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Substrate specificity and inhibitory study of human airway trypsin-like protease

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ABSTRACT

Human airway trypsin-like protease (HAT), also referred to as TMPRSS11D, is an important physiological enzyme with the main activity pronounced in an airway. In this work we have described the substrate specificity and selectivity study of the protease, performed by the combinatorial approach. Fluorogenic/chromogenic tetrapeptide library was used for this purpose. The most efficiently hydrolyzed substrates' sequences that we selected were ABZ-Arg-Gln-Asp-Arg(Lys)-ANB-NH_2. The most active inhibitor with C-terminal Arg residue underwent detectable proteolysis action in the presence of 35 pM of HAT. Based on the selected sequences the two peptide aldehydes were synthesized and (Abz-Arg-Gln-Asp-Arg(Lys)-H) were found to be an effective HAT inhibitor, working in nanomolar range with inhibition constant 54 nM and 112 nM, respectively.

1. Introduction

Human airway trypsin-like protease, also referred as TMPRSS 11D, was first identified by the Yasuoka et al. in fluid secreted in human airways (trachea and bronchi).[1] This proteinase displays trypsin-like activity and belongs to the new family of proteolytical enzymes associated with cell membrane called type II transmembrane serine protease (TTSP). Later the same group confirmed that this proteinase has human origin by finding its gene sequence and cloning it.[2] Within the molecule of HAT there are two segments identified. One is responsible for its function as serine proteinase, whereas second for anchoring the enzyme into the cell membrane. Amino acid sequence of catalytical region of this enzyme reveals high structural homology with other members of human serine protease such as mast cell tryptase,[3] hepsin[4] or acrosin.[5] The further studies revealed that this protease is involved in several important physiological functions such as fibrinogen processing that yields the complete loss of its clotting capacity[6] and stimulates proliferation of human bronchial fibroblasts.[7] It is also engaged in the protease-activated receptor 2[8] and the proteolytic shaving of urokinase receptor.[9]

The elevated levels of this enzyme are observed in patient with chronic skin and airway disease like psoriasis vulgaris,[10] as well as lower respiratory track inflammation and is responsible for proteolytical activation of hemaglutinin antigen of influenza virus.[11]

However, to the best author's knowledge so far there has not been published full substrate specificity study of this enzyme. Only primary specificity has been identified.[1] HAT is found to hydrolyze the Arg containing peptide substrates and is inhibited by soybean trypsin inhibitor and aprotinin but hardly any inhibition is observed for secretory leukocyte protease inhibitor. Also the Yasuoka group proposed the fluorogenic coumarin based (initially design for trypsin) substrate to follow up the HAT activity. However, in their study[1] the investigated substrates sequence did not include all amino acid residues but was limited to several ones.

To fulfill this gap, the tetrapetide library presumably containing fluorogenic/chromogenic substrates of HAT with general formula given below was synthesized using the split and mix method:

ABZ-X_4-X_3-X_2-X_1-ANB-NH_2

where ABZ—2-aminobenzoic acid; in position X_4–X_2 the set of proteinogenic amino acid except Cys; X_1 basic amino acid residues (Lys or Arg); ANB-NH_2—amide of 5-amino-2-nitrobenzoic acid.

ABZ moiety serves as donor of fluorescence since (ANB-NH_2) acts as a quencher, whose presence in a single peptide chain results in fluorescence resonance energy transfer called FRET. Upon enzymatic hydrolysis of peptide bond (when the enzyme displays affinity towards amino acid sequence) the distance between donor and quencher increases and the boost of the fluorescence is observed. An alternative method could be a monitoring of the increase of absorbency resulting from the release of chromophore (ANB-NH_2) at 405 nm.

Moreover, based on the amino acid sequence of the most active substrates selected by the combinatorial approach, two peptide

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aldehydes were synthesized and their inhibitory potency against HAT was determined.

2. Results and discussion

The deconvolution of the library was performed in solution using iterative approach as it is shown in Figure 1. The most rapidly hydrolyzed sublibraries with defined substrate P_4 position corresponding to X_4 in the synthesized library contained basic Arg residue, followed by hydrophobic Tyr, Val and Phe. No significant hydrolysis was observed for sublibraries with acidic residues Glu, Asp and its amides Asn and Gln. Also Pro strongly affects proteolysis of the peptides investigated by the experimental enzyme. For the next deconvolution step, Arg was fixed in position P_4.

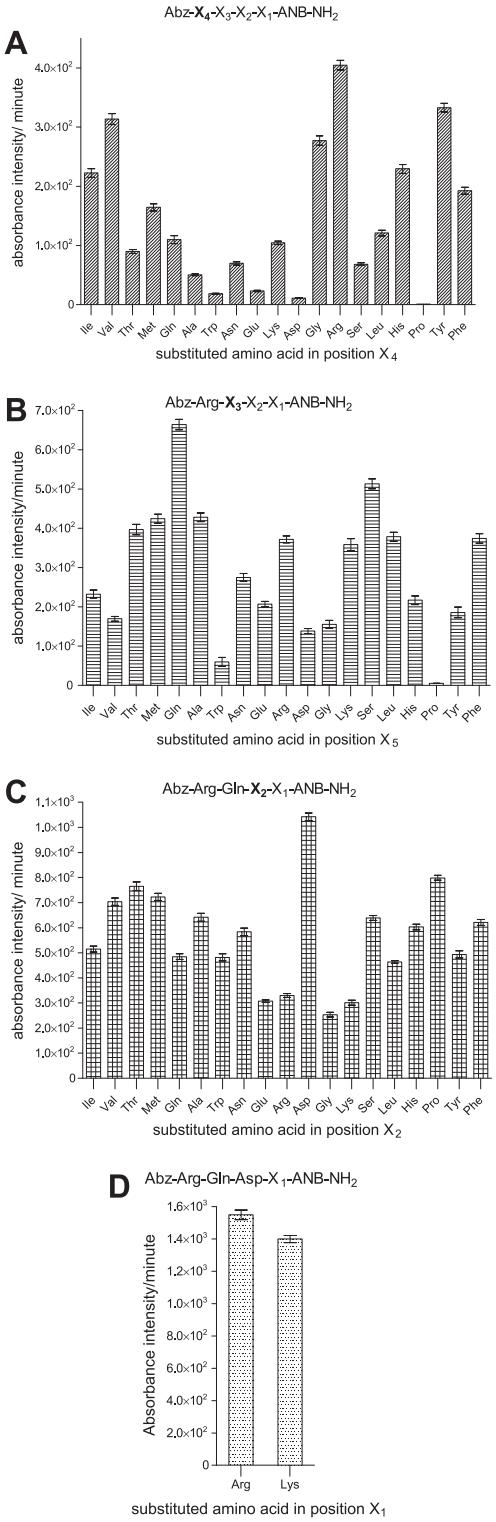

Figure 1. Deconvolution of tetrapeptide library against HAT.

In position P_3 outstanding fluorescence liberation was observed for sublibrary containing Gln. It dominated over all other amino acid residues in discussed position. In this position polar charged Arg, Lys and uncharged Thr, Ser and hydrophobic Ala, Leu and Phe amino acid residues can be also accommodated in enzyme substrate subside. Libraries with Pro and Trp were practically inactive.

In position P_2 the highest proteolytic susceptibility was detected for sublibrary with Asp residue. It is worth emphasizing that Glu residue in discussed position yielded peptides with low HAT substrate activity. Substantially, significantly lower activity was observed for the sublibrary that contained Pro, Val and Met. Sublibraries with basic residues (Arg, Lys) were among the less efficient. This is a surprising result, since only few proteolytic enzymes prefer acidic residues in this position. According to the best author's knowledge, only one serine protease—human enteropeptidase is likely to prefer the Asp in this position. This residue has been identified as a highly deleterious to catalysis for trypsin and other trypsin-like proteinases like tissue plasminogen activator.[12]

In position P_1 as expected, since the enzyme is called trypsin-like, the basic residues Arg and Lys were hydrolyzed almost equally, with a 15% preference of peptide with Arg in position P_1.

The study performed by Beliveau et al.[13] on specificity of a few members of the type II transmembrane serine proteases (matriptase I, II hepsin and DESCI) using similar approach (FRET peptide set based on fragments of natural protein substrates) led to the discovery of a sequence ABZ-Arg-Gln-Ala-Arg-XXX, which is homologous to one obtained by our group. It could indicate that studied proteases and HAT share a partial specificity and are able to hydrolyze the same protein substrate set. However, when Ala in position P_2 is replaced by negatively charged Glu, obtained peptide is hardly hydrolyzed by any of the enzymes studied by French group.

Finally, the peptides ABZ-Arg-Gln-Asp-Arg-ANB-NH_2 (**1**) and ABZ-Arg-Gln-Asp-Lys-ANB-NH_2 (**2**) have been selected for further studies. It is also worth underlining that Arg-Gln-Asp-Arg is a unique sequence. Among proteins found using NCBI Blast, this tetrapeptide was identified in amino acid sequence of human fibronectin segment (1371–1374) called cell binding domain.[14] Fibrinogen is a large, adhesive glycoprotein which is found in a number of locations, most notably on cell surfaces, in extracellular matrixes, and in blood.[15] This could suggest that HAT is taking part in fibronectin processing.

Since in the selected sequence there are two basic residues, it can be assumed that two potential sides of cleavage are present in obtained peptides. However, the ANB-NH_2 release indicates that the first peptide bond that undergoes proteolysis is located at the C-terminus. The LC–MS experiments have been performed to confirm our expectations. The obtained results shown in Figure 2 clearly indicate that upon incubation of peptide **1** with HAT, ANB-NH_2 is released and the remaining fragment ABZ-Arg-Gln-Asp-Arg-OH is further cleaved into the ABZ-Arg-OH and H-Gln-Asp-Arg-OH fragments.

Kinetic parameters determined for the two selected peptides are listed in Table 1. The obtained values were compared to the substrate Boc-Phe-Ser-Arg-4-methylcoumaryl-7-amide, routinely used for kinetic assay for bovine β-trypsin and tryptase.[16] Determined values of specificity constant (k_{cat}/K_M) for all three FRET peptides indicate that peptides **1** and **2** display eight and fivefold, respectively, higher substrate HAT activity than the aforementioned reference coumaryl substrate (**3**). It should be emphasized

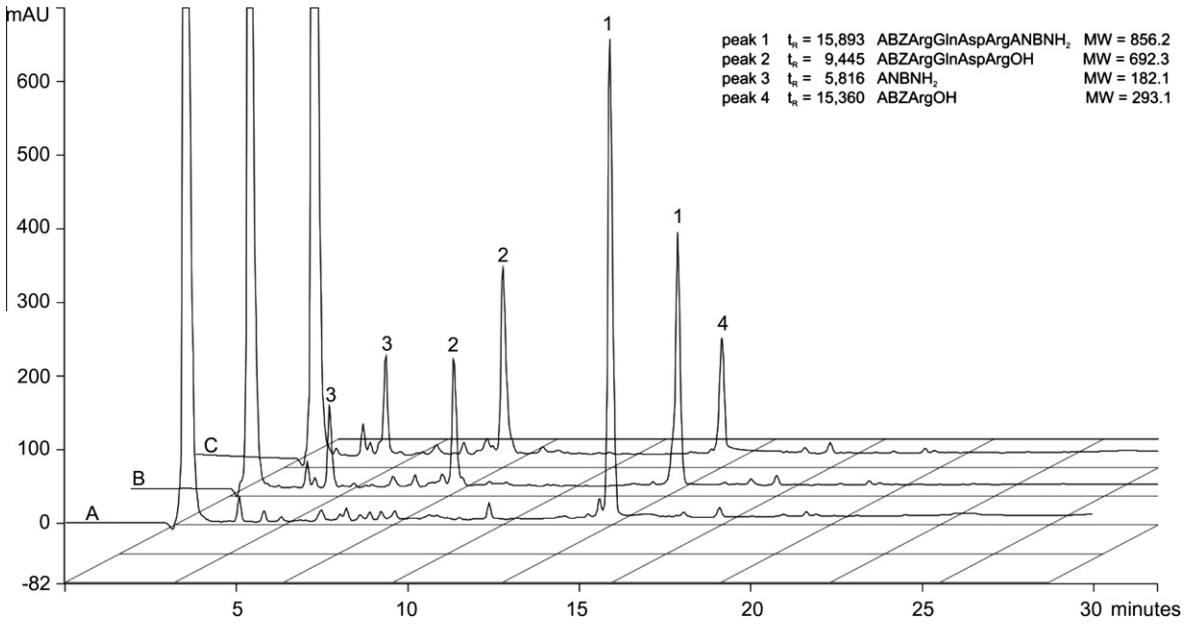

Figure 2. Proteolytic digestion pattern for substrate ABZ-Arg-Gln-Asp-Arg-ANB-NH$_2$ (**1**). After 1 min (A), 15 min (B), and 1 h (C) incubation with human HAT. Within first 15 min the single cleavage site is observed, and the resulting fragments were identified by MS (MALDI-TOF). Peak 1 corresponds to intact peptide (MW = 856.2), peak 2 corresponds to ABZ-Arg-Gln-Asp-Arg-OH (MW = 692.3) fragment, and peak 3 corresponds to amide of ANB (MW = 182.1). After 1 h the peak 2 decrease and peak 4 ABZ-Arg-OH (MW = 293.1) appeared. The fragment Gln-Asp-Arg was eluted in void volume.

Table 1
Physicochemical and kinetical characterization of substrates

Peptide	MW calcd/found	t_R[a] (min)	Enzyme	k_{cat} (s^{-1}) × 10^{-1}	K_M (μM)	k_{cat}/K_M ($M^{-1} \times s^{-1}$) × 10^3
ABZ-Arg-Gln-Asp-Arg-ANB-NH$_2$ (**1**)	855.3	9.56	HAT[b]	**115.2 ± 0.3**	**25.4 ± 2.1**	**454.4 ± 22.7**
	856.2		Matriptase[b]	67.4 ± 4.3	68.5 ± 4.2	98.4 ± 12.6
			Tryptase[b]	42.2 ± 3.8	154.7 ± 8.3	27.4 ± 3.2
			Trypsin[c]	1.4 ± 0.2	1120.4 ± 81.8	0.2 ± 0.03
ABZ-Arg-Gln-Asp-Lys-ANB-NH$_2$ (**2**)	827.4	8.75	HAT[b]	**24.1 ± 0.8**	**12.34 ± 1.2**	**195.2 ± 13.7**
	828.0		Matriptase[b]	16.3 ± 2.1	70.61 ± 6.5	23.1 ± 2.6
			Tryptase[b]	11.8 ± 2.3	269.1 ± 28.7	6.7 ± 0.9
			Trypsin[c]	0.8 ± 0.1	1842.5 ± 79.3	0.1 ± 0.01
Boc-Phe-Ser-Arg-4-MCA[22] (**3**)	N/A	N/A	HAT[b]	**25.4 ± 1.8**	**43.6 ± 3.7**	**58.2 ± 4.9**
			Matriptase[b]	42.8 ± 2.6	12.1 ± 1.9	356.2 ± 21.8
			Tryptase[b]	53.6 ± 7.8	19.4 ± 1.5	275.7 ± 22.1
			Trypsin[c]	72.3 ± 11.6	16.5 ± 0.9	442.5 ± 37.6

[a] Linear gradient 10–90% of B within 40 min has been applied; detection at 226 nm.
[b] Human.
[c] Bovine.

that substrate (**1**) was hydrolyzed significantly faster (about five times higher value k_{cat}) than the remaining two peptides. Both (**1**) and (**2**) displayed also higher affinity towards HAT (2 and 3.4 times, respectively) as compared to substrate **3**. The experiments performed with β-trypsin, human matriptase and human mast cell tryptase indicate that reference peptide (**3**) is selectively hydrolyzed by these enzymes, whereas peptides **1** and **2** were highly selective substrates of HAT. Selectivity ratio of (**1**) to bovine β-trypsin, human matriptase and human mast cell tryptase measured as quotient of corresponding specificity constants (k_{cat}/K_M) was 2000, 5 and 16, respectively. Peptide (**2**) was even more selective (although less active); the corresponding values of specificity constants were 2000 (trypsin), 8 (matriptase) and 29 (tryptase) fold lower than for HAT.

Titration of decreasing amount of HAT by peptide **1** and **2** (Fig. 3) clearly indicates that detectable fluorescence can be measured at concentration of HAT 53 pM and 93 pM, respectively. In

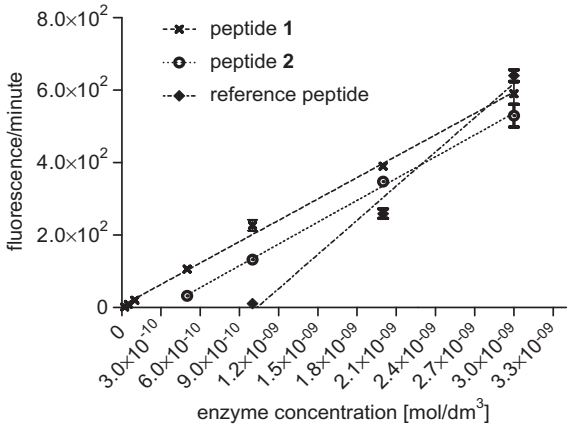

Figure 3. Titration of selected peptides (1, 2 and 3) by decreasing amount of enzyme. The method detection limit 3:1 (signal to noise ratio) was used.

the case of reference compound **3,** the determined value was almost 20 times higher than obtained for substrate **1** (982 pM).

One of commonly utilized approach to design proteinase inhibitors is the conversion of the C-terminal moiety of potent substrates into aldehyde. Recently, we have successfully utilized that approach to obtain potent chymotrypsin inhibitors.[17] Bearing this in mind, we synthesized two peptide aldehydes based on the sequences of substrates **1** and **2**. Determined inhibition constants of these peptides (numbered as **1a** and **2a**) with HAT (see Table 2) showed that they are potent (in nano molar range) HAT inhibitors with calculated values of inhibition constants (K_i) 54 nM and 112 nM, respectively. The obtained compounds are also able to inhibit (although the activity was at least two orders of magnitude lower) other members of serine protease family like bovine β-trypsin (392 μM (**1a**); 723 μM (**2a**)), human matryptase (927 nM (**1a**); 1.71 μM (**2a**)) and tryptase (21.2 μM and 37.5 μM, respectively). In early studies that dealt with HAT characterization it was shown that the enzyme was inhibited by leupetin (Ac-Leu-Leu-Arg-H) at micromolar level.[1] However, in comparison with other studied serine proteases it displayed no selectivity. The main influence on selectivity seems to play the Asp residue in position P_2 of the inhibitor.

In summary we would like to underline that this is the first report describing the substrate specificity of human airway trypsin-like proteinase (HAT). Using combinatorial approach, we selected two potent chromogenic/fluorogenic substrates of this enzyme. One of this substrates, (ABZ-Arg-Gln-Asp-Arg-ANB-NH_2 (**1**)) with high value of specificity constant (k_{cat}/K_M 454000 $M^{-1} \times s^{-1}$), undergoes detectable proteolysis in presence of 53 pM of experimental enzyme and is a good candidate for utilization in clinical diagnostic. Converting sequences of these substrates into the corresponding aldehydes by replacing the chromophore moiety (ANB-NH_2) by aldehyde group, yielded the potent and selective inhibitors of this enzyme. Moreover, such inhibitor contains the fluorescence moiety (ABZ) and can be potentially used as an active site probe for this proteinase.

3. Materials and methods

3.1. Peptide synthesis

All peptides were synthesized manually by the solid-phase method using Fmoc chemistry, as described previously.[18] TentaGel S RAM (substitution 0.25 mequiv/g) (RAPP Polymere, Germany) was used as a solid support. The α-amino groups of amino acids derivatives were Fmoc protected. The Fmoc protected amino acid residues were attached to the resin using DIPCI/HOBt method. Briefly the mixture of N-protected amino acid derivative, DIPCI and HOBt (molar ratio, 1:1:1) was dissolved in DMF/NMP solution (1:1, v/v) and added into the resin. The three fold excess to the resin active sites was used. This step was followed by the coupling of 5-amino-2-nitrobenzoic acid (ANB) to the previously deprotected (with 20% piperidine in the mixture of DMF/NMP (1:1, v/v)) α-amino groups using the TBTU/DMAP method.[19] Shortly the 3 equiv of ANB was dissolved in 5 ml DMF and 3 equiv of TBTU was added followed by 2 equiv of DMAP. Obtained solution was added to the resin and after 30 s, the 6 equiv of DIPEA was added. The whole mixture was stirred for 3 h. The solution was filtered off and the resin was washed with DMF. The procedure was repeated three times. Fmoc protected amino acid derivative of Lys or Arg was attached to the amino group of ANB using $POCl_3$ as the coupling reagent.[19] In the next step, the peptide chain was elongated by the appropriate amino acid derivatives using the DIPCI/HOBt method. The Boc-*o*-aminobenzoic acid (Boc-ABZ) was coupled to the N-termini of peptide using DIPCI/HOBt method with three molar excess. After completing the synthesis, the peptides were cleaved from the resin using a TFA/phenol/triisopropylsilane/H_2O mixture (88:5:2:5, v/v).[20] Purity of peptides was checked on RP-HPLC Pro Star system (Varian, Australia) equipped with a Kromasil 100 C_8 column (8 × 250 mm) (Knauer, Germany) and a UV–VIS detector. A linear gradient from 10% to 90% B within 40 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm. Mass spectra of peptides synthesized and ANB derivates were recorded using a Biflex III MALDI TOF mass spectrometer (Bruker, Germany) and α-cyano-4-hydroxycinnamic acid as a matrix. NMR study of final compounds were performed on Mercury VX 400 spectrometer (Varian, USA).

3.1.1. Compound 1

^{1}H NMR (DMSO-d_6) δ: 10.54 (s, 1H), 8.32 (d, 1H), 8.19–8.09 (m, 2H), 8.05 (d, 1H), 8.01 (s, 1H), 7,85–7.74 (m, 2H), 7.60 (s, 1H), 7.56 (d, 2H), 7.27 (s, 2H), 7.11 (t, 2H), 6.80 (s, 2H), 6.67 (d, 1H), 6.51 (t, 1H), 4.57 (q, 1H), 4.44–4.31 (m, 2H), 4,27 (q, 1H), 3.17–2.99 (m, 4H), 2.7 (q, 2H), 2.54 (q, 2H), 2.18–2.07 (m, 2H), 1.89–1.79 (m, 2H), 1.76 (d, 2H), 1.67–1.55 (m, 10H), 1.52–1.50 (m, 1H).

3.1.2. Compound 2

^{1}H NMR (DMSO-d_6) δ: 10.55 (s, 1H), 8.31 (d, 1H), 8.20–8.09 (m, 2H), 8.05 (d, 1H), 7,85–7.74 (m, 2H), 7.60 (s, 1H), 7.56 (d, 2H), 7.27 (s, 2H), 7.11 (t, 2H), 6.80 (s, 2H), 6.67 (d, 1H), 6.51 (t, 1H), 5.01 (s,2H), 4.57 (q, 1H), 4.42–4.31 (m, 2H), 4.28 (q, 1H), 3.18–3.00 (m, 2H), 2.7 (q, 2H), 2.54–2.60 (m, 6H), 2.49 (t, 2H) 2.18–2.07 (m, 2H), 1.89–1.79 (m, 2H), 1.76 (d, 2H), 1.67–1.55 (m, 6H), 1.52–1.46 (m, 1H).

3.1.3. Compound 1a

^{1}H NMR (DMSO-d_6) δ: 10.56 (s, 1H), 9.47 (s, 1H), 8.32 (d, 1H), 8.19–8.09 (m, 2H), 8.05 (d, 1H), 8.01 (s, 1H), 7.85–7.74 (m, 1H), 7.11 (t, 2H), 6.80 (s, 2H), 6.67 (d, 1H), 6.51 (t, 1H), 5.09 (s, 2H), 4.57 (q, 1H), 4.44–4.31 (m, 2H), 4.27 (q, 1H), 3.17–2.99 (m, 4H), 2.7 (q, 2H), 2.54 (q, 2H), 2.18–2.07 (m, 2H), 1.89–1.79 (m, 2H), 1.76 (d, 2H), 1.67–1.55 (m, 10H), 1.52–1.50 (m, 1H).

Table 2
Physicochemical and kinetic properties of peptide aldehydes

	Peptide	MW calcd/found	t_R[a] (min)	Enzyme	K_i (nM)
1a	ABZ-Arg-Gln-Asp-Arg-H	676.3/677.1	11.78	HAT[b]	54.3 ± 2.1
				Matryptase[b]	972.9 ± 82.2
				Tryptase[b]	2127.5 ± 171.9
				Trypsin[c]	3925.1 ± 94.8
2a	ABZ-Arg-Gln-Asp-Lys-H	648.3/649.2	12.17	HAT[b]	112.3 ± 7.5
				Matryptase[b]	1710.7 ± 110.1
				Tryptase[b]	3753.3 ± 282.5
				Trypsin[c]	7232.2 ± 252.9

[a] Linear gradient 10–90% of B within 40 min has been applied; detection at 226 nm.
[b] Human.
[c] Bovine.

3.1.4. Compound 2a

^{1}H NMR (DMSO-d_6) δ: 10.55 (s, 1H), 9.35 (s, 1H), 8.31 (d, 1H), 8.20–8.09 (m, 2H), 8.05 (d, 1H), 7,85–7.74 (m, 1H), 7.11 (t, 2H), 6.80 (s, 2H), 6.67 (d, 1H), 6.51 (t, 1H), 5.01 (s, 2H), 4.57 (q, 1H), 4.42–4.31 (m, 2H), 4.28 (q, 1H), 3.18–3.00 (m, 2H), 2.7 (q, 2H), 2.54–2.60 (m, 6H), 2.49 (t, 2H) 2.18–2.07 (m, 2H), 1.89–1.79 (m, 2H), 1.76 (d, 2H), 1.67–1.53 (m, 6H), 1.52–1.46 (m, 1H).

3.2. Preparation of the peptide library

The peptide library was synthesized by the portioning–mixing method.[21,22] Initially, 5 g of the solid support (TentaGel S RAM) was used. Threefold amino acid molar excess was used for the coupling. Other synthetic methods employed were as described above. The deconvolution of obtained library was performed in solution using iterative approach.

3.3. Peptide aldehydes

All peptides with the aldehyde group at their C-termini were synthesized manually by the solid-phase method using Fmoc chemistry, applying previously described method using Weinreb AM resin (NovaBiochem, Switzerland) as a solid support.[23] α-Amino groups of the amino acid derivatives were protected by (Fmoc) moiety. Amino acid derivatives were coupled to the solid support by the DIPCDI/HOBt method. The peptide aldehydes with fully protected side chains were detached from the resin using $LiBH_4$. Protecting groups were removed using a TFA/phenol/triisopropylsilane/H_2O mixture (88:5:2:5, v/v).

4. Enzymatic studies

4.1. Initial screening

The catalytic domain of recombinant human airway trypsin-like protease was purchased in R&D System (USA). The active site titrant NPGB and substrate Boc-Phe-Ser-Arg-4-methylcoumaryl-7-amide comes from Sigma Aldrich (USA). The aliquot of HAT 10 μl (0.1 mg/ml) were used for activity assay. Deconvolution of the sublibraries was performed applying UV–vis method using the 12 mg/ml DMSO stock solution of each compound. All UV measurements were performed using a Cary 3E spectrophotometer (Varian, Australia). The 10 μl of each sublibrary was injected into 1.5 ml cuvette containing 0.1 M Tris–HCl (pH 7.8). The velocity of the chromophore liberation was measured within 5 min at 405 nm.

4.2. Determination of Michaelis constants (K_M), catalytic constants (k_{cat}), and specificity constants (k_{cat}/K_M)

Enzymatic hydrolysis of the peptide was performed in 0.1 M Tris–HCl (pH 7.8) buffer at 25 °C. The velocity of the chromophore liberation was measured within 5 min at 405 nm using a Cary 3E spectrophotometer (Varian, Australia). Measurements were carried out at enzyme concentration 2.76×10^{-9} M. Three to five measurements were carried out for each compound (systematic error expressed as a standard deviation never exceeded 20%). The calculated initial hydrolysis rates were used as a measure of substrate activity of the investigated peptides. All details of kinetic studies and the method of calculating kinetic parameters, K_M, k_{cat}, and k_{cat}/K_M have been described in our previous papers.[24,25]

4.3. Inhibition constants determination

The values of K_i for peptides with aldehyde groups at the C-termini were determined as described elsewhere.[23] Increasing concentrations of the inhibitor (from 0 to 100 nM) were added to the buffered (Tris–HCl pH 7.8) enzyme solution (concentration 20 nM) and incubated for 60 min. In the next step, to each inhibitor–enzyme solution, various concentrations of chromogenic substrates ranging from 1.8 μM to 100 μM were added. For HAT assay, the chromogenic substrate with the sequence ABZ-Arg-Gln-Asp-Arg-ANB-NH_2 was used. The increasing absorbency was measured as a function of time. Based on the measured initial velocities of the substrate hydrolysis, the K_i values were calculated using a Prism GraphPad software.

4.4. Fluorescence studies

All fluorescence measurements were performed using Fluorostar Omega (BMG Labtech, Germany). All peptides investigated were dissolved in DMSO to achieve final concentration of 3 mg/ml and then further diluted 2–100 times followed by addition of HAT solution. For ABZ the excitation and emission wavelengths were λ_{ex} = 325 nm and λ_{em} = 400 nm, respectively. The substrate Boc-Phe-Ser-Arg-4-methylcoumaryl-7-amide (peptide **3** purchased from Sigma Aldrich (USA) and used as reference compound) 360 nm and an emission wavelength of 440 nm was used.

4.5. Proteolytic cleavage pattern determination

Selected substrate was mixed with a twofold molar excess of the enzyme in a buffer used for kinetic studies. HPLC analysis of this mixture was performed after the following incubation times: 0, 15 min and 1 h. A linear gradient from 5% to 30% B within 30 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm. The appearing peaks were collected and analyzed by MALDI MS as described above.

4.6. Sensitivity curve

Constant amount of peptide **1, 2** or **3** (3.3 μM) was added into buffered solution of HAT in Tris–HCl pH 7.8. The amount of assayed enzyme ranged from 3.2×10^{-9} M to 2.2×10^{-11} M. The fluorescence increase at 400 nm versus time was measured. All obtained values were measured against substrate concentration with no enzyme added. The threshold limit for all measurements was 3:1 expressed as signal to noise ratio.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.059.

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