Effect of pea and bovine trypsin inhibitors on wild-type and modified trypsins

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Abstract In order to modify the catalytic properties of trypsin, lysine-188 (S1) of the substrate binding pocket was substituted by an aromatic amino acid residue (Phe, Tyr, Trp) or by a histidyl residue. Two other mutants were obtained by displacement or elimination of the negative charge of aspartic acid-189 (K188D/D189K and G187W/K188F/D189Y, respectively). The high affinity inhibitors, like PSTI II and BPTI, behaved as specific substrates of the trypsin and its mutants. Their inhibiting effect toward modified trypsins was studied. The bovine inhibitor had a higher affinity for all tested enzymes than pea inhibitor. The inhibition constants differed according to the mutations on the protease.

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Key words: Trypsin; Trypsin inhibitor; Site-directed mutagenesis

1. Introduction

The current understanding of the physiology and biochemistry of nutritional nitrogen shows that some of the peptide fragments resulting from the action of the digestive tract proteases function not only as suppliers of amino acids to the organism but also as physiological regulators. It is believed that several peptides arising from hydrolysis of β-casein have important physiological functions. Opioid activity was assigned by Brantl et al. [1] to peptide fragment 60–66, called β-casomorphin. The first 20 residues of β-casein contain phosphopeptide binding calcium [2]. Anti-hypertensive activity was also reported for a β-casein 177–183 peptide [3]. Opiate peptides can also be excised from β-lactoglobulin (β-lactorphin: residues 102–105) during its hydrolysis by trypsin in vitro [4]. Many dietetic and pharmaceutic uses of milk proteins and derived peptides were reviewed by Maubois and Léonil [5]. Consequently, the study of chemical and/or enzymatic modifications of milk proteins in order to produce such peptides are especially interesting [6–11]. The search for enzymes with novel specificities is one of the main goals of protease engineering. The substrate specificity differences in serine proteases, such as trypsin, chymotrypsin and elastase, result from relatively simple structural changes in their substrate binding site. Several studies were carried out to determine the crucial role of negative charge of the Asp-189 residue in binding and catalysis using site-directed mutagenesis [12]. The study of mutants such as D189K [13], D189S [14], K188D/D189K [15] and G187W/K188F/D189Y [16] showed that the presence of a negative charge is essential for a good orientation of the substrate and efficient catalysis by trypsin. Alignments of amino acid sequences revealed that porcine pancreatic elastase, fiddler crab collagenase and rat anionic trypsin all have either Lys or Arg in position 188 [17]. However, all these enzymes present different specificities. Consequently, the amino acid in position 188 also plays an important structural role differing from outright definition of protease specificity.

In order to modify the catalytic properties of trypsin, three mutants were produced by substituting Lys-188 with an aromatic amino acid (Phe, Tyr or Trp). These substitutions induce a modification of electrostatic and hydrophobic interactions in the neighborhood of the Asp-189 residue. The study of β-casein hydrolysis by these aromatic mutants showed that, in addition to cleavage of peptide bonds involving Lys or Arg, new sites of cleavage appeared, involving glutaminyl and asparaginyl residues [18].

The substitution of Lys-188 by a histidyl residue created a chelation site in the substrate binding pocket and allowed the study of the inhibitory effect of a metal ion (Cu^{2+}) [19] on the catalytic activity of trypsin.

Tryptic inhibitors are often rich in amide residues (Asn, Gln) and it seemed interesting to study the effect of trypsin inhibitors on these mutant trypsins and to compare it with the inhibition of wild-type enzyme.

Serine protease inhibitors are found in the plant and animal kingdoms. They are classified into different families according to amino acid sequence homology, cysteine number and the position of the active site. Inhibitors present in plants were studied extensively because of their antinutritional properties [20–22] and their protective functions in plants [23–26]. In the present work two inhibitors belonging to two different families (Kunitz and Bowman-Birk) but displaying competitive properties toward trypsin [27] were used.

Pisum sativum trypsin inhibitor (PSTI II), taken from winter pea seeds (cv. Frilene), is a member of the Bowman-Birk family. It is characterized by a low molecular weight (6807 Da), the presence of 14 cysteinyl residues (forming seven disulfide bridges) and of two distinct binding sites for two different proteases (trypsin and chymotrypsin) inhibited independently [28–30] (Fig. 1A). Its three-dimensional structure is being studied.

Bovine pancreatic trypsin inhibitor (BPTI) belongs to the Kunitz family. It is characterized by a low molecular weight (6516 Da), the presence of six cysteinyl residues (forming three disulfide bridges) and of a single protease binding site (Fig. 1B). This inhibitor was chosen since its activity and its three-dimensional structure are well known [31–33].

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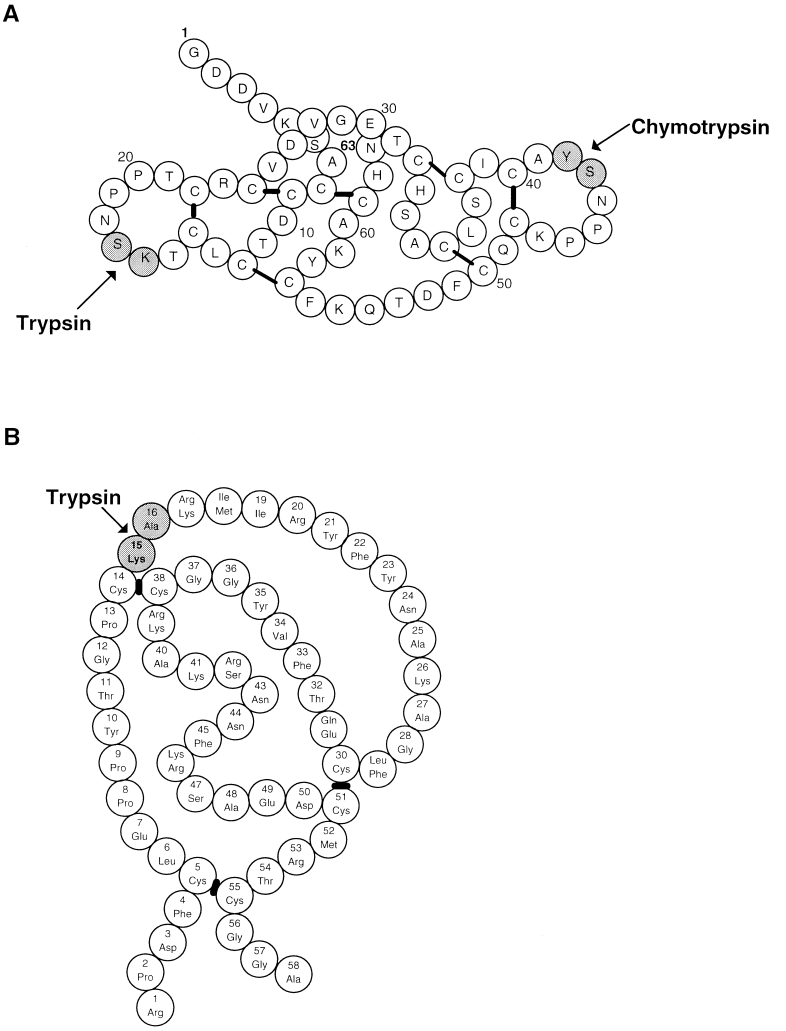

Fig. 1. A: Primary structure of the *Pisum sativum* inhibitor PSTI II [27]. B: Primary structure of the bovine inhibitor BPTI [48].

2. Materials and methods

Bovine trypsin TPCK-treated (10 000–13 000 U/mg), bovine inhibitor (BPTI), succinyl-Ala-Ala-Pro-X-*p*-nitroanilide (X = Arg or Lys), 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) were from Sigma Chemical Co.

Pisum sativum inhibitor (PSTI II), purified according to Ferrasson [34], was a kind gift from Eric Ferrasson (INRA LBTP, Nantes).

Trypsin mutants were prepared by oligonucleotide-directed mutagenesis carried out by the method of Kunkel et al. [35], using single-stranded, uracil-containing DNA templates, as previously described [19]. Six mutants were studied. K188F, K188Y, K188W and K188H are the results of single substitutions. Trypsin mutant K188D/D189K resulted from an inversion of the charge in the substrate binding pocket. The triple mutant G187W/K188F/D189Y introduced three aromatic amino acid residues in the substrate binding pocket with suppression of the negative charge.

2.1. Protein production and purification

Culture of *Escherichia coli* strain HB2151 was carried out on Luria broth medium overnight at 37°C in the presence of ampicillin (50 mg/l) and 0.5 mM IPTG to induce expression. Wild-type and mutant trypsins were secreted in the periplasm space of cells. Periplasmic extracts were prepared by treatment with lysozyme [36] and were subsequently dialyzed against 10 mM sodium citrate pH 2.2, at 4°C for 24 h. The resulting solution was then applied to a CM-Sepharose fast-flow column (2.6 i.d. × 30 cm) in a volume equal to the volume of the column equilibrated with a 0.1 M citric acid, 0.1 M trisodium citrate, pH 4.0 buffer. Fractions containing trypsin were monitored by the following method. An aliquot of each fraction was mixed with an equal volume of 2 × Laemmli buffer [37] lacking 2-mercaptoethanol. The sample was then electrophoresed on a 12.5% polyacrylamide, 1% SDS running gel with a 4.75% polyacrylamide stacking gel. After electrophoresis, the separating gel was soaked in 2.5% Triton X-100 for 35 min then in a 50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0 solution. This gel was overlaid with an 8% polyacrylamide gel in 40 mM Tris-HCl, 15 mM $CaCl_2$, pH 8.0 containing 0.1% whole bovine casein. The 'gel sandwich' was incubated overnight at 37°C then dyed with Coomassie blue. Trypsin was therefore detected by digestion of the casein leading to a lack of coloration at a level corresponding to the molecular weight of this protein. The pH of the fractions from chromatography on CM-Sepharose and containing trypsin was adjusted at 6.0 with 6 N NaOH. The solution was applied to a benzamidine-Sepharose column (1.6 i.d. × 4.0 cm) and chromatography carried out in 50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0. After complete elution of unbound proteins as indicated by the return of the absorbance to its initial value, bound trypsin was eluted with 10 mM HCl. All enzyme purifications were carried out at 4°C.

2.2. Active site titration

A stock (10 mM) solution of MUGB was prepared in *N*-methylpyrrolod-2-one. The stock solution was diluted 1/10 000 in 10 mM HCl. 10 μl of the dilution was added to 980 μl of a 50 mM Tris-HCl, 100 mM NaCl, 20 mM $CaCl_2$, pH 8.0 buffer in a fluorometer cuvette. Then, 10 μl of bovine trypsin (0.01 M) was added to the solution. Fluorescence was measured on a SLM4800 Aminco with an excitation wavelength of 365 nm and an emission wavelength of

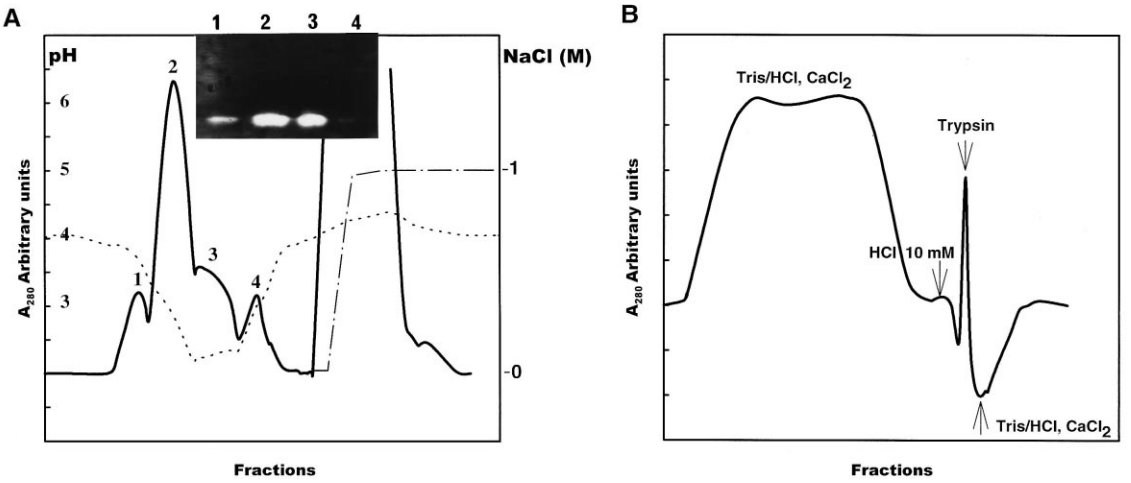

Fig. 2. A: Cation exchange chromatography on CM-Sepharose of periplasmic extract. Solid line: absorbance at 280 nm; dashed line: NaCl molarity; dotted line: pH. Inset: Zymogram of fractions 1–4 (a lack of coloration is observed at a level corresponding to the molecular weight of trypsin). See Section 2 for conditions. B: Affinity chromatography on benzamidine-Sepharose.

445 nm [38]. Titration was performed at 20°C and the reaction was followed for 10 min. A standard scale was made with bovine trypsin of known concentration ([E] ≪ [S]) and the MUGB solution. During active site titration of trypsin mutants, 100 μl of enzyme solution was added to 890 μl of 50 mM Tris-HCl, 100 mM NaCl, 20 mM $CaCl_2$, pH 8.0 buffer and to 10 μl of MUGB (0.1 μM).

2.3. Molecular modelling

Molecular modelling studies were carried out on Silicon Graphics computers with Biosym/Molecular Simulation packages. Molecular display and energy minimizations were performed with Insight II and Discover packages, respectively. Two force fields were used at different stages of the calculations. The Amber force field was initially employed for energy measurements and in minimization procedures.

The initial structure of trypsin was extracted from X-ray data of its complex with ecotin [39] accessible from the Protein Data Bank. Although two segments are missing in this structure (114–117 and 146–148), this trypsin structure was chosen as the starting geometry because of its high crystallographic resolution (1.8 Å). The missing residues do not significantly perturb the meaning of this molecular modelling study since all energy calculations were performed by keeping fixed the backbone of heavy atoms not involved in the binding pocket contour (essentially residues 182–195 and 213–228).

The mutant model was obtained by replacing Lys-188 with Phe, Tyr, Trp or His but also by inversion of Lys-188 and Asp-189. Wild-type and mutant geometries were optimized with the same protocol. A first minimization for 1000 iterations fixing the backbone heavy atoms was followed by a second minimization step for 3000 iterations fixing all heavy atoms except those involved in the substrate binding pocket.

2.4. Hydrolysis of synthetic substrates by wild-type and mutant trypsins, in the presence or absence of inhibitors

Succinyl-Ala-Ala-Pro-Arg-pNA and succinyl-Ala-Ala-Pro-Lys-pNA were used for the kinetics studies. Synthetic substrates and inhibitors were dissolved in 100 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0 buffer. Bovine trypsin was previously solubilized in 10 mM HCl and the recombinant trypsins were conserved at pH 2.0 and −20°C. The cleavage of *p*-nitroanilide derivatives was measured by following *p*-nitroaniline released at 405 nm with a Varian Cary 13E spectrophotometer. During the testing of the proteolytic activity inhibition of each inhibitor (BPTI and PSTI II), enzymes (various concentrations according to mutants and substrate) were preincubated for 4 min at 37°C and pH 8.0 with each inhibitor at the indicated concentration. The substrate was then added to the protease inhibitor solution and the volume was adjusted to 500 μl with 50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0 buffer.

2.5. Determination of inhibition constants

The Lineweaver-Burk representation ($1/v = f(1/[s])$) is a current method to determine inhibition constants:

$$K_i = \frac{[i]}{\left[\frac{K'_m}{K_m} - 1\right]}$$

However, when using high affinity inhibitors, a deviation between theoretical and experimental curves appears. Consequently, three other representations, which are more specific for high affinity inhibitors, were used: the Dixon ($1/v = f([i])$), the Henderson ($[i]_t/[1-(v_i/v_0)] = f(v_0/v_i)$) and the Dixon ($v = f([i])$) representations.

3. Results and discussion

3.1. Purification of mutant trypsin

Mutant trypsin was separated from the other molecules of periplasmic extract by cation exchange chromatography (Fig. 2A). The drop of pH from 4.0 to 2.0 allowed the dissociation of the trypsin/ecotin complex (ecotin is an inhibitor of trypsin, secreted by *E. coli*, present in the periplasmic extract). Trypsin collected in the non-retained fractions (as determined from the zymogram) was further purified by affinity chromatography on benzamidine-Sepharose (Fig. 2B). Aliquots of 100 μl were kept frozen at −20°C until use.

3.2. Active site titration

MUGB reacts irreversibly with a 1:1 stoichiometry with the Ser-195 of the catalytic site of trypsin. This reaction induces

Table 1
Active site concentrations of trypsins

Enzyme	Wild-type	K188D/D189K	K188F	K188Y	K188W	K188H	WFY[a]
Active site (nM)	55	27	53	48	56	81	22

[a]WFY: G187W/K188F/D189Y.

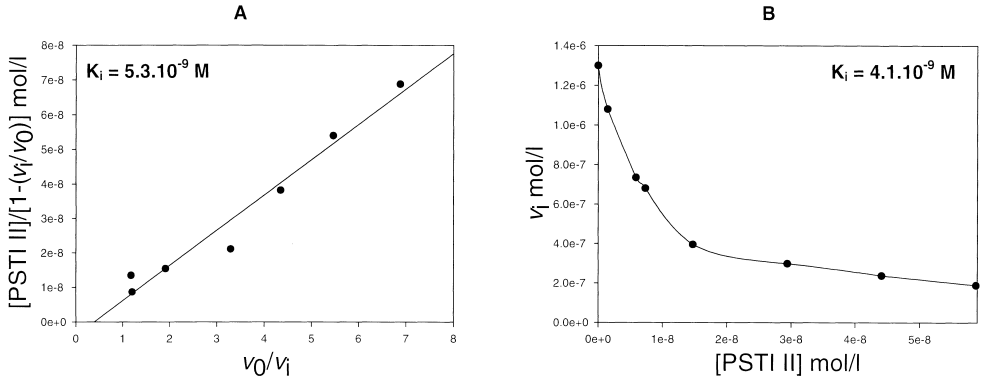

Fig. 3. A: Henderson representation for PSTI II toward K188F. B: Dixon representation for PSTI II toward K188F. Arg = Suc-Ala-Ala-Pro-Arg-pNa.

the formation of a trypsin/guanidinobenzoate complex and the release of 4-methylumbelliferone which is a fluorescent compound. The fluorometer was calibrated with a bovine trypsin solution, the concentration of which was in excess, in such a way that all the molecules of MUGB were hydrolyzed. The active site concentrations of mutant trypsins are given in Table 1.

3.3. Inhibition constants

Inhibitors enter into competition with the specific substrates of trypsin. More precisely, they decrease the enzyme affinity for their substrates, increasing the Michaelis-Menten constant (K_m). The inhibition constant is defined as the equilibrium existing between inhibitor and enzyme.

The determination of K_i is a very important step for the characterization of inhibiting activity of inhibitors. Several methods of calculation exist. The Lineweaver-Burk representation in the presence of inhibitor (PSTI II and BPTI) loses the linear part of the curve for low concentrations in substrate (data not shown). According to Morrisson [40], such a type of curve in the presence of inhibitor is characteristic of a high affinity inhibitor.

The inhibition of mutant K188F is shown in Fig. 3A,B. Lost linearity of the representation v_0=f([i]) [41] for a strong inhibitor concentration is proof that these inhibitors are very specific (Fig. 3B).

Since the inhibitors studied have high affinity for mutant trypsins, the fraction of inhibitors bound to enzyme is important in comparison with the whole concentration of inhibitor. Consequently, the Morrisson equations were used because they show the effect of very specific inhibitors on the initial velocities of hydrolysis. The determination of K_i according to Henderson derives from the Morrisson equations. The equations, making it possible to plot the second Dixon representation (Fig. 3B), take the concentration of bound inhibitor into account. Thus, they can be applied to competitive high affinity inhibitors.

The reaction of inhibition is strictly competitive when the enzyme activity is inhibited totally by the formed enzyme-inhibitor complex [42]. The competitive inhibitors behave as highly specific substrates of the enzymes they inhibit. However, in contrast to enzyme-substrate complexes, which dissociate rapidly, the enzyme-inhibitor complex is highly stable ($K_{ass} \approx 10^8$–10^{13} M^{-1}). This complex, maintained by van der Waals forces and hydrogen bonds, can dissociate releasing native or modified inhibitor. Inhibitors are cleaved specifically between the residues P1 and P′1 (nomenclature of Schechter and Berger [43]), but the localization of the cleaved site on an external loop, closed by a disulfide bridge, allows the modified inhibitor to maintain its three-dimensional structure.

From the calculated inhibition constants (Table 2) it can be concluded that the inhibition of each mutant trypsin by every studied inhibitor is always very powerful ($K_i \gg 10^{-7}$ M). The bovine inhibitor had a higher affinity for all tested enzymes than the pea inhibitor. This could be a consequence of the animal origin of the modified enzyme (rat anionic trypsin). This finding raises an interesting question from the nutritional point of view. It would be worth while to test other trypsin inhibitors of plant origin. The inhibition constants differ according to the mutations on the protease (Table 2). Despite the similarity of the K_m, k_{cat} and k_{cat}/K_m values of K188H, K188F and K188Y, the K_i values of K188H and K188F are

Table 2
Inhibition constants and kinetic parameters of trypsins

Enzyme	Inhibition constant		Kinetic parameters		
	PSTI II K_i (mol/l)	BPTI K_i (mol/l)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Bovine trypsin	$1.3\times10^{-9}\pm2.10^{-10}$	$1\times10^{-10}\pm2.10^{-11}$	37.2 ± 4.3	143 ± 2.4	3.85 ± 1.1
Wild-type enzyme	$2.0\times10^{-9}\pm1.10^{-10}$	$4\times10^{-10}\pm2.10^{-11}$	32.8 ± 7.1	163 ± 31	4.97 ± 1.21
K188D/D189K	$1.47\times10^{-8}\pm1.10^{-9}$	not determined	50.8 ± 4.2	4.7 ± 0.1	0.11 ± 0.01
K188F	$5.2\times10^{-9}\pm1.10^{-10}$	$4\times10^{-10}\pm8.10^{-11}$	55.4 ± 1.3	144 ± 15	2.63 ± 0.31
K188Y	$2.4\times10^{-8}\pm2.10^{-9}$	$3.3\times10^{-9}\pm3.10^{-10}$	53.4 ± 2.2	114 ± 12	2.14 ± 0.33
K188W	$3.5\times10^{-8}\pm4.10^{-9}$	$3.7\times10^{-9}\pm1.10^{-10}$	98.0 ± 8.1	58 ± 3	0.59 ± 0.01
K188H	$2.1\times10^{-8}\pm3.10^{-9}$	$3\times10^{-10}\pm8.10^{-11}$	50.2 ± 4.1	128 ± 3.3	2.56 ± 0.5
G187W/K188F/D189Y	$5.0\times10^{-8}\pm5.10^{-9}$	$8\times10^{-10}\pm5.10^{-11}$	46.6 ± 6.8	5 ± 0.45	0.11 ± 0.01

Substrate: Suc-Ala-Ala-Pro-Arg-pNa.

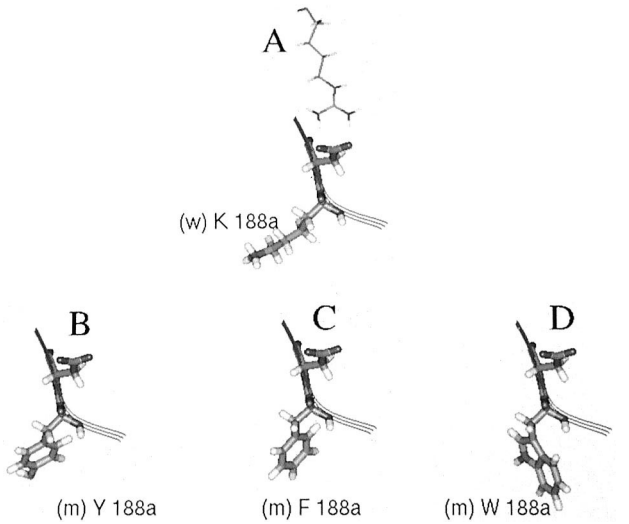

Fig. 4. Molecular modelling representation of Asp-189 and amino acid in position 188 of substrate binding pocket for (A) wild-type enzyme, (B) K188Y, (C) K188F, and (D) K188W mutant trypsins. The segment 187-188-189 is represented by a ribbon. The model substrate is in the upper part.

one order of magnitude lower than that of K188Y. Inhibition of K188H and K188F by PSTI II shows K_i values differing by a factor of four. Generally, the mutants with Asp in position 189 and K188 substituted by aromatic groups remained strongly inhibited by both PSTI II and BPTI but it should be noticed that K188Y was less inhibited by BPTI even though it presented a k_{cat}/K_m value similar to that measured with K188F. The low catalytic activity of the mutants in which Asp-189 is substituted made inhibition measurements difficult in K188D/D189K and G187W/K188F/D189Y. In this case, inhibition was measured during longer intervals of time. With a k_{cat}/K_m value smaller than that of wild-type enzyme (45-fold decrease), the K188D/D189K mutant trypsin showed a K_i value for PSTI II identical with those observed in aromatic mutants. In spite of the experimental conditions used, the determination of K_i values for K188D/D189K inhibition by BPTI was impossible. The lower inhibition by PSTI II was observed with the G187W/K188F/D189Y mutant. This mutant has the same k_{cat}/K_m value as K188D/D189K but is devoid of any negative charge in the substrate binding pocket.

In contrast, the inhibition of mutant G187W/K188F/D189Y by BPTI was roughly equal to that observed with wild-type enzyme or K188F. In the case of this inhibitor, K_i values were less discrepant with the values of K_m of each enzyme measured with Suc-Ala-Ala-Pro-Arg-pNa substrate. The K_m value of the K188D/D189K mutant was similar to that of G187W/K188F/D189Y mutant but, surprisingly, as stated above, K_i could not be measured in the presence of BPTI.

Molecular modelling of the substrate binding pocket of aromatic mutants (Fig. 4) shows that these mutations do not perturb the binding site structure and thus they do not prevent substrate or inhibitor fixation. The same observations can be made with the K188H mutant trypsin (data not shown).

Graf et al. [13] found that D189K is devoid of trypsin activity but possesses residual chymotryptic activity. BPTI, with its protruding reactive K15, could be repelled by K189 of the D189K binding pocket. Molecular modelling of the substrate binding pocket of K188D/D189K mutant shows a modification of the pocket structure due to the displacement of the negative charge of Asp-189→188. This modification can force the side chain of the substrate to change its orientation (Fig. 5). Surprisingly, the inhibition of this mutant by PSTI II is relatively strong in comparison with its k_{cat}/K_m value. It is possible that K188D/D189K could also, like D189K, possess chymotryptic activity and that its inhibition by PSTI II is due to the presence in this inhibitor of a separate reactive site for chymotrypsin. However, K188D/D189K showed no chymotryptic activity when measured with β-casein as natural substrate [15].

The disparity between the affinity of two inhibitors is reversed and less pronounced in G187W/K188F/D189Y. Molecular modelling of the substrate binding pocket of G187W/K188F/D189Y mutant is under investigation. With this mutant, K15 of BPTI would not be repelled by Y189 of the trypsin mutant.

Thus, except for the K188D/D189K mutant, the inhibition

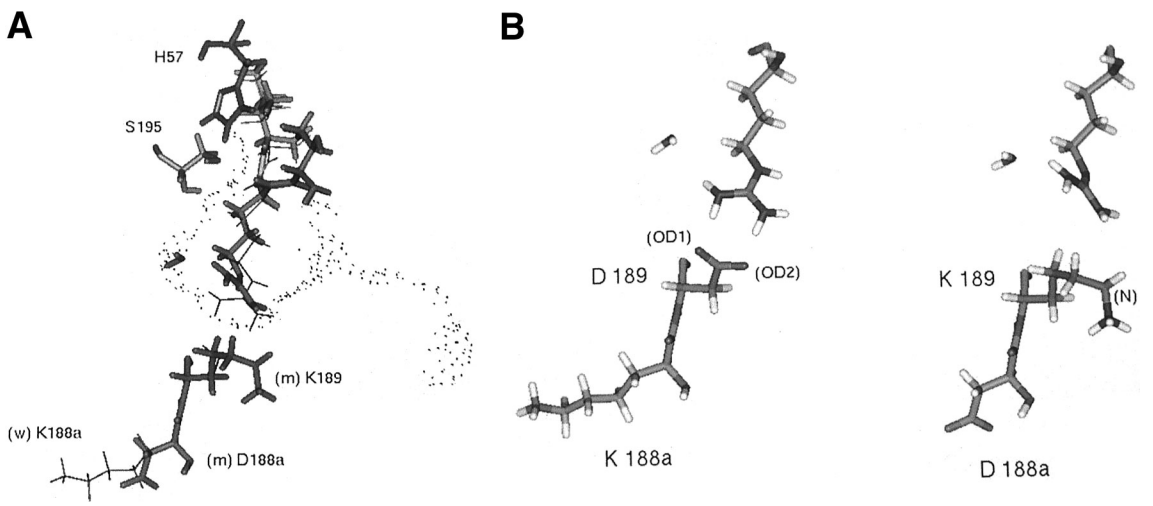

Fig. 5. Molecular modelling representation (A) of substrate binding pocket (dots) of wild-type trypsin (thin lines) and K188D/D189K mutant (thick lines), in the presence of a tripeptide substrate; (B) of residues 188 and 189 of the substrate binding pocket in wild-type and K188D/D189K trypsins.

differences seem to be due to variations of affinity of modified enzymes for their substrate (the competitive inhibitors behave like substrates and are fixed in the same place) in comparison with the wild-type enzyme.

Since the studied inhibitors present a lysyl residue in position P1, the reasons why these high affinity inhibitors (PSTI II and BPTI) are not good substrates are not completely understood [44–47].

In order to decrease or eliminate the antinutritional quality of these inhibitors, it is of interest to follow their catabolism by the different mutant trypsins.

Previous results obtained by hydrolysis of β-casein by the aromatic mutants showed that they are able to cleave peptide bonds involving Asn and Gln residues. This may be interesting since inhibitors are often rich in amide residues.

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