30. This suggests that the cleavage products of some of the peptides are able to inhibit trypsin activity, and thus bind to trypsin.

Replacement of individual amino acids of the TRY3-X10-1-derived peptide by Ala suggest that, in addition to Arg, Trp and Pro, also Ile and Phe are crucial for the activity. The same amino acids were found to be important for complex formation in modeled trypsin-3/peptide complexes, confirming the importance of these residues for the activity of TRY3-X10-1-derived peptides (Figure 4). It is somewhat surprising that Phe could not be changed to Ala without greatly decreasing the bioactivity, as most of the other identified peptides have an Ala in that position. Phe was predicted to be important for the stability of the trypsin-peptide

complex as it makes extensive contacts with Leu99 of trypsin-3 and is part of the hydrophobic patch of the N-terminal residues Trp, Pro and Ile (Figure 4). Thus, Phe is in a special position in TRY3-X10-1-derived peptides and could be one of the reasons explaining the higher activity of this series of peptides compared with the other ones. Structural study of trypsin-3 (Katona et al., 2002) has suggested that the charge and size of the amino acid in P2′ position is important for substrate specificity. We made TRY3-X10-1 variants in which we replaced Gly in this position (Arg7 is in P1 position) by Phe, Ser or Asp, but did not find any big effect of these on the activity. Amino acid changes in the C-terminal side of the R residue do not greatly affect the activity of the peptides (peptide #1 vs. #8–10, #21 vs. #22, and #23 vs. #24), which is also supported by the modeling showing that these amino acids are not crucial for interaction with trypsin-3. This is not surprising as some of the cleavage products itself seem to be inhibitory, as noted above.

Most of the peptides were found to be fairly selective inhibitors of trypsin-1 and -3, inhibiting both with similar efficiency. The best inhibitors showed significant inhibition at submicromolar concentrations. Trypsin-2 and -C were also inhibited, but only at over 10-fold higher concentrations. Significantly, one of the peptides (#37) that inhibited trypsin-1, showed no effect at all on trypsin-3. This could result from the difference in active sites of these trypsin-isoenzymes (Nyaruhucha et al., 1997; Katona et al., 2002; Szmola et al., 2003); in particular, the presence Arg193, instead of Gly193 in trypsin-1, in close vicinity to the Ser of the active site in trypsin-3 might prevent binding of larger peptides. The presence of Arg193 explains the inability of trypsin-3 to react with polypeptide inhibitors and to hydrolyze some substrates (Nyaruhucha et al., 1997; Szmola et al., 2003). Although small molecule inhibitors are equally effective inhibitors for different trypsin-isoforms, trypsin-3 is not inhibited by several polypeptide inhibitors that inhibit trypsin-1 and -2, including soybean trypsin inhibitor and pancreatic secretory trypsin inhibitor (also called tumor associated trypsin inhibitor, TATI or SPINK1) (Sahin-Toth, 2005). However, these differences do not explain the isoform-selectivity of our peptides as most of them inhibited trypsin-1 and -3 efficiently, but not trypsin-2. The explanation for this selectivity remains to be studied.

Several of the peptides having Phe before Arg inhibited plasma kallikrein, a trypsin-like enzyme cleaving after arginine and lysine bonds. Not surprisingly, Phe is frequently observed in this position in plasma kallikrein substrates (Gosalia et al., 2005). However, other trypsin-like proteases, i.e., KLK2 and plasmin, were only weakly, if at all, inhibited by the tested peptides. Chymotrypsin was weakly inhibited by some of the peptides, whereas PSA, which has chymotrypsin-like activity, was not inhibited. This could be explained by the restricted chymotrypsin-like activity of PSA (Coombs et al., 1998).

Although peptides inhibiting trypsin from bovine pancreas or from undefined sources have been found previously by phage display or by substrate analogy (Eichler and Houghten, 1993; Fang et al., 1996), these do not share sequence similarity with our peptides, and to our knowledge, peptides showing isoenzyme-selective inhibition have not been described previously. Furthermore, the sequences of the inhibitors described here are unique, although some similarity with several human proteins was found.

In conclusion, we have identified novel peptides that inhibit the activity of trypsin-1 and -3. A synthetic peptide variant significantly inhibiting only trypsin-1 was also found. These peptides could be useful for the establishment of assays measuring active trypsins similar to those developed for PSA (Wu et al., 2004; Zhu et al., 2006). Such assays have not so far been established using antibodies. Specific trypsin isoenzyme assays might be useful for diagnosis of cancer, certain neurological and pancreatic diseases, and other diseases/disorders associated with increased trypsin activity. These peptides might also be useful leads for development of imaging agents and treatment of such diseases.

Materials and methods

Materials

Monoclonal anti-trypsin antibody (MAb 6D11; Itkonen et al., 1990) and recombinant trypsinogen-1, -2, -C and -3 were produced as described previously (Koistinen et al., 2009). For quantification of the concentrations of active trypsins, active sites were titrated using 4-nitrophenyl 4-guanidinobenzoate hydrochloride (*p*-NPGB, Sigma, St. Louis, MO, USA) as described previously (Chase and Shaw, 1970; Koistinen et al., 2009). The activity towards S-2222 was determined from the same preparations, which allowed calculation of concentrations of active trypsins in each experiment based on the activity towards S-2222. Turnover number (k_{cat}, i.e., moles of reacted substrate/mol of active trypsin/second) was 23.5 s^{-1} for trypsin-1 (Koistinen et al., 2009), 32.7 s^{-1} for trypsin-2, 51.7 s^{-1} for trypsin-C and 70.5 s^{-1} for trypsin-3 (in keeping with this, the k_{cat} value for trypsin-3 has previously been reported to be around 3-fold that of trypsin-1 and -2; Szmola et al., 2003). Enterokinase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Other proteases used were kallikrein-related peptidase 2 (KLK2, also known as human kallikrein 2, hK2) (Lovgren et al., 1999), prostate specific antigen (PSA or KLK3, isoform-B) (Mattsson et al., 2008), bovine chymotrypsin (Boehringer Mannheim, Mannheim, Germany), human plasmin and plasma kallikrein (both from Sigma). Chromogenic substrates S-2586, S-2222, S-2302, S-2403 and S-2266 were from Chromogenix Instrumentation Laboratory (Milan, Italy).

Selection of phage peptides

The construction of phage display peptide libraries in fuse 5-phage has been described previously (Koivunen et al., 1994). In the present study, we used libraries with structures X7, X10, CX8C, CX10C, CX3CX4CX2C and CX3CX3CX3C, where C is cysteine and X is any of the 20 naturally occurring amino acids. The screening of phage libraries was performed essentially as described previously (Wu et al., 2000). In total, 100 ng of recombinant trypsinogen-3 was captured by MAb 6D11 attached onto microtiter wells, and incubated for 60 min at 22°C in 100 μl of TBS (0.05 M Tris buffer, pH 7.7, 0.154 M NaCl), containing 1 g/l bovine serum albumin

(BSA). The wells were washed to remove unbound trypsinogen and 300 ng of enterokinase in 100 μl of TBS was added for 90 min to activate trypsinogen. After a washing step, an aliquot of each phage library containing 10^{10}–10^{11} transducing units was added to the wells in 100 μl TBS containing 1 g/l BSA, and incubated for 3 h at 22°C with gentle shaking in the first round and for 1 h in the subsequent rounds. The wells were washed ten times with TBS containing 0.5% Tween 20. The bound phage were eluted by adding 100 μl of 0.1 M glycine-HCl, pH 2.2, containing 1 g/l BSA. The eluted phage were neutralized with 1 M Tris buffer, pH 9.0, and amplified by infection of *Escherichia coli* K91 cells and purified by polyethylene glycol precipitation. After three rounds of selection and amplification, single-stranded DNA from individual phage clones was prepared and the peptide sequences were determined by sequencing the relevant part of the viral DNA. The sequencing was performed with an ABI 310 Genetic analyzer and Dye Terminator Cycle Sequencing core kit (PE Applied Biosystems, Foster City, CA, USA) using the oligonucleotide 5′-CCC TCA TAG TTA AGC GTA ACG-3′ as a primer.

Phage binding assay

The binding of individual phage clones to trypsin-3 was tested by an immunofluorometric assay (IFMA) essentially as described previously (Wu et al., 2000). In the phage IFMA, 10 ng trypsinogen-3 was incubated for 1 h in microtiter wells coated with MAb 6D11. After washing with 150 mM NaCl, containing 7.7 mM NaN_3 and 0.2 g/l Tween 20, 300 ng of enterokinase in 200 μl of TBS was added for 90 min. For controls, addition of trypsinogen or enterokinase was omitted. After washing, 10 μl of phage (approximately 10^8–10^9 infectious particles) and 200 μl assay buffer (PerkinElmer Wallac, Turku, Finland) was added to the wells and incubated for 1 h. The wells were washed and filled with 200 μl of assay buffer containing 50 ng europium-labeled anti-phage polyclonal antibody (Abcam, Cambridge, UK). After incubation for 60 min, the wells were washed four times and enhancement solution (PerkinElmer Wallac) was added. The fluorescence was quantified with Victor^2 multilabel counter (PerkinElmer Wallac).

Construction of trypsin-binding peptide fusion proteins and immunopeptidometric assay

Production of trypsin-binding peptides as GST fusion proteins was performed as previously described (Wu et al., 2000). Briefly, single-stranded phage DNA was purified and the insert region was amplified by PCR (Rajotte et al., 1998). The amplified DNA was isolated and subcloned between the BamHI and EcoRI sites of the PGEX-2TK vector (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to express the selected peptides as GST fusion proteins. Recombinants were verified by DNA sequencing. The fusion proteins were expressed in *E. coli* BL21 cells and purified by glutathione affinity chromatography (GE Healthcare Bio-Sciences) as described previously (Smith and Johnson, 1988). The purity of the fusion proteins and the release of correctly sized peptides by thrombin cleavage were analyzed by SDS-polyacrylamide gel electrophoresis in 12.5% gels on the PhastSystem (GE Healthcare Bio-Sciences), followed by silver staining of the gel. The binding of the GST-peptide fusion proteins (GST-peptides) to trypsin was measured by an immunopeptidometric assay (IPMA) essentially as described previously for PSA, except MAb 6D11 was used as a capture antibody (Wu et al., 2000, 2004). Briefly, trypsin-isoforms were captured on microtiter wells and activated as described for the phage binding assay. Different amounts of GST-peptides were added to the wells and their binding was quantified by using a europium-labeled anti-GST antibody. The effects of GST-peptides (4 μM) on enzymatic activity of trypsins was also determined as described below.

Peptide synthesis

Peptides were synthesized using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY, USA) with Fmoc strategy, TBTU/DIPEA as coupling reagent. Fmoc-Gly-Wang, Fmoc-Ala-Wang and Rink resins were used as solid phase (Novabiochem, Läufelfingen, Switzerland). The side chain protecting groups used in the synthesis were t-Butyloxycarbonyl (Boc) for Trp and Lys, tert-Butyl (tBu) for Ser and Thr, acetamidomethyl (Acm) for Cys, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. Peptides were cleaved from the solid support with 95% trifluoroacetic acid (TFA), 3% ethane dithiol (EDT), 1% triisopropylsilane (TIS) and 1% H_2O. During cleavage from the resin the Acm-protection group remains in the Cys side chain. EDT and TIS were used as scavengers.

The peptides were purified by HPLC (Shimadzu, Kioto, Japan) on a C_{18} reversed phase column (xTERRA, Waters, Milford, MA, USA) using a 0–90% acetonitrile (ACN) gradient in 0.1% TFA for elution. Peptide purity was verified with an ABI QStar LC-ESI mass spectrometer (Applied Biosystems) and by analytical HPLC on a 240×1.4 mm C18 column (xTERRA, Waters) with a 5–90% ACN gradient for 30 min. Some peptides were acetylated on the resin by treatment of free amine groups with 20% acetic anhydride in dimethyl formamide (DMF) twice for 10 min before cleavage.

Cyclization of the peptides

Peptides containing cysteines (Acm) were cyclized by the iodination method. Lyophilized peptides were dissolved in 50% acetic acid (AcOH) in H_2O at a concentration of 2 mg/ml. Then, 1 M HCl (0.1 ml/mg of peptide) was immediately added, after which 0.1 M iodine solution in 50% AcOH in H_2O (5 eq./Acm) was added. The solution was stirred vigorously at room temperature for 40 min and the reaction was stopped with 0.1 M sodium thiosulfate. After filtering (0.45 μm) peptides were purified by HPLC as described above. The cyclization of the peptides was verified by analytical HPLC and mass spectrometry.

Effect of peptides on the enzymatic activity of different trypsin isoforms

The inhibitory effect of the peptides and GST-peptides on trypsin-1, -2, -C and -3 was tested by using the chromogenic substrate S-2222. Before inhibition tests, trypsinogens were activated for 30 min at room temperature using 0.75 μg enterokinase (Roche) per 15 μg of trypsinogen in 200 μl 50 mM Tris, pH 8.0, containing 150 mM NaCl and 10 mM $CaCl_2$. For initial screening, active trypsins (5.0±0.6 nM trypsin-1, 2.8±0.3 nM trypsin-2, 2.1±0.4 nM trypsin-C and 1.8±0.2 nM trypsin-3, mean±SE) were incubated with 1 μM peptides or 4 μM GST-peptides for 60 min at room temperature in 100 μl 50 mM Tris, pH 8.0, containing 0.1% BSA. Finally, substrate S-2222 was added to a final concentration of 0.1–0.2 mM and absorbance measured at 405 nm every 1–5 min for 15–30 min.

For determination of IC_{50} values (the concentration of peptide inhibiting trypsin activity by 50%), peptides, at concentrations ranging from 0.25 to 20 μM, were preincubated for 15 min with trypsins (2.1, 1.7, 1.5 and 1.4 nM active trypsin-1, -2, -C and -3, respectively) before addition of substrate. The effect of peptides on the Michaelis-Menten constant (K_m) and the maximal reaction rate (V_{max}) was

studied using 1 and 10 μM peptides, 1.25 and 2.2 nM trypsin-1 or 1.6 and 1.85 nM trypsin-3, and 100–750 μM S-2222. Here, no preincubation of trypsins with peptides was used. K_m and V_{max} values were determined from Hanes-Woolf plots.

For degradation of α-casein, trypsin-1 (17 nM final concentration) was preincubated 15 min with or without peptides (154 μM) before addition of 0.33 mg/ml α-casein (Sigma). After incubation for 3 h at 37°C, fragmentation was analyzed by reducing SDS-PAGE and silver staining of the gel.

Inhibition of other proteases

The specificity of selected peptides (10 μM) was studied by measuring their effect on PSA (4 μM), plasmin (20 nM), plasma kallikrein (20 nM), KLK2 (193 nM) and chymotrypsin (10 nM) using the chromogenic substrates S-2586 for PSA and chymotrypsin, S-2403 for plasmin, S-2266 for plasma kallikrein and S-2302 for KLK2 (all at 0.2 mM final concentration, except S-2403 which was 0.4 mM). The activity of PSA, chymotrypsin and KLK2 was measured in Tris buffer, pH 7.7, containing 0.1% BSA and 9 g/l NaCl. For plasmin and plasma kallikrein, NaCl was omitted and pH values used were 7.4 and 9.0, respectively.

Degradation of peptides by trypsin

Selected peptides (0.2–0.25 mM) were incubated with trypsin-3 (0.3 μM) in 200 μl of 200 mM NH_4CO_3, pH 8, at 37°C for 60 min. Samples of 30 μl were taken every 15 min. Enzyme reaction was stopped by acidification of the solution with 30 μl of 2% ACN, 0.5% TFA in H_2O. Samples were analyzed by HPLC and mass spectrometry using the same protocol as for the intact peptides above. Degradation was evaluated by the formula: degradation %=[degraded/(degraded+nondegraded)]×100%, using peak heights for calculation of concentrations.

Binding of peptides to trypsin-1 and -3

Peptides #1 and #12 (8 μM) were incubated with trypsin-1 and -3 (0.036 μM of active trypsin-1 and 0.014 μM active trypsin-3) at room temperature for 30 min in TBS containing 1 g/l BSA. Duplicate samples were used. After incubation, the solution was transferred to a Nanosep Centrifugal Device (10 kDa molecular mass cut-off value; Pall Corporation, Port Washington, NY, USA) and centrifuged at 14 000 *g* for 10 min. The filter was washed three times with 100 μl of TBS by centrifugation as above. The bound peptide was eluted from trypsin with 10 μl of 50% ACN in H_2O. The peptides were analyzed with an ABI QStar mass spectrometer equipped with an orthogonal MALDI ionsource (Applied Biosystems), using α-cyano-4-hydroxycinnamic acid as matrix. Peak heights were used for semiquantitative estimation of relative abundance of fragmented and intact peptides.

Molecular modeling

Binding of cyclic peptide #1 and its linear variant peptide #12 to trypsin-3 was predicted using molecular modeling and computer simulation techniques. The initial coordinates for trypsin-3 were obtained from the X-ray structure of the trypsin-3/benzamidine complex (PDB code 1H4W; Katona et al., 2002) determined to 1.7 Å resolution. Crystallographic water molecules of the X-ray structure were included in the simulation systems. Docking of the peptides was done stepwise starting with a tetrapeptide WFRT, assuming that the arginine residue of the peptide binds to the specificity pocket of trypsin. The tetrapeptide was docked to the catalytic site using the coordinates of the cyclic peptide inhibitor of bovine trypsin-1/inhibitor complex (PDB code 1OX1, determined to 2 Å resolution). In this complex, the backbone of the tetrapeptide sequence CTRS is bound in a highly similar way compared with the corresponding residues of BPTI in the trypsin-BPTI complex (PDB code 3TGI). After docking, MD simulation of 0.5 ns was started to relax the trypsin-3/tetrapeptide complex. To keep the peptide in the correct position during the simulation, harmonic constraints (force constant=5 kcal/mol, when the O to N distance was <3.0 Å or >3.3 Å) were applied to maintain the four backbone hydrogen bonds between the tetrapeptide and the protein. Then, the rest of the residues were added to peptide #1 by keeping the geometry (average structure from the last 0.2 ns of simulation) obtained from the MD simulation unchanged. The added residues were built using the Maestro program (version 8.0, Schrödinger, LLC, New York, NY, USA) and geometries optimized using AMBER force field (Ponder and Case, 2003). Then, peptide #1 was docked to trypsin-3 by fitting the backbone atoms of peptide #1 to the corresponding atoms of the simulated tetrapeptide. MD simulation of 2.0 ns was started for this initial trypsin-3/peptide #1 complex. The end structure of the simulation was used as a starting structure for two parallel simulations (different equilibration simulation lengths) using the locally enhanced sampling (LES) method to efficiently sample the conformational space of peptide #1 and to dock the peptide to trypsin-3. The LES method is a mean-field technique providing ability to focus on the interesting part of the system (Simmerling and Elber, 1995). In practice, multiple copies are employed on the part of the system where conformational sampling can be critical and the rest of the system is treated as a single structure. The LES method can sample conformational space by an order of magnitude more efficiently than ordinary MD. In LES calculations (1.5 ns) of trypsin-3/peptide #1 complex, the peptide was represented as seven copies. LES simulations resulted in five distinct conformations for trypsin-bound peptide #1. For each of these conformations, MD simulations of 14.0 ns were started to properly dock the peptide to trypsin-3. Inspection of the simulation results revealed that in two of the cases the peptide did not stay in contact with the protein. To quantitatively evaluate the relative binding affinities of the three other bound conformations, Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) free energy calculations (Kollman et al., 2000) were done using the single-trajectory approach implemented in AMBER. For MM/PBSA calculations, 100 snapshot structures were collected from the last 1.0 ns of the 14.0 ns simulations. The conformation of peptide #1, having the most favorable MM/PBSA binding free energy (-82.6 kcal/mol vs. -73.9 kcal/mol and -71.1 kcal/mol), was used as a peptide template for initial trypsin-3/peptide #12 simulation. A production MD simulation of 4.0 ns was done for the trypsin-3/peptide #12 complex. The average structure of the last 0.5 ns of the 14 ns simulation of the energetically most favorable peptide #1 conformation and the 4.0 ns peptide #12 simulation were generated for structural analyses.

For MD simulations, the protein-ligand complexes were solvated by TIP3P water molecules (approximately 5500) in a truncated octahedral periodic box with dimensions of 64×64×64 Å. The water molecules of the complexes were first energy-minimized for 1000 steps, heated to 300 K in 15 ps and equilibrated for 60 ps at a constant temperature of 300 K and pressure of 1 atm. Then, the whole simulation system was minimized for 1000 steps, the temperature of the system was increased to 300 K in 15 ps and equilibrated for 30 ps. The production simulations of various lengths were then started. In the simulations, the electrostatics were treated using the particle mesh Ewald method. A time step of 1.5 fs was

used and bonds involving hydrogen atoms were constrained to their equilibrium lengths using the SHAKE algorithm (Ryckaert et al., 1977). From the production simulations, structures were saved every 0.75 ps for analyses, which were done using the ptraj program of the AMBER9.0 simulation package (Case et al., 2005). The MD simulations were done using SANDER and PMEMD programs of the AMBER package. In all the simulations, f99 force-field (Cornell et al., 1995; Wang et al., 2000) augmented with backbone corrections by Hornak et al. (2006) was used.

Sequence similarity searches

Proteins harboring similar sequences as the peptides identified in this paper were searched using BLASTP 2.2.20+ (http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1997). The search was restricted to human proteins in the Swiss-Prot database.

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