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How the Substitution of K188 of Trypsin Binding Site by Aromatic Amino Acids Can Influence the Processing of β-Casein

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Aspartyl 189 residue of trypsin is known to be essential for specific lysis of Arg-X and Lys-X bonds. Undertaking to modulate the catalytic properties of this protease, otherwise highly conserved K188 was replaced with aromatic amino acid residues aiming the perturbation of the electrostatics and the amplifying of hydrophobic interactions of the substrate binding site. The catalytic properties of the mutants K188F, K188Y, and K188W were measured at pH 7, 8, 9, and 10 using a pair of synthetic tetrapeptide *p*-nitroanilide substrates and beta-casein. The kinetic analysis reveals that all the mutants conserve the native trypsin capacity to split peptide bonds containing arginyl and lysyl residues. Surprisingly, however, depending on mutation, the optimum pH of activity changes. As demonstrated only by proteolysis of a natural substrate, all mutants cleave also peptide bonds involving asparagine and glutamine. These stuttered cleavage sites are close to the β-casein fragments in β-sheet according to Hydrophobic Cluster Analysis. © 1998 Academic Press

Key Words: **trypsin; serine protease; site-directed mutagenesis; substrate specificity; β-casein; proteolysis; pH effect.**

It is known that the protein nutrition in children and adults may be a limiting factor if the availability of the nitrogen required for the biosynthesis of proteins and nucleic acids is restrictively small. 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 either directly, in the form of neurotransmitters, or indirectly stimulating the secretion of hormones and enzymes from the intestinal receptors. It is believed that several peptides arising from hydrolysis of β-casein have important physiological functions [1-3]. Many of dietetic and pharmaceutic uses of milk proteins and derived peptides were reviewed by Maubois and Léonil [4]. Consequently, proteolysis of these proteins *in vitro* with gastric or pancreatic proteases may present significant nutritional and nutraceutic interest and as such was investigated thoroughly [5-9].

Various proteases are used in primary structure determination or as probes of protein conformation and of its changes [10]. It is well known that the cleavage of all peptide bonds does not occur at the same rate and/or at the same time and may be highly structure dependent. It is influenced by the proteinase binding substrate site specificity and the ease of positioning of the substrate on the enzyme molecule, and further, by the presentation of cleavable peptide bond.

The quest to design enzymes with novel specificities is one of the major challenges of protease engineering. The best characterized examples of substrate specificity determinants which do not explain however the character of substrate/protease contact are available for the serine proteases. Their substrate specificity is quite different. Trypsin hydrolyzes peptides with arginine or lysine in the P1 position, according to the nomenclature of Schechter and Berger [11]. Chymotrypsin splits peptides containing large hydrophobic residues in this position, and elastase prefers small aliphatic P1 residues. These different substrate specificities result from apparently simple structural changes of the substrate binding pockets of these proteases. A series of studies have addressed the role of the negatively charged Asp189 residue in binding and reactivity by site-directed mutagenesis as well as by genetic selection for the isola-

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tion of new variants [12, 13]. Kinetic characterization of these proteases, as well as characterization of the mutants D189K [14], D189S [15], K188D/D189K [16] and G187W/K188F/D189/Y [17] indicate that the presence of a negative charge at the base of the binding pocket is essential for efficient catalysis by trypsin. However, the mutation of Asp189 to Ser in trypsin does not convert trypsin into a protease with chymotrypsin-like specificity. D189S mutant gives rather sluggish and non specific proteinase. Additional mutations of the residues forming substrate binding site of trypsin into the analogous residues of chymotrypsin do not induce chymotrypsin-like specificity in trypsin either. Trypsin mutant containing chymotrypsin-like binding site acquires chymotrypsin-like specificity only after replacement of two surface loops [loop 1 (residues 185-188) and loop 2 (residues 221-225)] with analogous residues of chymotrypsin [18]. Chymotryptic activity of this mutant enzyme was enhanced to 2-15% by additional mutation Tyr172Trp [19].

Alignments of amino acid sequences revealed that porcine pancreatic elastase, fiddler crab collagenase and rat trypsin have all either Lys or Arg in position 188 [20]. However, all these enzymes show different specificities. Consequently, the amino acid in position 188 plays an important role differing, however, from outright definition of protease specificity determination. In search for modifications of catalytic properties without radical decrease of the trypsin activity, a substitution of Lys188 was executed recently. Replacement of Lys188 with histidine created a metal chelation site in the substrate binding pocket of trypsin, build in a metal binding AE switch AF, and extended the maximum of its activity towards lower pH [21]. The present study aimed at charge neutralization and the creation of possibility for stacking interactions between aromatic moieties of the protease binding site and the substrate. Three mutants were produced substituting Lys188 with aromatic amino acids (Phe, Tyr or Trp). This modifies also hydrophobic interactions in the neighborhood of the Asp189 residue. The catalytic properties of the mutated enzymes, determined in respect to hydrolysis of synthetic substrates and of a protein (β-casein) at different pH are described and compared.

EXPERIMENTAL PROCEDURES

Trypsin mutant were prepared by oligonucleotide-directed mutagenesis carried out by the method of Kunkel [22], using single-stranded, uracil containing DNA templates as previously described [21]. The 30-base oligonucleotide primers directing the phenylalanine, 5′-GCCCTGGCAGGAATCAAAGCCTCCCTCTAG-3′, tyrosine, 5′-GCCCTGGCAGGAATCATAGCCTCCCTCTAG-3′ and tryptophane, 5′-GCCCTGGCAGGAATCCCAGCCTCCCTC-TAG-3′, substitution of Lys-188 were synthesized with a Biosearch Cyclone™ DNA synthesizer (mismatched bases are underlined). Wild-type, K188F, K188Y and K188W trypsins were secreted in the periplasm space of *E. coli* and purified by chromatography on CM-Sepharose fast-flow column and benzamidine-Sepharose column as previously described [23].

Kinetic measurements. Succinyl-Ala-Ala-Pro-Arg-*pNA*, succinyl-Ala-Ala-Pro-Lys-*pNA*, succinyl-Ala-Ala-Pro-Phe-*pNA* and succinyl-Ala-Ala-Pro-Leu-*pNA* were used for the kinetic studies. Their hydrolysis was monitored spectrophotometrically at 405 nm in a Varian Cary 13E spectrophotometer. Twelve substrate concentrations from the appropriate range were used for determining initial rates. The initial slopes of the reaction curves were measured to yield ΔA/min. Michaelis-Menten parameters were calculated from Eadie-Hofstee plots. Hydrolysis were performed at 37°C using 0.2 M imidazole, 20 mM $CaCl_2$, pH 7; 50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8 and 0.2 M glycine-NaOH, 20 mM $CaCl_2$, pH 9 and 10.

β-Casein hydrolysis and peptide assignment. Crude β-casein A1 was prepared as described by Zittle and Custer [24]. It was purified further by chromatography according to the method of Mercier et al. [25] on a Q-Sepharose fast-flow column. β-Casein was dissolved (1 mg/ml, initial concentration) in the appropriate buffer (0.2 M Tris-HCl, for pH 7.0 - 9.0; 0.1 M glycine-NaOH, pH 10). The final concentration of β-casein was determined spectrophotometrically using for calculations the molecular absorption coefficient E_{280} = 11,000 $M^{-1} \cdot cm^{-1}$. Active enzyme was added to β-casein solution at an enzyme/substrate ratio (E/S) of 0.005, 0.01, 0.02 and 0.02% (W/W) for wild-type, K188F, K188Y and K188W mutants, respectively. The reaction mixture was incubated at 37°C. Aliquots were taken at intervals (30 min, 1, 2, 4, 8, 24 and 48 h) and the hydrolysis was stopped by addition of 0.5 volume of 0.2 N HCl. Tryptic peptides of β-casein were separated by reversed-phase HPLC on a Nucleosil C_{18} column (4.6 mm i.d. × 25 cm, SFCC, Gagny, France) with a linear gradient from solvent A (0.11% TFA in H_2O) to 100% solvent B (60% acetonitrile, 40% H_2O, 0.09% TFA) in 62.5 min [26]. The peptides produced were identified by electrospray mass spectrometry on Perkin-Elmer Sciex (Thornhill, Ontario, Canada) API III^+ mass spectrometer.

Computer modeling method. Molecular modeling studies were carried out on Silicon Graphics computers with Biosym/MSI packages. Molecular displays and energy minimization were performed with Insight II, Biopolymer and Discover modules. For all calculations, the CFF91 forcefield was selected. The calculation conditions (especially the complete restoration of the trypsin model from two sets of X-ray data) have been described in detail in a previous paper [16]. We shall just mentioned here the specificities of the calculations concerning the mutants discussed in this paper.

These calculations were done in two steps. The first one consisted in building the different mutants (F, Y and W) on residue 188a (amino acid labeling according to the X-ray crystal structure of Brinen et al. [27]) and performing energy comparisons with the wild-type in the absence of substrate. Then, the effects of these mutations were estimated on the binding of the substrate. In the first stage, Lys 188a was replaced by Phe, Tyr and Trp residues using Insight II facilities. For each case, all essential side chain orientations were tested and the best solution was kept for further calculations (Biopolymer facility). Finally, the addition of hydrogen atoms was done with respect of pH 8 (Insight II facility) and both wild-type and mutant molecules were optimized with a minimization of 3000 iterations fixing all heavy atoms except those belonging to the side chains of residues involved in the substrate binding site. During the second step, the docking of a small substrate fragment (Thr-Arg-Ile, simulating segment Thr24-Arg25-Ile26 of β-casein) with the wild-type and the mutants was performed according to the procedure already described [16]. Finally the interaction energies (enzyme+water/substrate) were used to estimate the enzyme/substrate affinity. For sake of comparisons, we have also included the results obtained for the K188D/D189K mutant [16].

RESULTS

Despite the importance of β-casein in human nutrition and technology, diversification of its processing by mutated proteinases was not studied until the recent work of Briand et al. [21]. On the other hand, the activities and specificities of the majority of recombinant proteinases were studied with the help of artificial substrates only. A metal-assisted trypsin N143H, E151H, D189S with a specificity towards an octapeptide that has a Tyr at P1 and a His at P2′ was described recently [28]. Synthetic substrates allow to calculate rapidly catalytic constants of an enzyme. All this was done despite the knowledge that the environment of the peptide bond depends largely on physico-chemical conditions of the applied media, and imposed steric hindrance. Since these parameters are important, the hydrolysis of purified β-casein was studied at different pHs. The use of β-casein as a test substrate presents, besides the importance of this protein in food industry, advantages of releasing the hydrolysis from several structural limitations characteristic of many other potential native protein substrates. The use of protein allows the understanding of the scope and validity of the results obtained with synthetic substrates. Additionally, the harnessing of mutated trypsins into the processing of β-casein diversifies the peptide products obtained.

Hydrolysis of Synthetic Substrates

Kinetic analysis of wild-type and mutant trypsins at pH 8. The trypsin mutants were characterized first analyzing the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m for comparable artificial tetrapeptide arginine and lysine substrates. Compared with trypsin, mutants K188F, K188Y and K188W exhibit a 1.6- to 3-fold increase of K_m for arginine and lysine containing substrates, respectively (Table 1). These mutants have k_{cat} similar to one observed with wild-type except for a 3- to 4-fold decrease for K188W mutant. While the second-order rate constant k_{cat}/K_m of K188W trypsin mutant decreases ≈ 7- and 12-fold for substrates containing arginine and lysine, respectively, 2-fold decrease is observed for K188F and K188Y trypsin mutants. Wild-type and K188Y trypsins show 6-fold arginine/lysine preference whereas K188F and K188W trypsins prefer the arginine substrate over the lysine substrate by a factor 8 and 10, respectively.

Influence of pH on the kinetic parameters of wild-type trypsin (Table 1). The kinetic study realized with succinyl-Ala-Ala-Pro-Arg-*pNA* substrate showed pH-dependence of K_m. The smaller value is obtained at pH 8. It increases at pH 9 then at pH 7 and is 3-fold higher at pH 10. Study realized with succinyl-Ala-Ala-Pro-Lys-*pNA* substrate indicates that K_m are identical at pH 8 and 9, and increase 2- and 8-fold at pH 10 and 7, respectively. k_{cat} values are similar at pH 8, 9 and 10 but they decrease at pH 7, whatever the substrate used. Consequently, the second-order rate constant k_{cat}/K_m shows the highest value at pH 8 for succinyl-Ala-Ala-Pro-Arg-*pNA* substrate, and at pH 8 and 9 for succinyl-Ala-Ala-Pro-Lys-*pNA* substrate. Wild-type trypsin displays a 4 to 6-fold arginine/lysine preference at pH 8, 9 or 10 but prefers the substrate containing arginine over lysine by a factor 23 at pH 7.

Influence of pH on the kinetic parameters of K188F mutant trypsin (Table 1). K_m of hydrolysis of synthetic substrate containing arginine measured at pH 8 and 10 are a half lower from those obtained at pH 7 and 9.

TABLE 1

Kinetic Parameters of Wild-Type and Mutant Trypsins According to the pH

pH	Substrate*	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} μM^{-1})
Wild-type				
pH 7	Arg	87 ± 5	95 ± 7	1.1 ± 0.05
pH 8		33 ± 7	163 ± 31	5.0 ± 1.2
pH 9		57 ± 6	144 ± 20	2.5 ± 1.0
pH 10		99 ± 3	145 ± 1	1.5 ± 0.04
pH 7	Lys	839 ± 100	40 ± 7	0.05 ± 0.02
pH 8		105 ± 15	74 ± 15	0.71 ± 0.21
pH 9		101 ± 10	59 ± 8	0.60 ± 0.12
pH 10		184 ± 4	64 ± 1	0.35 ± 0.06
K188F				
pH 7	Arg	101 ± 10	54 ± 5	0.52 ± 0.08
pH 8		55 ± 1	144 ± 15	2.63 ± 0.31
pH 9		113 ± 10	185 ± 22	1.69 ± 0.43
pH 10		63 ± 5	164 ± 42	2.62 ± 0.32
pH 7	Lys	1883 ± 250	105 ± 5	0.06 ± 0.004
pH 8		188 ± 20	61 ± 12	0.32 ± 0.07
pH 9		208 ± 15	57 ± 3	0.27 ± 0.05
pH 10		169 ± 30	69 ± 4	0.44 ± 0.15
K188Y				
pH 7	Arg	503 ± 17	108 ± 4	0.21 ± 0.05
pH 8		53 ± 2	114 ± 12	2.14 ± 0.3
pH 9		68 ± 5	146 ± 2	2.16 ± 0.2
pH 10		63 ± 3	170 ± 16	2.72 ± 0.3
pH 7	Lys	1612 ± 90	38 ± 8	0.02 ± 0.008
pH 8		107 ± 10	43 ± 4	0.41 ± 0.03
pH 9		156 ± 12	39 ± 7	0.28 ± 0.06
pH 10		278 ± 8	89 ± 3	0.31 ± 0.02
K188W				
pH 7	Arg	706 ± 9	51 ± 5	0.07 ± 0.002
pH 8		98 ± 8	58 ± 3	0.59 ± 0.01
pH 9		87 ± 6	57 ± 4	0.66 ± 0.05
pH 10		848 ± 49	50 ± 2	0.06 ± 0.003
pH 7	Lys	2837 ± 58	24 ± 3	0.008 ± 0.0002
pH 8		292 ± 25	17 ± 4	0.059 ± 0.013
pH 9		595 ± 27	27 ± 4	0.045 ± 0.004
pH 10		3660 ± 56	—	—

* Substrates: Suc-Ala-Ala-Pro-Arg-*pNA* and Suc-Ala-Ala-Pro-Lys-*pNA*.

k_{cat} values are identical at pH 8, 9 and 10 being strongly reduced at pH 7. Hydrolysis of a synthetic substrate containing lysyl residue demonstrates K_m values identical at pH 8, 9 and 10 increasing 10-fold at pH 7. As observed with succinyl-Ala-Ala-Pro-Arg-*pNA*, k_{cat} values are identical at pH 8, 9 and 10 and they increase at pH 7. The values of k_{cat}/K_m show a comparable evolution for each substrate indicating a 9- to 6-fold arginine/lysine preference from pH 7 to pH 10.

Influence of pH on the kinetic parameters of K188Y mutant trypsin (Table 1). While K_m for succinyl-Ala-Ala-Pro-Arg-*pNA* substrate increase 10-fold at pH 7 (as compared with K_m measured at pH 8), they are almost unchanged at other pHs. K_m measured for succinyl-Ala-Ala-Pro-Lys-*pNA* substrate increase 16-, 1.5- and 2.8-fold at pH 7, 9 and 10 when compared to K_m measured at pH 8. k_{cat} increase with pH, giving values 1.6- to 2.4-fold higher than one observed at pH 7. k_{cat}/K_m determined with both synthetic substrates display comparable pH dependence, showing the 10-fold drop at pH 7. A 5- to 9-fold arginine/lysine preference can be observed in the pH range 7-10.

Influence of pH on the kinetic parameters of K188W mutant trypsin (Table 1). K188W mutant shows large increase in K_m at pH 7 and 10. Whatever the pH, k_{cat} values are similar and very low. This constant was impossible to measure at pH 10 with synthetic substrate containing lysyl residue. K188W mutant prefers arginine containing substrate over the lysine substrate by a factor 15 at pH 9 while wild-type trypsin has 4-fold preference at this pH. However, this modulation of preference is obtained at the expense of its dramatically reduced catalytic activity.

Comparison of catalytic properties of native and mutant trypsins (Table 1). Wild-type trypsin and mutants K188F and K188Y show similar pH dependence in K_m, reaching the highest value at pH 7, when measured with succinyl-Ala-Ala-Pro-Lys-*pNA* substrate. Mutant K188W displays the highest K_m at pH 7 and 10. The K_m determined with succinyl-Ala-Ala-Pro-Arg-*pNA* is less influenced by the pH except for the mutant K188Y which has an increased K_m at pH 7 and for K188W mutant manifesting the highest K_m at pH 7 and 10.

The mutant K188W reveals the lowest k_{cat} values independently of the pH for both synthetic substrates. In contrast to the wild-type and other studied mutants, K188F trypsin reveals its highest k_{cat} value at pH 7 for succinyl-Ala-Ala-Pro-Lys-*pNA* substrate. This value is the highest from the values measured with all other trypsin mutants.

The introduction of a tyrosyl residue in position 188 decreases the k_{cat} of the enzyme, when measured with a lysine containing substrate at pH 7, 8 and 9, but the k_{cat} increases at pH 10. That large increase of k_{cat} at pH 10 of K188Y mutant is the highest observed at this pH. The second-order rate constant k_{cat}/K_m of wild-type trypsin, shows a marked maximum at pH 8, while K188F and K188Y mutants display a plateau from pH 8 to 10 with a drop at pH 7 only. The K188W substitution reduces the activity toward these substrates (Arg or Lys at the P1 site) by a 7- to 12-fold at pH 8 and gives rise to the less efficient protease.

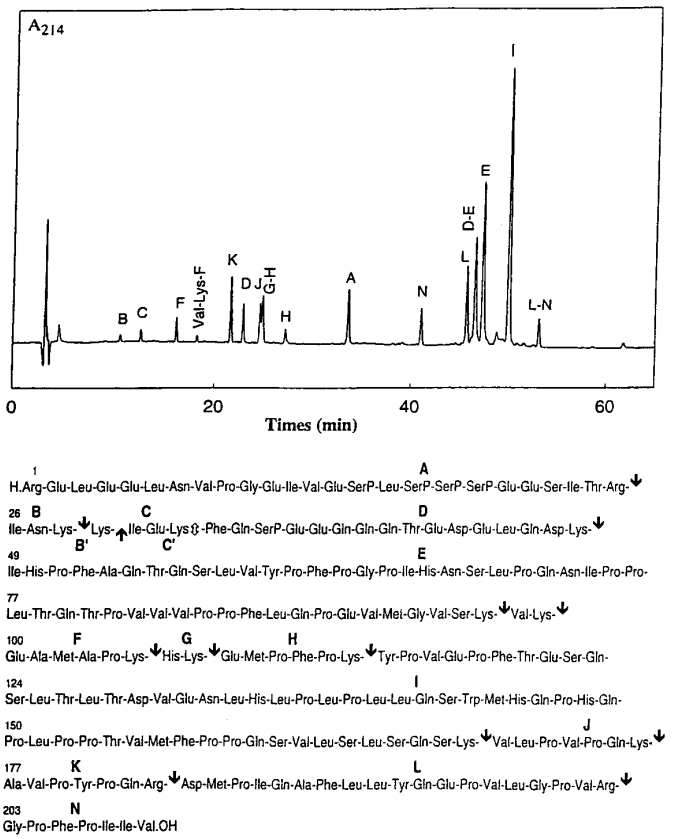

FIG. 1. HPLC profile of tryptic peptides from bovine β-casein, and their localization in the sequence of the protein.

Hydrolysis of β-Casein by Wild-Type and Mutant Trypsins

Bovine β-casein was incubated at pH 8 with wild-type and mutant trypsins at E/S ratios (0.005, 0.01, 0.02 and 0.02% (W/W), for wild-type, K188F, K188Y and K188W trypsins, respectively) adapted in the way that the substrate disappeared with the same velocity. The E/S ratios used for hydrolysis at pH 7, 9 or 10 were the same than those used at pH 8. Peptide mixtures obtained after 48h of hydrolysis of β-casein by the studied trypsin mutants were separated on a C_{18} column and identified by mass spectrometry. The major peptides detected were named in alphabetical order according to the potential tryptic cleavage sites (Fig. 1).

pH dependence of tryptic profiles. During the hydrolysis of β-casein by the proteases studied, the pH affects their catalytic activities and may also modify the conformation and electrostatic charges of the substrate. In the case of the comparative study of wild-type and mutant trypsin activities at the same pH, the role of the conformation of the substrate can be neglected (since comparable) and the observed differences can be assigned to the effect of mutation only.

The kinetics of β-casein fragmentation by wild-type trypsin demonstrate that a maximum of its activity is reached at pH 8 and 9. A lower activity is observed at pH 7 and the lowest efficiency at pH 10 (Fig. 2).

The variation of hydrolysis of β-casein as function of the pH can be well followed by the detection of peptides products such as *B* (Ile26-Lys28), *C* (Lys29-Lys32), *H* (Glu108-Lys113), *I* (Tyr114-Lys169), Val98-Lys99-*F* (Val98-Lys105) and *G-H-I* (His106-Lys169). In spite of decreases of catalytic efficiency with pH observed with synthetic substrates, the cleavage of peptide bonds leading to the liberation of peptides *A* (Arg1-Arg25) and *N* (Gly203-Val209), produced by hydrolysis of peptide bonds involving an arginine, and of peptides *F* (Glu100-Lys105), *J* (Val170-Lys176) and *K* (Ala177-Arg183), produced by hydrolysis of peptide bonds containing lysine is unchanged. The yield of some peptides is reduced at pH 10 only. This is the case of peptides *E* (Ile49-Lys97), *D-E* (Phe33-Lys97), *G-H* (His106-Lys113) and *L-N* (Asp184-Val209). At this pH, the hydrolysis of the bond Lys48-Ile49 is significantly reduced.

Hydrolysis of β-casein with K188F trypsin (Figs. 2 and 3). Compared with hydrolysis by wild-type trypsin, the liberation of the N-terminal phosphopeptide *A* (Arg1-Arg25) is delayed (still undetected after 30 minutes hydrolysis). The main reason seems to be the persistence until 8 h of hydrolysis of the peptide *A-B* (Arg1-Lys28). This peptide was never obtained during hydrolysis of β-casein by wild-type trypsin because the Arg25-Ile26 bond is rapidly splitted yielding the peptides *A* and *B*. However, the processing of β-casein C-terminal is the same since the appearance of the C-terminal *L-N* peptide (Asp184-Val209) after the cleavage of Arg183-Asp184 bond by K188F is identical as with wild-type enzyme. The subsequent hydrolysis of Arg202-Gly203 bond is largely delayed since after 48 h of hydrolysis only small quantity of the peptide *N* is obtained.

In the first hour of hydrolysis, hydrophobic peptides (with a high retention time on C_{18} column) are produced before the major tryptic peptides. The peptide bond Lys105-His106 is resistant to hydrolysis by K188F mutant since peptide *F-G* (Glu100-Lys107) is observed, leading to a delayed appearance of peptide *G-H* (His-106-Lys113) and an earlier obtaining of peptide *H* (Glu108-Lys113). The low yield of peptide *C* (Ile30-Lys32) could be explained by the persistence of peptide *C-D-E* (Ile30-Lys97) even after 8 h of hydrolysis. Between 8 and 48 h of incubation, peptide *C-D-E* is fragmented in *C* (Ile30-Lys32) and *D-E* (Phe33-Lys97) the last being completely hydrolyzed thereafter as observed by the increase of the peak corresponding to peptide *E*, showing higher susceptibility of the peptide bond Lys48-Ile49.

The kinetics of hydrolysis of β-casein reveal that K188F mutant, in contrast to wild-type trypsin, has optimum activity at pH 7, which decreases slightly at pH 8 and 9 and is largely lowered at pH 10 whatever the kind of peptide bond (Lys-X or Arg-X) is aimed at. The most important feature during the hydrolysis of β-casein by K188F trypsin is the obtaining at pH 7, in smaller amounts at pH 9 and 10, of novel peptides. Surprisingly, none of them is obtained at pH 8.

At pH 7 and 9, the hydrolysis of the peptide bond Pro51-Phe52 was determined by the detection of peptides Phe33-Pro51 and Phe52-Lys97, and the cleavage of peptide bond Ser57-Leu58 was indicated by the presence of peptides Phe33-Ser57 and Leu58-Lys97. The cleavage of the bond Ala189-Phe190 was revealed by identification of peptide Phe190-Arg202. The detection at pH 10 of the peptide Phe33-Gln56 allowed to confirm the hydrolysis of the peptide bond Gln56-Ser57 (Fig. 4).

β-casein hydrolysis with K188Y trypsin (Figs. 2 and 5). The hydrolysis of β-casein shows that K188Y as wild-type trypsin presents an activity which is maximal at pH 8 and 9, smaller at pH 7 and lowered significantly at pH 10. In contrast with what was observed in case of K188F mutant, the phosphopeptide *A* (Arg1-Arg25) is obtained rapidly. The peptide *L-N* (Asp184-Val209) is fragmented after 4 h of hydrolysis in peptides *L* (Asp184-Arg202) and *N* (Gly203-Val209) while the peptide bond Arg202-Gly203 is particularly resistant to hydrolysis by K188F mutant. The peptides resulting from cleavage of bonds involving lysyl residues are obtained in comparable yields as those produced by wild-type trypsin.

The decreased yield of peptides *D-E* (Phe33-Lys99), *G-H-I* (His106-Lys169) and *L-N* (Asp184-Val209) is partly due to the cleavage of peptide bonds containing lysyl or arginyl residues and in part to non tryptic cleavages as it was identified by mass spectrometry. When the hydrolysis is carried out at pH 8, the following peptide bonds are attacked: SerP18-SerP19, SerP35-Glu36, Gln38-Gln39, Gln40-Thr41, Gln56-Ser57, Leu58-Val59, Ile66-His67, His67-Asn68, Phe119-Thr120, Gln141-Ser142, Trp143-Met144, Ser161-Val162, Leu163-Ser164, Phe190-Leu191, Leu191-Leu192, Tyr193-Gln194 and Gln194-Glu195. Some of these bonds are splitted also at other pHs. However, the hydrolysis of peptide bonds Thr120-Glu121 and Val155-Met156 is observed only at pH 10 and 9, respectively. The peptide bond Ile208-Val209 is only cleaved at pH 7 and 9 (Fig. 4).

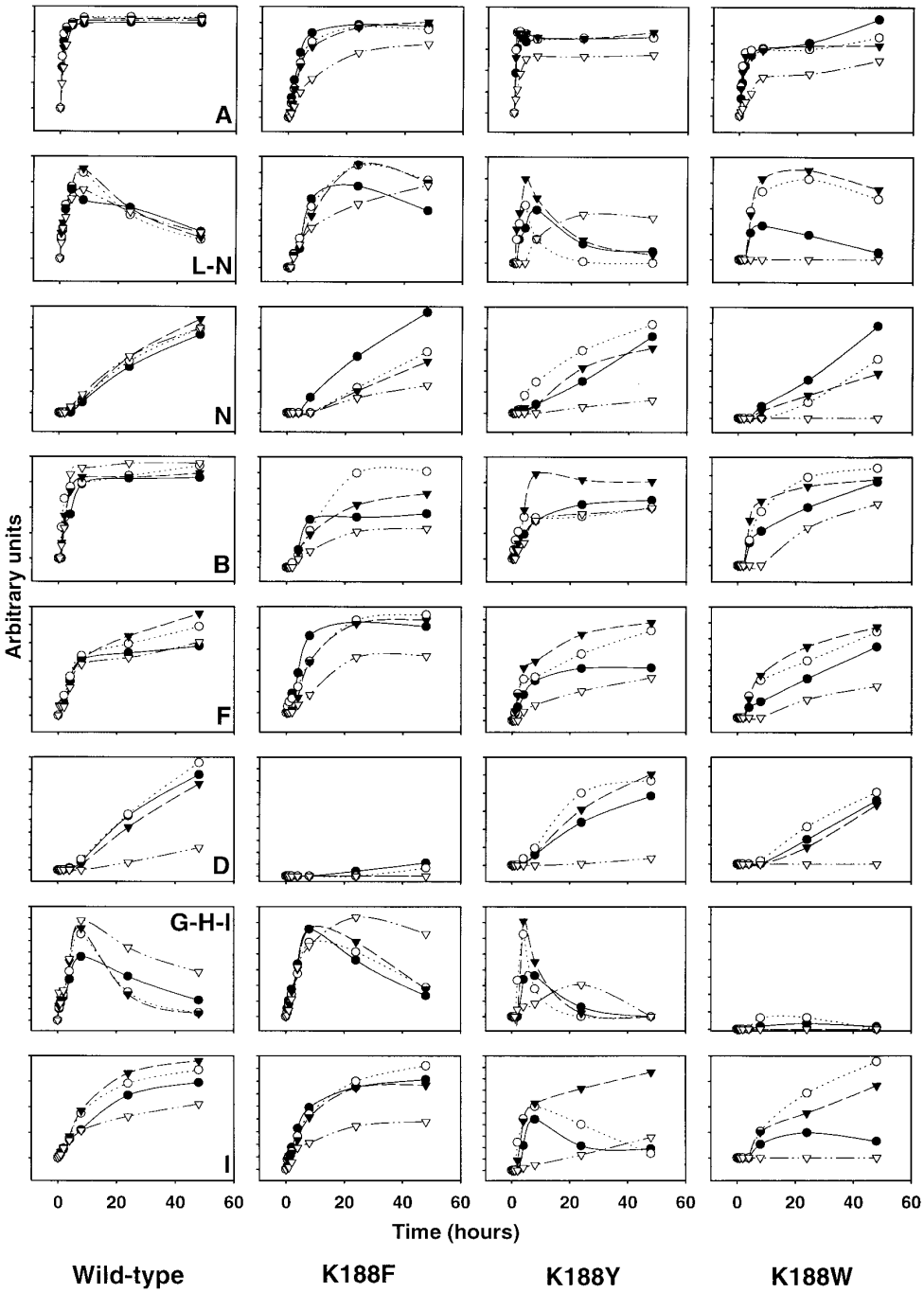

FIG. 2. Evolution of some tryptic peptides obtained from hydrolysis of β-casein at pH 7 (●), 8 (○), 9 (▼) and 10 (▽) with wild-type, K188F, K188Y, and K188W trypsins. Peptides are names as in Fig. 1.

Hydrolysis of β-casein with K188W trypsin (Figs. 2 and 6). The hydrolysis of β-casein at pH 8 by K188W trypsin mutant is similar to one observed with wild-type trypsin although the most of hydrophobic peptides are hydrolyzed slower during the initial phase of incubation. Compared with mutants K188F and K188Y, trypsin mutant K188W differs by the difficulty to process the large tryptic peptides (*G-H-I*, *D-E*, *L-N*) towards the major wild-type end products. The maximum of its catalytic activity is observed at pH 8 and 9. It lowers at pH 7. This enzyme has a very low activity at pH 10 since peptides *G-H-I* (His106-Lys169), *G-H* (His106-Lys113), *D-E* (Phe33-Lys97), *L-N* (Asp184-Val209), *D* (Phe33-Lys48), *E* (Ile49-Lys97), *H* (Glu108-

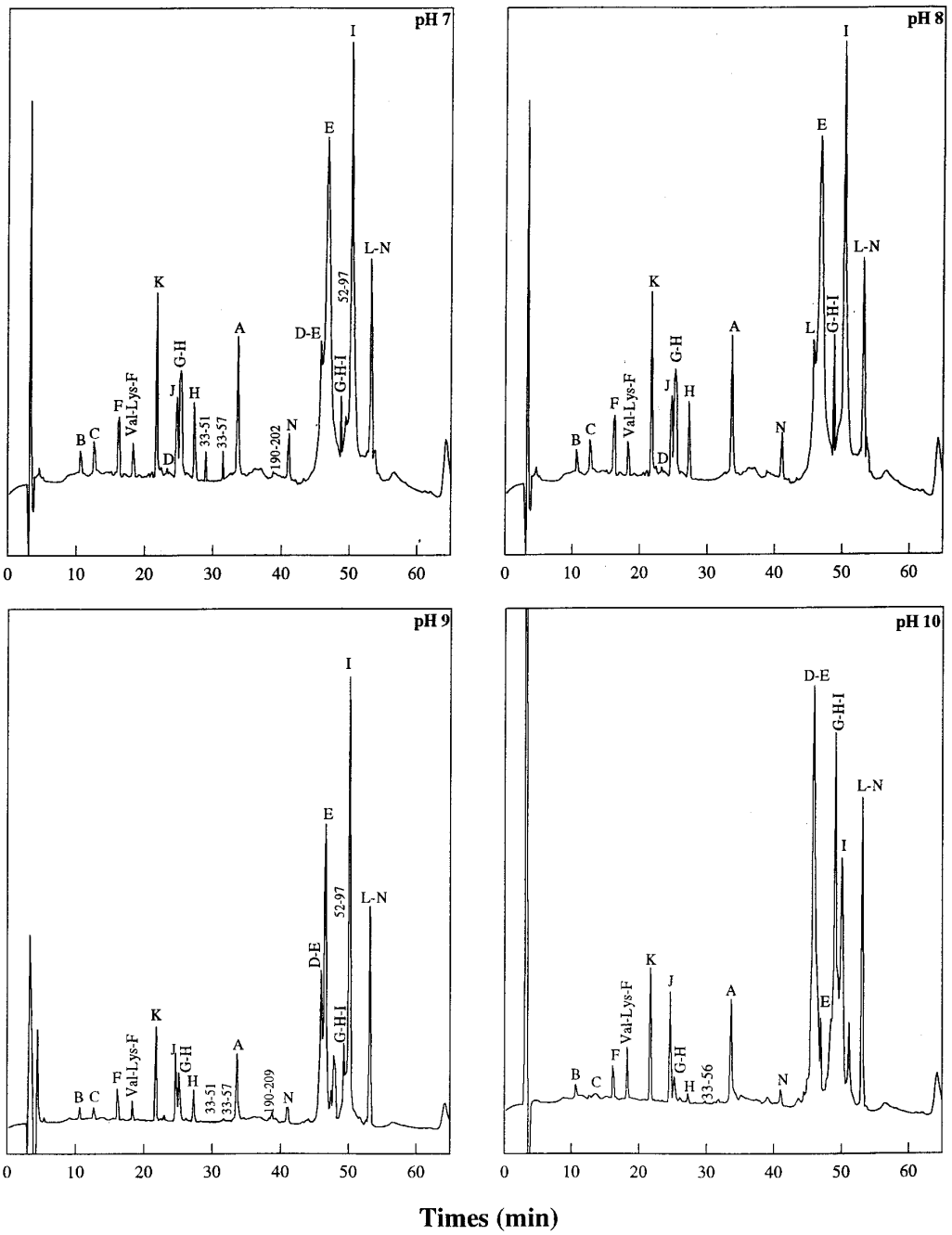

FIG. 3. HPLC profiles of tryptic peptides obtained from 48 h of hydrolysis at pH 7, 8, 9, and 10 of β-casein with K188F trypsin.

Lys113), *I* (Tyr114-Lys169) and *N* (Gly203-Val209) are not obtained at all.

At pH 7, the decreased output of peptides *L-N* (Asp184-Val209) and *I* (Tyr114-Lys169) is partly due to their rapid hydrolysis. As observed in case of K188Y mutant trypsin, peptide bonds not containing basic lysyl or arginyl residues are hydrolyzed too. They were identified by mass spectrometry as: Gln38-Gln39, Gln40-Thr41, Phe52-Ala53, Gln56-Ser57, Leu58-Val59, Ile66-His67, Asn68-Ser69, Gln123-Ser124, Leu137-Pro138, Leu140-Gln141, Gln141-Ser142, Trp143-Met144, Gln160-Ser161, Leu163-Ser164, Phe190-Leu191, Leu191-Leu192, Gln194-Glu195 and Ile207-Ile208 (Fig. 4).

DISCUSSION

As demonstrated by activity profiles of the tryptic mutants studied, it is possible to generate new profiles

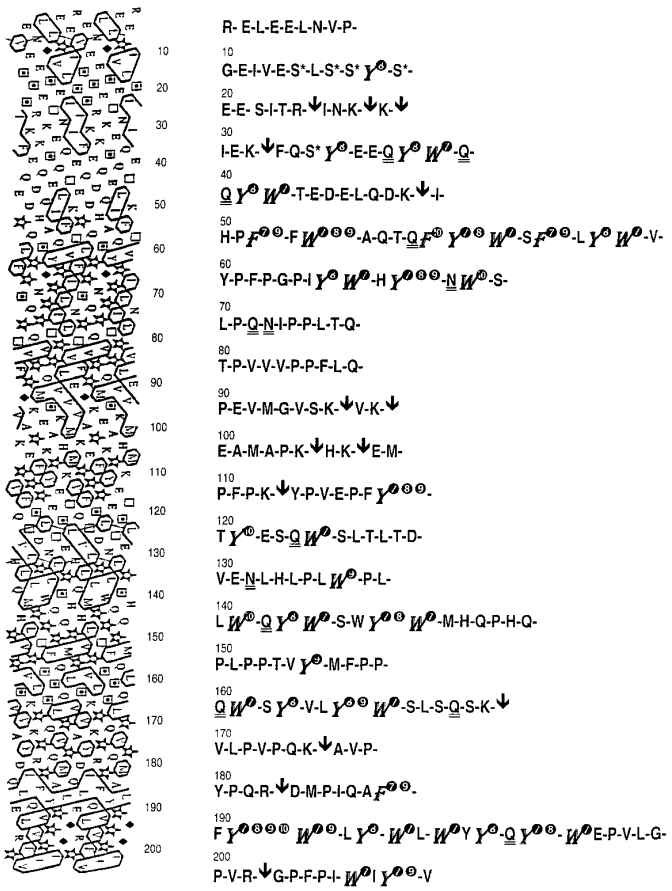

FIG. 4. Left : HCA plots of β-casein ; symbols are : Pro (★); Gly (♦); Ser (□) ; Thr (□). Right : Cleavage sites obtained from 48 h of hydrolysis of β-casein by K188F (***F***), K188Y (***Y***) and K188W (***W***) at pH ❼, ❽, ❾ and ❿.

of specificity and to maintain high lytic activity by modifying polar and apolar enzyme/substrate interactions. However, the precise assessment of the degree to which hydrophobicity, steric exclusion, and substrate-induced conformational changes function in determining specificity profiles, requires many additional structural informations about the mutant enzymes. What appears clear is that steric, electrostatic and hydrophobic effects (water organization) play altogether important roles in determination and modulation of specificity. Previous attempts to transform subtilisin to more specific protease by altering hydrophobic and steric environment [31] showed that an increase in the side-chain volume at the base of the substrate binding pocket, which reduced its size, caused substantial decrease (up to 5,000-fold) in k_{cat}/K_m toward substrates with large amino acids in P1. Presumably, this happens because of steric hindrance dominating the effect of a more hydrophobic pocket.

Replacement of lysyl residue 188 by phenylalanyl, tyrosyl or tryptophanyl residue did not lead to important modification in catalytic efficiency of resulting enzymes since the hydrolysis of β-casein by mutant trypsins could be performed with E/S ratios comparable to wild-type trypsin. Almost all expected tryptic peptides were obtained in comparable yields.

Until recently, the impact of pH on specificity of mutant trypsins was not studied in broad context of large protein sequences. While the catalytic activities of wild-type, K188F and K188Y trypsins measured by hydrolysis of the synthetic substrates succinyl-Ala-Ala-Pro-Arg-*pNA* and succinyl-Ala-Ala-Pro-Lys-*pNA* indicate higher activities at pH 10 than at pH 7, hydrolysis of β-casein showed surprisingly higher catalytic activities of these proteases at pH 7.

Kinetic parameters measured at pH 8 with synthetic substrates showed that K188F trypsin prefers substrates containing arginine over lysine by a factor 8 compared to a factor 5.7 measured with wild-type trypsin. However, this mutant enzyme (in contrast to K188Y and K188W trypsin mutants) was forestalled in splitting peptide bonds Arg25-Ile26 and Arg202-Gly203 as observed by the identification of peptide *A-B* (Arg1-Lys28) and by reduced hydrolysis of peptide *L-N* (Asp184-Val209). Only in the case of mutant K188W both methods gave comparable catalytic parameters, with exception of pH 7.

Despite that peptide bonds of β-casein involving phenylalanine and leucine were hydrolyzed by the three mutants studied, no hydrolysis of the synthetic substrates such as succinyl-Ala-Ala-Pro-Phe-*pNA* and succinyl-Ala-Ala-Pro-Leu-*pNA* could be observed. This may be due to the particular conformation of these model substrates. Surprisingly, as shown only by proteolysis of a natural substrate, the produced mutants cleaved efficiently well peptide bonds containing asparagine and glutamine (Fig. 4). Some of the new cleavage sites can be related to the nature of the amino acid residue introduced in position 188. An important factor which can be seen from the primary structure of β-casein is that it is an amphiphilic protein with many unpolar amino acids. Their distribution is relatively random along the whole sequence. The majority of polar amino acids is situated in the stretch of 40 N-terminal amino acids. The remainder of the molecule is strongly hydrophobic. This makes β-casein an amphiphilic protein with hydrophilic and hydrophobic termini, as shown from the linear sequence of amino acids. Most of observed new cleavage sites are located in the hydrophobic portion of the protein (Fig. 4). The interpretation of cleavage of β-casein is difficult despite some visible pattern. First of all, the classical hydrophobicity / polarity plots are useless in this case. Hydrophobic Cluster Analysis [32] points to the preference of mutated enzymes to concentrate their stuttered and may

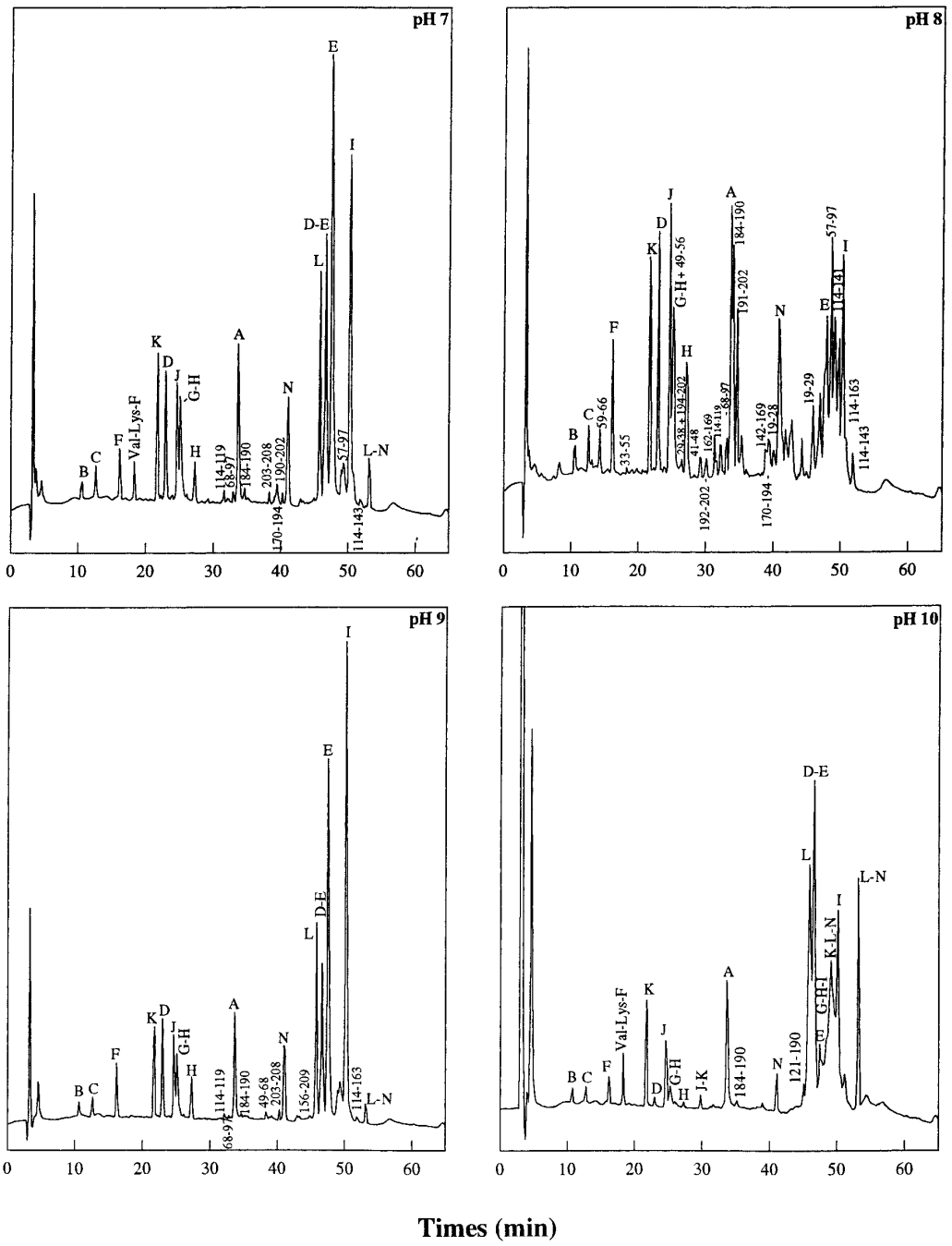

FIG. 5. HPLC profiles of tryptic peptides obtained from 48 h of hydrolysis at pH 7, 8, 9, and 10 of β-casein with K188Y trypsin.

be somehow progressive attack around the fragments showing β-sheet arrangement, besides amidated amino acids and, what is even more surprising, highly negatively charged SerP and Glu areas. Some fragments of β-casein are not processed at all even by the amide specific mutants for reasons which, in absence of well defined β-casein structure are hard to understand. Consequently, only the combination of several methods (synthetic substrate, protein substrate, influence of pH) can help to define better the differences of catalytic properties of wild-type and mutant proteases.

As presented in Table 2, computer modeling shows that in the absence of substrate in the binding pocket, the conformational energies of the mutants are quite comparable but higher than those of the wild type

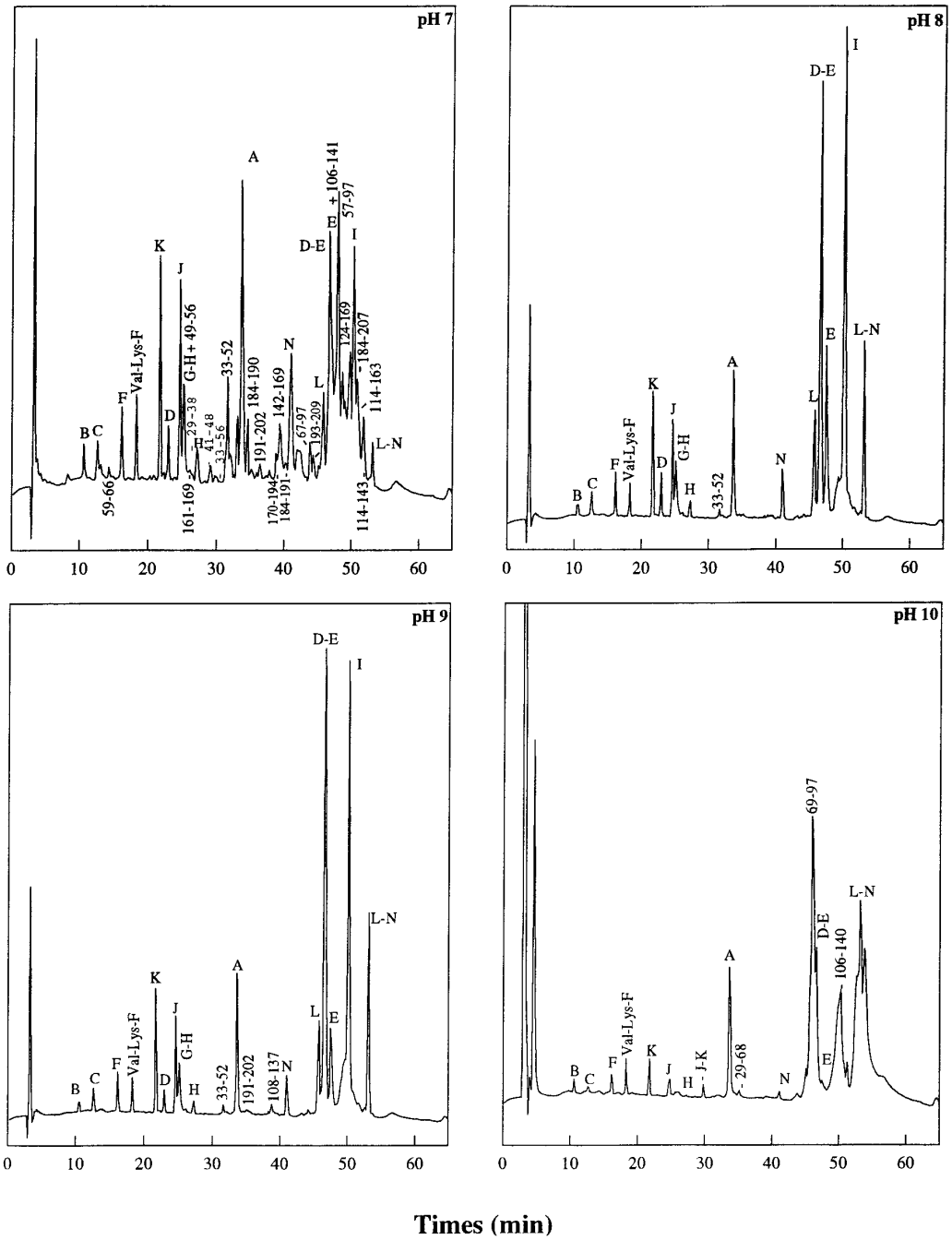

FIG. 6. HPLC profiles of tryptic peptides obtained from 48 h of hydrolysis at pH 7, 8, 9, and 10 of β-casein with K188W trypsin.

and the K188D/D189K mutant. Compared to the wild type, the differences seem important but can be explained by rather strict minimization conditions perturbing the full relaxation of the mutants after their construction. As for the K188D/D189K mutant, no dramatic steric constraints were revealed. However, these high energies are clearly related to less favored conformations (compared to the wild type) even if the backbones of segment (188, 188a, 189), in all cases, are quite well superimposed as shown in Figure 7 with a ribbon representation. Consequently, this suggests that the side-chains of the substituted aromatic amino acids conserve the wild type-like orientation and their hydrophobic side-chain protrudes outside

the primary binding pocket according to their hydrophobicity (Fig. 7). Considering the apolar character of these residues, this extrapolation is certainly open to discussion.

In contrast with K188D/D189K mutant [16], these AE aromatic AF mutants maintained the same general shape of the substrate binding pocket. Therefore, in the presence of the model substrate (Thr-Arg-Ile), the docking calculations yielded comparable results with the wild-type with a good steric complementarity in the binding site. The small differences of interaction energies between the wild-type and the mutants indicate that the enzyme/substrate affinity for basic amino acids is not perturbed by these mutations. This was confirmed also experimentally.

Another puzzling observation (tyrosine mutant at pH 10 taken apart) is high pH-dependence of the activity of these otherwise ionicaly neutral mutants compared to wild-type. These observations would rather suggest that bulky and apolar side chains of substituted amino acids influence the organization of polar residues changing thus the mutant pH activity optima. If this is true, the medium and long range effects would rather be at the origin of these phenomena. The unexpected appearance of cleavage of amidated amino acids is difficult to explain on the basis of the increase of so-called "hydrophobic interactions" only.

Modifications introduced by these mutations are at the origin of the broadening of the specificities of these enzymes studied which are cleaving β-casein in many new sites, hydrolyzing well also the fragment Arg1-Lys105, reported to be an efficient trypsin inhibitor [33]. Since many tryptic inhibitors contain amidated Glu and Asp, and form amyloid structures, the mutants of this type could be used for the hydrolysis of the lyticaly resistant protein structures. Structural modifications induced by the variations of pH can give some insight into the impact of structural and electrostatic phenomena on lytic functions.

TABLE 2

Conformational Energies of Trypsins

Trypsins	Relative global energies (kcal/mol)	Relative interaction energies (enzyme/substrate) (kcal/mol)
wild-type	0.0	22.4
K188D/D189K	81.8	106.0
K188F	249.7	0.6
K188Y	233.2	0.8
K188W	394.4	0.0

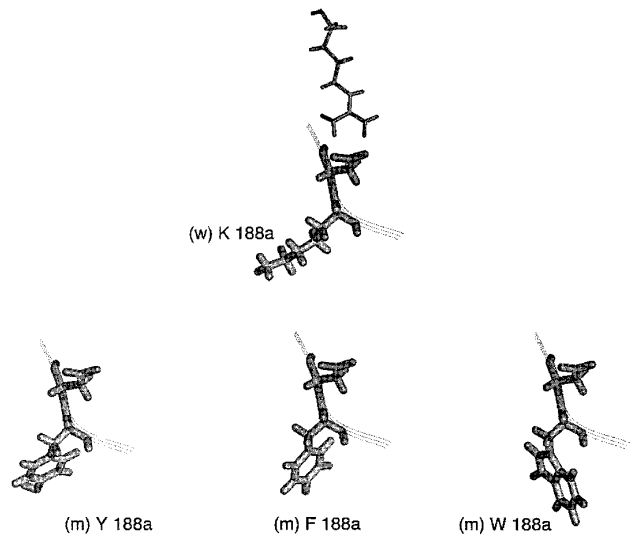

FIG. 7. Molecular modeling of Asp189 and amino acid in position 188a (amino acid labeling according to the X-ray crystal structure of Brinen et al., 1996). The segment 188-188a-189 is represented by a ribbon. The model substrate is in the upper part.

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