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The influence of substrate peptide length on human β-tryptase specificity

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Abstract: Combinatorial chemistry approach was applied to design chromogenic substrates of human β-tryptase. The most active substrate, Ala-Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2, was selected from among over 9 million heptapeptides. The amide of 5-amino-2-nitrobenzoic acid (ANB-NH_2) attached at the *C*-terminus served as a chromophore. In order to determine the optimal length of the tryptase substrate, a series of *N*-terminally truncated fragments of this substrate was synthesized. Pro-Ile-Arg-Asn-Lys-ANB-NH_2, with the determined value of the specificity constant (k_{cat}/K_M) above 9×10^6 M^{-1} s^{-1}, appeared to be the most specific substrate of tryptase. This substrate was twice as active as the parent heptapeptide substrate. We postulate that the optimal size of the pentapeptide substrate for the interaction with human β-tryptase is associated with the unique structure of this proteinase, comprising four almost identical monomer subunits arranged in a square flat ring with its substrate pockets faced inside, forming a tetramer with a central pore that can be penetrated by this short peptide. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide library; human tryptase; chromogenic substrate

INTRODUCTION

Human β-tryptase is the main form of tryptase that belongs to the serine protease family and displays trypsin-like specificity [1]. It cleaves peptide bonds after Arg and Lys residues, but requirements regarding the amino acid sequence in the vicinity of this substrate specificity P_1 position are much stricter. This enzyme is involved in numerous physiological processes such as asthma, arthritis, inflammatory reactions and allergic response [2,3]. Tryptase is synthesized, stored and released upon mast cell activation. It is worth mentioning that tryptase accounts for almost 10% of protein content in the secreted mast cells [2]. Its active form comprises four almost identical subunits arranged in a flat ring with its substrate pockets faced inside. This is the key factor that makes tryptase so unique. The whole structure is stabilized by a strong interaction with heparin chains. Only one natural inhibitor has so far been discovered, i.e. Leech-derived tryptase inhibitor (LDTI) [4]. It belongs to the Kazal-type family, consists of 46 amino acid residues and is able to inhibit tryptase at K_i 1.4 nM. There is also only one natural substrate identified by *in vivo* experiments (protease-activated receptor-2 (PAR-2)) [5]. Human tryptase activates this specific receptor leading to severe airway inflammation.

The enhanced specificity of human tryptase has been reported. Efficient hydrolysis requires positively charged residues in positions P_1 and P_3 (Arg or Lys). Positions P_2 and P_4 display a broader specificity [6]. Scarpi *et al.* [7] and Rice *et al.* [8] independently described the effect of the main chain length on β-tryptase activity in the case of peptide [7] or nonpeptide inhibitors [8].

Scarpi *et al.* focused their attention on a sequence from the binding loop of the Bowman–Birk inhibitor (BBI). The authors explored the impact of the *C*- and/or *N*-extension of this peptide on the inhibition of human β-tryptase. They introduced spacers with different lengths, containing positively charged Lys at the ends. The most active peptide with the spacers attached at the *N*- and *C*-termini acted probably as a tri-functional human β-tryptase inhibitor.

The influence of the chain length of dibasic nonpeptide inhibitors on the activity against tryptase was also reported by Rice *et al.* [8]. A series of urea derivatives that differ in the number of methylene groups has been investigated. The activity of such inhibitors was strongly correlated with the length of the main chain. The addition of two methylene groups resulted in a more potent inhibitor. Further elongation had a negative influence on the inhibitory activity.

The findings mentioned above motivated us to investigate the correlation between the length of the peptide and its tryptase substrate specificity. Combinatorial libraries of chromogenic substrates are excellent tools for fast examination of the specificity of most proteases [9]. In order to select the most active substrate of β-tryptase, a two-step procedure was applied. In the first step we decided to select a sequence of heptapeptides with the highest affinity

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towards the experimental enzyme. Therefore, a series of heptapeptide libraries was designed and synthesized on the solid phase by applying the portioning–mixing method. All peptides contained at their *C*-termini an amide of 5-amino-2-nitrobenzoic acid (ANB-NH_2) that upon enzymatic action is released from the peptide(s) displaying affinity towards proteinase. The concentration of ANB-NH_2 is proportional to the activity of the enzyme and therefore this derivative acts as a chromophore [10]. The general formula of the library synthesized is given below:

$$X_7\text{-}X_6\text{-}X_5\text{-}X_4\text{-}X_3\text{-}X_2\text{-}X_1\text{-ANB-}NH_2$$

X_1 = Arg, Lys, Arg(NO_2), Orn, Cit, Hci, Har, Phe(*p*-NO_2);
X_2 = Thr, His, Ala, Ser, Asn, Val, Phe;
X_3 = Arg, Lys, Arg(NO_2), Orn, Cit, Phe(*p*-NO_2);
X_4 = Asn, Ala, Phe, Glu, Lys, Ile, Pro;
X_5 = Ala, Tyr, Lys, Asp, Glu, Val, Asn, Ile, Ser, Phe, Pro;
In positions X_6 and X_7 all the proteinogenic amino acids except Cys were present.

The deconvolution of the peptide library was performed by the iterative approach utilizing a previously described procedure for selection of proteinase chromogenic substrates [9].

In the second step, the most susceptible substrate (**1**) of β-tryptase selected by the combinatorial approach was used as a starting structure to study the influence of the substrate length on its interaction with β-tryptase. The following shortened substrates were synthesized:

(**1**) Ala-Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2
(**2**) Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2
(**3**) Ac-Pro-Ile-Arg-Asn-Lys-ANB-NH_2
(**4**) Pro-Ile-Arg-Asn-Lys-ANB-NH_2
(**5**) Ac -Ile-Arg-Asn-Lys-ANB-NH_2
(**6**) Ile-Arg-Asn-Lys-ANB-NH_2
(**7**) Ac-Arg-Asn-Lys-ANB-NH_2
(**8**) Arg-Asn-Lys-ANB-NH_2

MATERIALS AND METHODS

Peptide Synthesis

The peptide library and individual peptides for kinetic studies were synthesized manually by the solid-phase method using Fmoc chemistry, as described previously [9]. TentaGel S RAM (substitution 0.25 meq g^{-1}) (RAPP Polymere, Germany) was used as a support. The amino acid α-amino groups were Fmoc-protected. 5-Amino-2-nitrobenzoic acid was attached to the resin using the TBTU/DMAP method [10]. The *C*-terminal amino acid residues were incorporated using $POCl_3$ as the coupling reagent [10]. The other amino acid derivatives were coupled by the DIPCI/HOBt method. 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/v/v) [11]. Peptide libraries were used for experiments without further purification.

Preparation of the Peptide Library

The peptide library was synthesized by the portioning–mixing method [12,13] as reported earlier [14], applying the same reagents as described above. Initially, 10 g of the solid support (TentaGel S RAM) was used. Five-fold molar excess of protected amino acid derivative was used for the coupling step.

Screening of the Peptide Library for β-tryptase Substrate Activity

β-Tryptase purchased from Elastin Product Company Inc. (USA) was isolated from mast cells [15]. Deconvolution of the peptide library synthesized was performed in solution by the iterative method [16]. The stock solution was prepared by dissolving 5 mg of the peptide library in 250 µl of dimethylsulfoxide (DMSO). Ten microliters of the substrate stock solution was used in the experiment. Enzymatic hydrolysis of the peptide library was performed in 0.1 M Tris–HCl (pH 7.5) buffer with 500 mM of NaCl at 25 °C. Measurements were carried out at an enzyme concentration of 6×10^{-10} M. The increase in absorbency at 405 nm due to the release of ANB-NH_2 was recorded as a function of time. The calculated initial rates were used as measures of the substrate activity of the peptide library investigated. Each experiment was repeated at least three times. Data differing from the mean value by more than 10% were rejected.

Physicochemical Properties of the Peptide Library

In order to prove that the library synthesized contained the intended peptides, MS analyses were performed for randomly chosen sublibraries at each stage of the synthesis. Mass spectra were recorded using a Biflex III MALDI TOF mass spectrometer (Bruker, Germany).

Kinetic Studies

All measurements were performed using a Cary 3E spectrophotometer (Varian, Australia). The concentration of both bovine β-trypsin and human β-tryptase stock solution was determined by titration with *p*-nitrophenyl-*p*-guanidinobenzoate (NPGB). Standardized enzyme solutions were further diluted. The stock solutions of resynthesized substrates were prepared by dissolving 5 mg of each peptide in 250 µl of DMSO, and were further diluted 2–20 times; enzyme concentrations ranged from 2.5×10^{-9} to 1.5×10^{-11} M. Three to five measurements were carried out for each compound (systematic error expressed as a standard deviation never exceeded 10%). All details of kinetic studies and the method of calculating kinetic parameters, Michaelis constants (K_M), catalytic rates (k_{cat}), and specificity constants (k_{cat}/K_M) have been described in our previous papers [17,18]. Values of k_{cat} were calculated based on single active site of each monomer of tryptase.

Cleavage Pattern Determination

Selected substrates synthesized were incubated with the experimental enzyme at a concentration of 10^{-9} M (molar ratio

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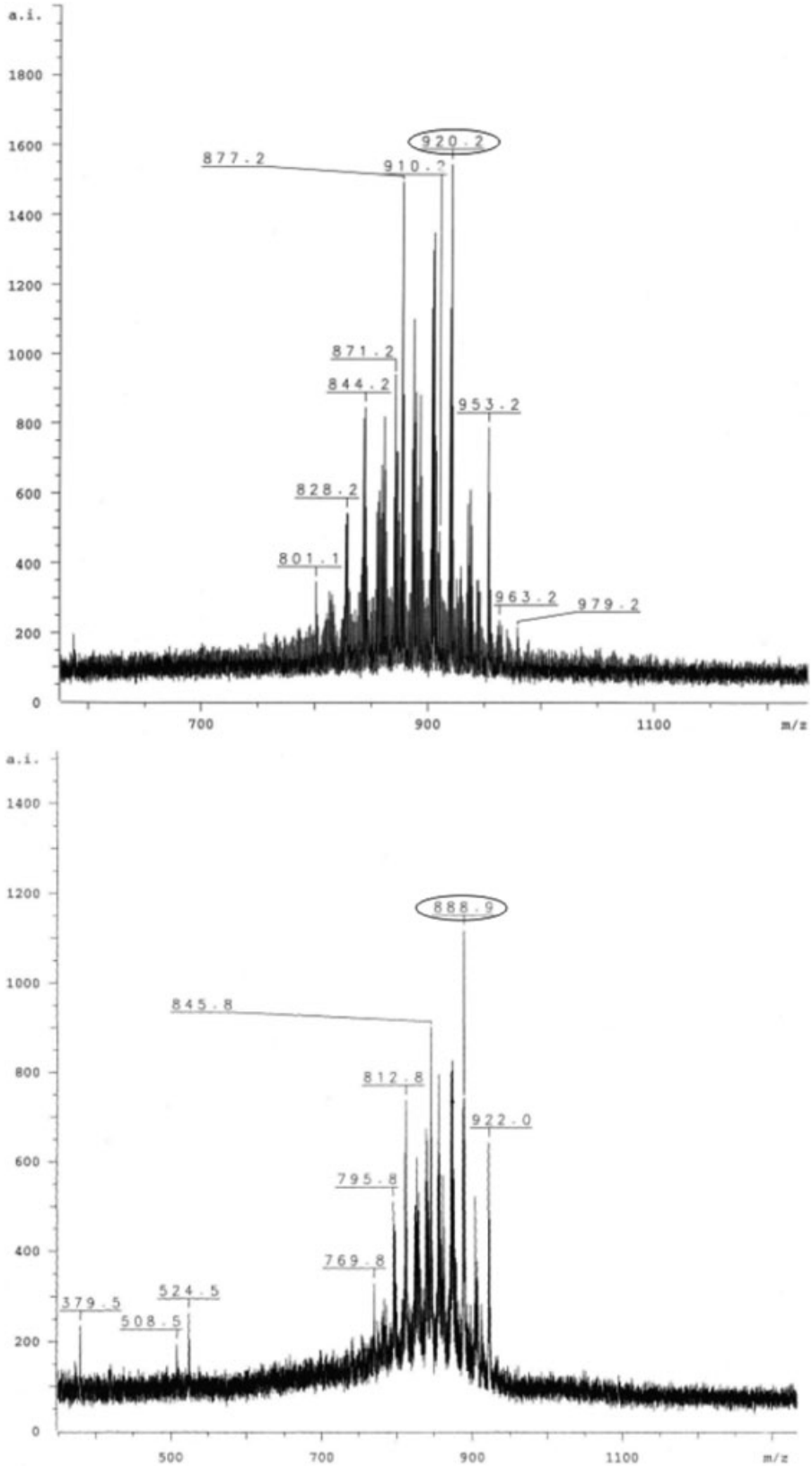

Figure 1 Mass spectra of selected sublibraries: (A) **Glu**-$X_4X_3X_2X_1$-ANB–NH_2 and (B) **Pro**-$X_4X_3X_2X_1$-ANB–NH_2 differing in position P_5 (Val/Ala). The mass shift between two most intense peaks (in circle) 920.2 – 888.9 = 31.3 is equal to the mass shift of amino acid residues in position P_5 $MW_{(Glu)} - MW_{(Pro)} = 31.9$.

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enzyme : substrate, 1 : 100) in the buffer used for the kinetic investigations. HPLC analysis of this mixture was performed 15 min and 1 h after incubation of the substrate with protease.

RESULTS AND DISCUSSION

The peptide library synthesized consists of over 9 million heptapeptides with the ANB-NH_2 moiety at their *C*-termini. An example of a MALDI TOF analysis of a randomly chosen sublibrary is shown in Figure 1. The method described by Furlong *et al.* [6] was used to check the quality of the library synthesized. The molecular shift between the most intense peaks present in the center of distribution of each sublibrary with fixed amino acid residues was equal to the mass difference of these residues. This finding confirmed integrity of the library. Details of its deconvolution against human β-tryptase using the iterative approach in solution are shown in Figure 2. In substrate positions P_7 and P_6, human tryptase displays a broad specificity. It tolerates

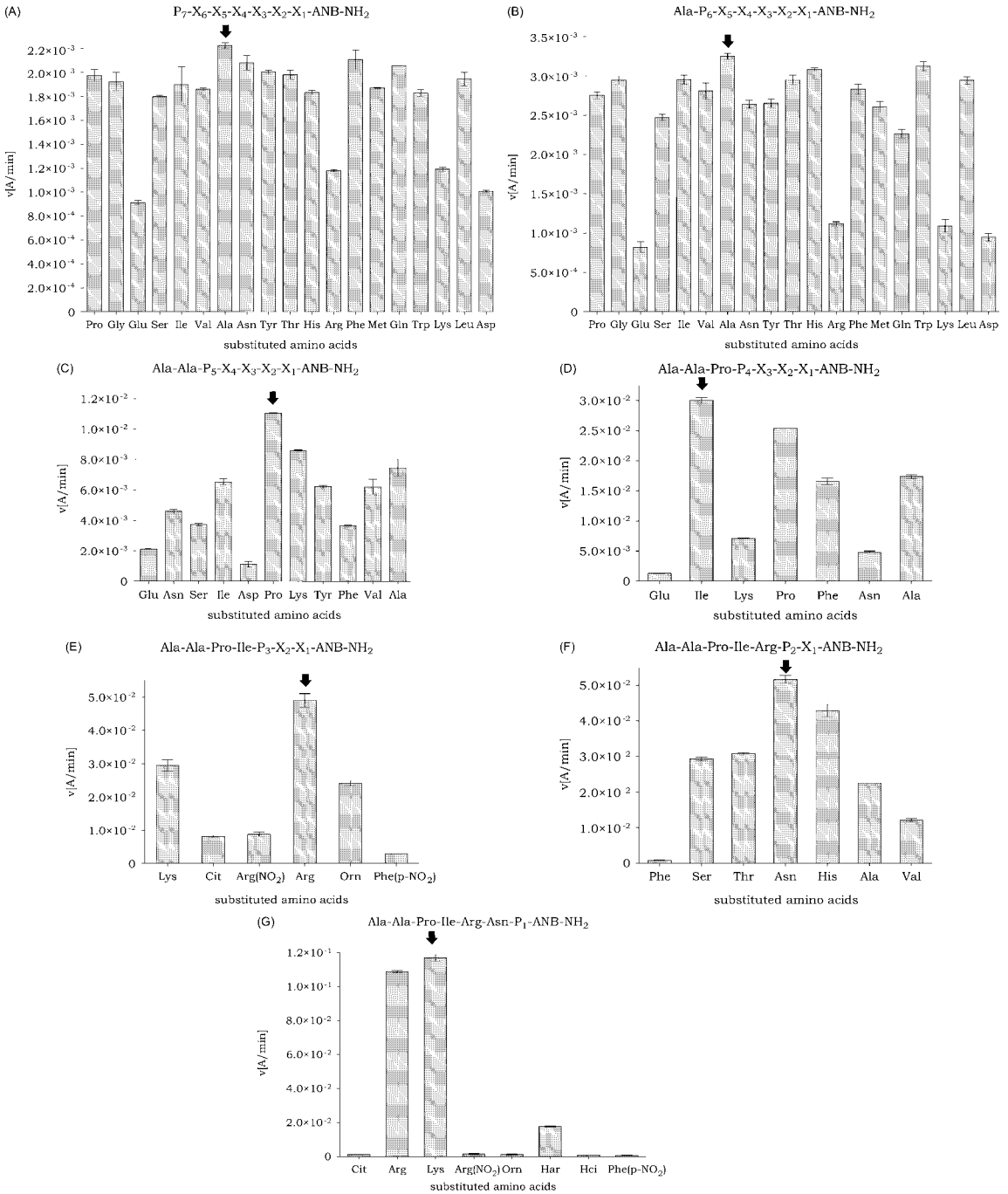

Figure 2 Deconvolution of heptapeptide library against β-tryptase using the iterative approach where (A) position P_7, (B) P_6, (C) P_5, (D) P_4, (E) P_3, (F) P_2, (G) P_1.

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Table 1 Physicochemical characteristics of synthesized chromogenic peptides

	Substrate	R_t [min]	MW calc./found
1	Ala-Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2	12.9	931.9/932.6
2	Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2	12.6	860.7/861.3
3	Ac-Pro-Ile-Arg-Asn-Lys-ANB-NH_2	15.2	831.7/832.5
4	Pro-Ile-Arg-Asn-Lys-ANB-NH_2	12.1	789.7/790.6
5	Ac-Ile-Arg-Asn-Lys-ANB-NH_2	14.5	734.6/735.3
6	Ile-Arg-Asn-Lys-ANB-NH_2	11.6	692.6/693.2
7	Ac-Arg-Asn-Lys-ANB-NH_2	11.3	621.6/622.2
8	Arg-Asn-Lys-ANB-NH_2	10.4	579.5/580.2

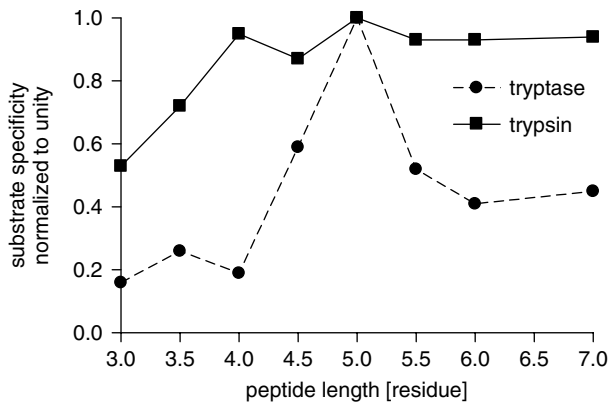

Figure 3 The activity of the chromogenic peptide substrate against tryptase and trypsin depending on the elongation of the peptide chain. Acetyl group was counted as a half residue. All specificity parameters were normalized to unity.

most proteinogenic amino acid residues with exception for the charged ones. In both positions the Ala residues yielded the most easily cleaved substrates. Therefore, for next steps of deconvolution, this simple amino acid was arbitrarily fixed in the sublibraries synthesized. In position P_5 of the substrate library, the Pro residue yielded the highest chromophore release. The highest hydrolysis rate was observed for substrates with Ile in position P_4, which is the only inconsistency in the data reported by Harris *et al.* [19], who found Pro to be optimal for this position. In our case this amino acid residue gave a chromogenic substrate with the second highest activity. Among sublibraries with fixed position P_3, the best effect was achieved with the Arg residue. The next step of deconvolution revealed that the highest proteolytic susceptibility was observed for heptapeptides with Asn in position P_2. Finally, in the substrate specificity P_1 position, both proteinogenic, basic amino acids Arg and Lys appeared to be optimal for the interaction with human β-tryptase. In consequence of these investigations, a heptapeptide Ala-Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2

Table 2 Kinetic studies of chromogenic peptide substrates

	Substrate	Human β-tryptase[a]			Bovine β-trypsin		
		K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1}) × 10^5	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1}) × 10^5
1	Ala-Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2	39.44 ± 2.28	186.98 ± 6.14	47.46 ± 1.19	26.55 ± 2.16	11.18 ± 0.76	4.21 ± 0.05
2	Ala-Pro-Ile-Arg-Asn-Lys-ANB-NH_2	53.00 ± 3.59	233.43 ± 13.18	44.03 ± 0.50	34.01 ± 2.43	13.42 ± 0.86	3.95 ± 0.03
3	Ac-Pro-Ile-Arg-Asn-Lys-ANB-NH_2	71.03 ± 4.65	378.00 ± 11.25	53.21 ± 1.78	37.38 ± 1.35	18.51 ± 0.28	4.96 ± 0.10
4	Pro-Ile-Arg-Asn-Lys-ANB-NH_2	43.75 ± 5.65	435.23 ± 23.10	99.53 ± 7.57	45.57 ± 3.56	18.95 ± 0.65	4.15 ± 0.18
5	Ac-Ile-Arg-Asn-Lys-ANB-NH_2	61.65 ± 5.10	367.19 ± 12.08	59.56 ± 2.97	46.96 ± 3.69	21.0 ± 0.87	4.48 ± 0.17
6	Ile-Arg-Asn-Lys-ANB-NH_2	111.12 ± 7.47	199.42 ± 6.30	17.95 ± 0.64	61.21 ± 5.13	24.2 ± 1.12	3.95 ± 0.15
7	Ac-Arg-Asn-Lys-ANB-NH_2	49.80 ± 4.15	140.65 ± 8.17	28.22 ± 0.71	76.22 ± 5.66	27.11 ± 0.93	3.56 ± 0.14
8	Arg-Asn-Lys-ANB-NH_2	99.90 ± 10.85	158.59 ± 15.41	15.87 ± 0.18	57.44 ± 4.23	11.80 ± 0.59	2.05 ± 0.05

[a] Specificity parameters were calculated based on a single active site.

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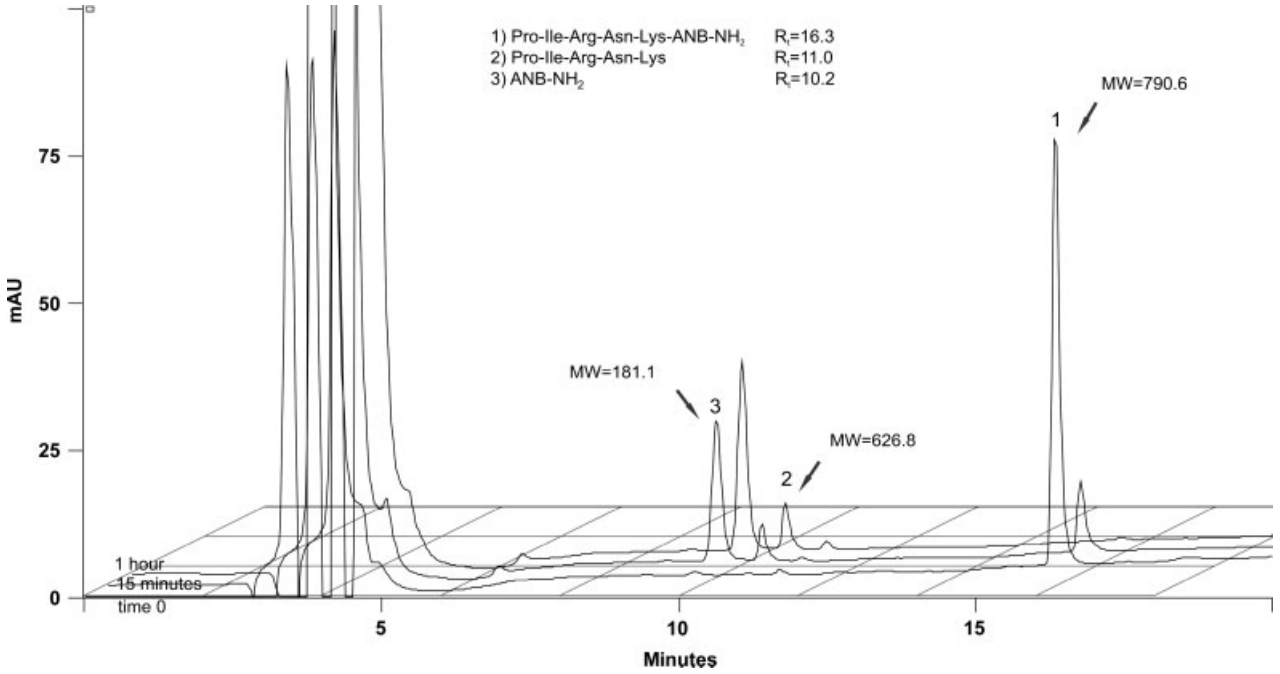

Figure 4 The MALDI TOF cleavage pattern for Pro-Ile-Arg-Asn-Lys-ANB-NH_2 (**4**).

(**1**), the most easily hydrolyzed human β-tryptase substrate, was selected.

In order to determine the influence of the length of the chromogenic substrate on its interaction with human β-tryptase, fragments of substrate (**1**) truncated at the *N*-termini were synthesized and subjected to kinetic investigations. The results of HPLC and MS analyses of the peptides are summarized in Table 1. All these peptides were assayed against human β-tryptase and bovine β-trypsin. Specificity parameters (K_M and k_{cat}), determined for all substrates, are listed in Table 2. Relations between the lengths of the substrates and their specificity constants are shown in Figure 3. In the case of trypsin, the removal of the first three *N*-terminal amino acids did not significantly change the calculated values of k_{cat}/K_M. Further shortening of the peptide length reduced the affinity towards trypsin. These results are consistent with the data reported by Case and Stein [20], who investigated the effect of the elongation of peptide substrates and their interactions with a cognate serine protease (chymotrypsin, leukocyte elastase). For all the enzymes studied, elongation of the peptide chain of the chromogenic substrate results in enhanced specificity. The influence of the peptide chain length on its interactions with the human β-tryptase substrate was much more pronounced. The removal of two *N*-terminal Ala residues significantly increased the affinity towards this proteinase. On the other hand, further shortening of this most susceptible substrate (**4**) by elimination of Pro resulted in a fivefold drop in the activity. The increase of specificity constant was observed when the *N*-terminal amino group in tetrapeptide substrate (**6**) was protected by the acetyl moiety. In all substrates synthesized, only one bond was hydrolyzed. The cleavage pattern of Pro-Ile-Arg-Asn-Lys-ANB-NH_2 (**4**) is presented in Figure 4. Upon incubation with tryptase, two peaks corresponding to the ANB-NH_2 moiety and the pentapeptide with a free *C*-terminal carboxyl group were observed.

We postulate that the highest specificity parameter observed for the pentapeptide substrate may by associated with the unique structure of human β-tryptase, which is a tetramer formed by four catalytically independent active tryptase monomers facing a central pore with dimensions 40 Å × 15 Å. Each peptide enters one active site and is independently hydrolyzed. The length of the pentapeptide in extended conformation amounts to about 18 Å, which is probably optimal for entering the substrate pocked with tryptase. Any elongation at its *N*-terminus strongly suppresses the proteolytic susceptibility to tryptase but not against trypsin. This should be taken into account when designing new substrates and inhibitors of human β-tryptase.

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