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# Reactivity of Lys($NH_2$)-containing Peptides Toward Endopeptidases

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Abstract: Lys($NH_2$)-containing peptides were subjected to various proteolytic enzymes which were selected for their well-documented specificity for arginyl and/or lysyl peptide bonds. Lys($NH_2$)-containing peptides were cleaved more rapidly by clostripain than the corresponding lysyl peptides. On the other hand, they proved to be resistant to *Achromobacter* protease I hydrolysis. The modified peptides synthesized in this study were more stable than the arginyl and lysyl analogues when incubated with trypsin or thrombin. The same tendency was observed when Lys($NH_2$)-containing peptides were incubated in diluted human serum, suggesting that the replacement of Arg or Lys by Lys($NH_2$) could be used to increase the stability of peptides *in vivo*. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Lys($NH_2$)-containing peptides; trypsin; thrombin; *Achromobacter* protease I; clostripain

## INTRODUCTION

We have described a new synthesis of Lys($NH_2$)-containing peptides (Scheme 1) using the Boc/benzyl strategy and the solid phase *N*-electrophilic amination of the $\varepsilon$ amino group of Lys with *N*-Boc-3-(4-cyanophenyl)oxaziridine [1–4]. This *N*-electrophilic amination was easily adapted to an automatic solid phase peptide synthesizer, allowing the rapid synthesis of a large variety of modified peptides [5]. More recently, this methodology was used for the conversion of the $\alpha$ and $\delta$ amino group of Gly and Orn, respectively, and was adapted to the Fmoc/*tert*-butyl strategy [6].

Lys($NH_2$) (Figure 1) is a new unnatural $\alpha$-amino acid which has interesting properties. Indeed, Lys($NH_2$)-containing peptides react readily with $\alpha$-oxo-aldehydes to give the corresponding hydrazones in good yield [5]. Moreover, we experienced that replacing a Lys residue in a peptide by Lys($NH_2$) led to minor changes of some physico-chemical properties such as the solubility, the RP-HPLC retention time, the CZE retention time at pH 3.0, the ionization propensity in ES-MS [6]. However, these physico-chemical similarities could not preclude Lys($NH_2$)-containing peptides and their unmodified counterparts to behave differently when engaged in biomolecular interactions. We thus decided to use endoproteases (such as trypsin, human $\alpha$-thrombin, *Achromobacter* protease I and clostripain), whose specificity was well documented, to characterize the side chain of Lys($NH_2$). These results, presented in the first part of this article, are supplemented in the second part by a study of the

Abbreviations: TFA, trifluoroacetic acid; MBHA, 4-methyl benzhydrylamine resin; Rink amide resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin; TOF, time-of-flight; PDMS, plasma desorption mass spectrometry; ES-MS, electrospray mass spectrometry; CZE, capillary zone electrophoresis; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; *t*Bu, *tert*-butyl; Mtt, 4-methyltrityl; DIEA, diisopropylethylamine; DMF, dimethylformamide; HBTU, *N*-[(1H-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, *N*-hydroxybenzotriazole; BCPO, *N*-Boc-3-(4-cyano--phenyl)oxaziridine; DTT, threo-1,4-dimercapto-2,3-butane-diol; DMAP, 4-dimethylaminopyridine; DIC, diisopropylcarbodiimide; PBS, phosphate buffered saline; API, *Achromobacter* protease I.

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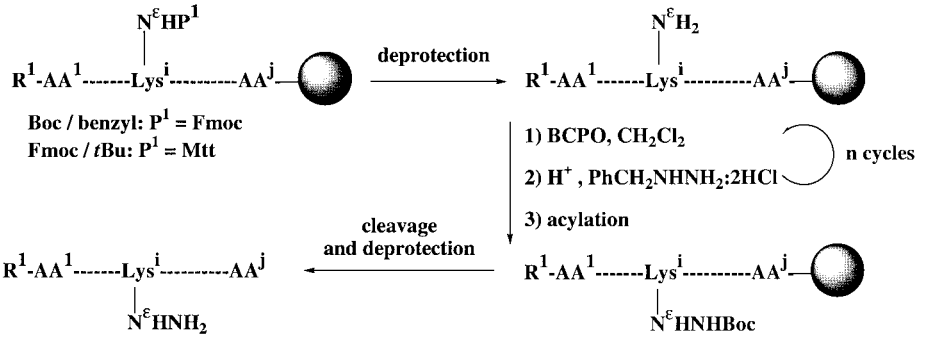


Scheme 1 Solid-phase synthesis of Lys($NH_2$) containing peptides.

stability of a model Lys($NH_2$) containing peptide in diluted human serum.

## MATERIALS AND METHODS

### Peptide Synthesis

Peptide **2** (Figure 2) was synthesized as described elsewhere [5,6] on a MBHA resin (0.57 mmol/g, Applied Biosystems, Foster City, USA), using the Boc/benzyl strategy [7] and the HBTU *in situ* protocol [8] in an Applied Biosystems 430A peptide synthesizer (Foster City, USA). Peptides **6** and **8** were elaborated as described elsewhere [6] on a Rink Amide resin (0.47 mmol/g; France Biochem, Meudon, France), using the Fmoc/*tert*-butyl strategy [9] and HBTU/HOBt activation in an Applied Biosystems 431A peptide synthesizer (Foster City, USA). The *N*-electrophilic aminations were performed in an Applied Biosystems 431A peptide synthesizer as described previously [5,6].

Peptide H-YGK($NH_2$)-OH was synthesized using the Fmoc/*tert*-butyl strategy. Fmoc-L-Lys(Mtt)-OH (10 eq) was attached to a *p*-benzyloxybenzyl alcohol resin (0.25 mmol, 0.7 mmol/g; Novabiochem) using DIC 5 eq/DMAP 1 eq (DMF, 1.5 h, 82% yield). After peptide elongation using successively Fmoc-Gly-OH and Fmoc-L-Tyr(*t*Bu)-OH (deprotection of the Fmoc group: piperidine 20% in DMF, 2 and 10 min; couplings: Fmoc-AA-OH/HBTU/DIEA: 4 eq/4 eq/8 eq, DMF, 30 min), the Mtt protecting group was removed with 1% TFA in $CH_2Cl_2$ (continuous flow, 1.25 h). After neutralization with DIEA 5% in $CH_2Cl_2$, the resin was subjected to the BCPO procedure (3 h for the *N*-electrophilic amination). The unreacted amino groups were capped with $Ac_2O$/DIEA/$CH_2Cl_2$, 10/5/85, by volume (5 min). After removal of the Fmoc group, the peptide was cleaved from the resin and deprotected using TFA/$H_2O$/anisole, 95/2.5/2.5, by volume, during 1.5 h. The crude peptide was purified by C18 RP-HPLC, yield 13%, TOF-PDMS $MH^+$, exp. 382.4, found 382.4.

### Peptides 1–3, Enzymatic Studies

Peptides **1**–**3** were 1.27 mM in the corresponding 100 mM Tris–HCl buffer containing 1.53 mM of H-L-Tryp-OH used as an internal standard. Concentrations were determined by total acid hydrolysis of aliquots in 6 N HCl (24 h, 110°C, sealed tube), followed by amino acid analysis using ninhydrin detection. All the experiments were performed at 22°C.

For the trypsic digests, 3 μl of 1.6 M $CaCl_2$ and 12 μl of trypsin (Sigma) (0.1 mg/ml) in water containing 0.01% TFA were added to 240 μl of peptide solution. The absence of chymotrypsin residual activity that could have cleaved the peptides at the tyrosine residues was confirmed by the total stability of the acetyl-peptide H-YGK(Ac)GYA-$NH_2$ toward enzyme digestion in the same conditions.

For API digests, 1.2 μg of API (Boehringer) dissolved in 12 μl of 50 mM pH 8.0 tricin buffer containing 10 mM EDTA were added to 240 μl of peptide solution. An experiment was performed using 4.8 μg of API dissolved in 48 μl of the same buffer.

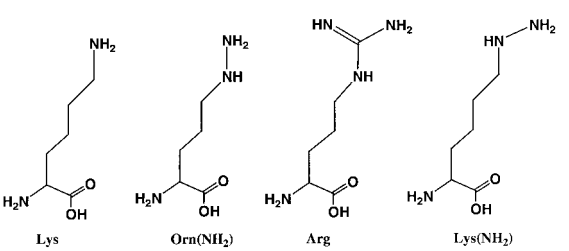


Figure 1 Comparison of the side chain length of Lys, Orn($NH_2$), Arg and Lys($NH_2$).

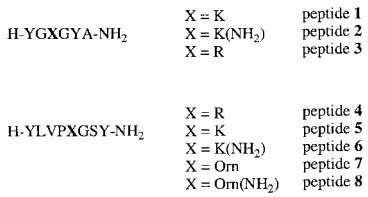


Figure 2 Peptides synthesized in this study.

For clostripain digests, 4.8 μl of enzyme (Sigma) (1 mg/ml) in water, 1 μl of 1.6 M $CaCl_2$ and 1.24 μl of DTT (0.2 g/ml) were added to 155 μl of 100 mM Tris–HCl buffer pH 7.40. The enzyme was activated during 2.5 h at 22°C and then added to a mixture of 240 μl of peptide solution, 3.9 μl of DTT (0.2 g/ml) and 1.5 μl of 1.6 M $CaCl_2$. Aliquots were diluted with an equal volume of acetic acid and analysed by RP-HPLC on a C18 Vydac column (4.6 × 250 mm). Eluent A: $H_2O$ containing 0.05% TFA; eluent B: MeCN/$H_2O$, 4/1, containing 0.0475% TFA, linear gradient 0–30% B in 20 min, flow 1 ml/min, detection at 215 nm. The amount of remaining peptide at a given time was determined by comparing the peak area ratio peptide/H-L-Tryp-OH to the same ratio obtained before the addition of the enzyme.

### Peptides 4–8, Enzymatic Studies

Peptides **4**–**8** were 1.27 mM in 100 mM Tris–HCl pH 7.36 buffer containing 1.53 mM of H-L-Tryp-OH used as an internal standard. Concentrations were determined as described above. All the experiments were performed at 24°C in triplicate. The initial hydrolysis rates presented in Table 2 are mean values ± the whole confidence interval [10]. For the following experiments: peptide **6** and clostripain, peptide **8** and trypsin/API/thrombin, the contribution of the contaminating native peptide to the initial hydrolysis rates could be neglected. For peptide **6** and trypsin/thrombin experiments, the rapid digestion of the contaminating native peptide led to a short non-linear part at the beginning of the kinetics which was omitted for the linear regression analysis.

For the trypsic digests, we used the above conditions except that the trypsin solution was 0.03 mg/ml. For the thrombin digests, 0.5 μl of 1.6 M $CaCl_2$, 2.1 mg of NaCl and 12 μl of α-human thrombin (ICN) (0.1 mg/l) in water/glycerol (1/1 by volume), were added to 240 μl of peptide solution. Additional experiments were performed using ten times more enzyme for trypsic and thrombin digests.

For API digests, 24 μl of API (Sigma) (9 units/ml) were added to 240 μl of peptide solution. Further experiments were performed using five times more enzyme.

For clostripain digests, 4.3 μl of enzyme (Sigma) (2 mg/ml in water), 0.9 μl of 1.6 M $CaCl_2$ and 1.14 μL of DTT (0.2 g/ml) were added to 144 μl of 100 mM Tris–HCl buffer pH 7.36. The enzyme was activated during 2.5 h at 24°C and then added to a mixture of 240 μl of peptide solution, 1.9 μl of DTT (0.2 g/ml) and 0.9 μl of 1.6 M $CaCl_2$.

Aliquots were diluted with an equal volume of acetic acid and analysed immediately by RP-HPLC on a C18 TSKgel Super ODS column (4.6 × 50 mm). Eluent A: $H_2O$ containing 0.05% TFA; eluent B: MeCN/$H_2O$, 4/1, containing 0.05% TFA, linear gradient 0–35% B in 3 min 30 s, flow 3 ml/min, detection at 215 nm.

### Peptides 4–6, Stability in Diluted Human Serum

The experiments were performed at 37°C in duplicate. 10.9 μl of decomplemented human serum (Sigma, SHN C′) were diluted with 109 μl of PBS (pH 7.2). To this mixture, 20 μl of peptide solution (see above) were added. Aliquots (20 μl) were diluted with 20 μl of water and immediately analysed by RP-HPLC using a C18 Vydac column (4.6 × 250 mm). Eluent system as above, linear gradient 0–60% B in 20 min, flow 1 ml/min, detection at 215 nm.

## RESULTS

### Peptide Synthesis

Peptides **1**–**3** (Figure 2) were chosen for the preliminary enzymatic studies aimed at evaluating the susceptibility of Lys($NH_2$)-containing peptides toward trypsin, API and clostripain-catalysed proteolysis. Two tyrosines were included in the sequence before and after the cleavage site to facilitate the RP-HPLC analysis. These substrates were also used to check the influence of buffer's pH on the cleavage profiles. Peptides **4**–**8** were then synthesized to further explore the properties of Lys($NH_2$). In particular, the sequence of peptides **4**–**8** was chosen to extend the study to human α-thrombin (E.C. 3.4.21.5), a trypsin-like serine protease that plays a major role in blood coagulation [11–13]. Thrombin, like trypsin, cleaves after arginyl and lysyl residues. However, thrombin displays distinct subsite

 

Table 1 Characterization of Hydrazinopeptides **2**, **6** and **8**

| Sequence | Peptide | ES-MS | | Isolated yield (%) | Amount of native peptide[a] | |
|---|---|---|---|---|---|---|
| | | Calc. | Found | | CZE | ES-MS |
| H-YGK($NH_2$)GYA-$NH_2$ | **2** | 672.8 | 672.7 | 17 | 9.2 ± 0.3 | 9.8 ± 0.4 |
| H-YLVPK($NH_2$)GSY-$NH_2$ | **6** | 941.1 | 940.9 | 30 | 2.0 ± 0.1 | 3.0 ± 0.3 |
| H-YLVPOrn($NH_2$)GSY-$NH_2$ | **8** | 927.1 | 926.9 | 24 | 3.0 ± 0.4 | 3.8 ± 0.3 |

[a] See Reference [6].

preferences, with optimum cleavage occuring at sites containing $P_4$-$P_3$-$P_2$-Pro-Arg-$P'_1$-$P'_2$, where $P_4$ and $P_3$ are hydrophobic residues and $P'_1$ and $P'_2$ non-acidic amino acids. The sequence Leu-Val-Pro-Arg-Gly-Ser is often inserted into GST fusion proteins to allow their isolation following thrombin digestion [14]. As for peptides **1**–**3**, tyrosines were added before and after the cleavage site to facilitate the RP-HPLC analysis. Peptides **7**–**8** were synthesized to evaluate the importance of the side chain length.

Peptide **2** was synthesized using the Boc/benzyl strategy. The purity of peptide **2** was carefully checked before the enzymatic studies were undertaken. CZE and ES-MS [6,15] revealed that peptide **2** was contaminated by 9% of native peptide **1**, despite the multiple acylation steps performed after the *N*-electrophilic amination (see Scheme 1), suggesting that the N–N bond was partially unstable during the HF treatment used for the cleavage of the peptide from the resin. This side reaction was studied in detail and will be reported elsewhere [6]. In particular, we observed that the scission of N–N bond was always significant when the final cleavage and deprotection step occurred in HF. On the other hand, the Fmoc/*tert*-butyl strategy, with a final cleavage and deprotection step in TFA, led to Lys($NH_2$)-containing peptides in much better isolated yields and purity. The scission of the N–N bond was marginal in TFA. With this knowledge, peptides **6** and **8** were synthesized using the Fmoc/*tert*-butyl strategy. The results of these syntheses are compiled in Table 1.

## Enzymatic Studies: Cleavage of Peptides 1–3 by Trypsin, API and Clostripain

Trypsin (E.C. 3.4.21.4) [16–18] is a serine protease that cleaves $P_1$–$P_{1'}$ peptide bonds with $P_1$ basic residues like Lys and Arg. As expected, peptides **1** and **3** were rapidly digested by the enzyme ($t_{1/2}$ 3.3 and 13.5 min, respectively, at a substrate/enzyme ratio of 5930 (mol/mol)). Alternately, peptide **2** was slowly cleaved by trypsin. The incubation of peptide **2** with trypsin during 6 h at pH 8.48 resulted in a 45% decrease of the RP-HPLC peak area, a value well above the amount of contaminating peptide **1**.

As the presence of a positive charge on the side chain of the $P_1$ residue is essential for the stability of the enzyme–substrate complex, the capacity of Lys($NH_2$) to fulfill the specificity of trypsin could be related to the protonation of *N*-alkylhydrazines at the alkylated nitrogen atom. The basicities of *N*-alkylamines and *N*-alkylhydrazines are nevertheless very different, with p$K_a$ values of 10.53 (25°C) [19] for the $\varepsilon$ amino group versus 7.82 (25°C) for *N*-butylhydrazine [20]. As the enzymatic studies were performed at pH values close to the p$K_a$ of the hydrazino group, we reasoned that the hydrolysis rate would be strongly influenced by the modification of the pH within the functional limits of the enzyme. The effect of pH was first examined between 8.48 and 7.90. As shown in Figure 3, the progress curves for the

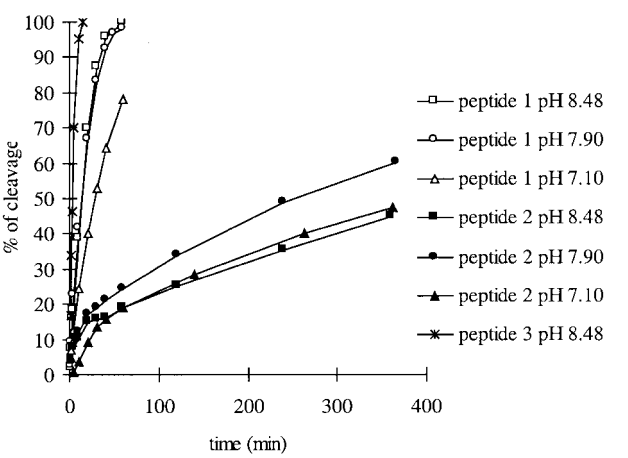


Figure 3 Trypsin-catalysed proteolysis of peptides **1**–**3** at 22°C (subs/enz ratio 5930 in mol/mol).

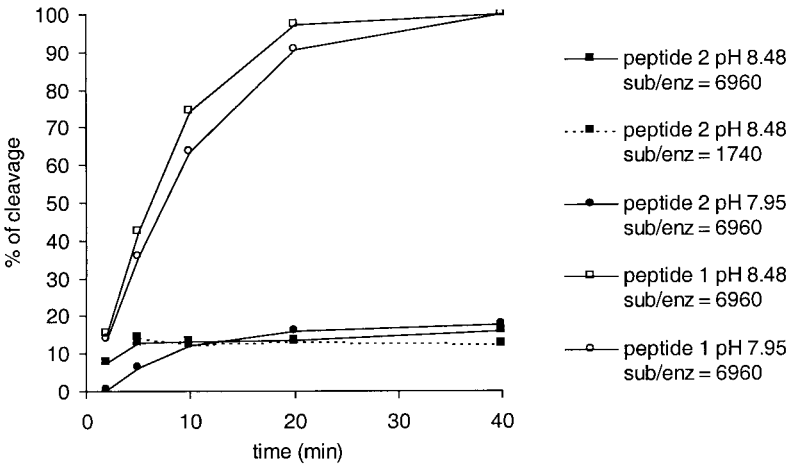


Figure 4 API-catalysed proteolysis of peptides **1** and **2** at 22°C (sub/enz ratios in mol/mol).

proteolysis of peptide **1** by trypsin were not altered when the pH was lowered from 8.48 to 7.90, while the proteolysis of peptide **2** after 6 h was increased by a factor of 1.4. As expected, the use of a lower pH value (7.10) modified the catalytic activity of the enzyme. Indeed, the proteolysis of peptide **1** at pH 7.10 was significantly reduced when compared to the experiment performed at pH 8.48. Despite the lower activity of trypsin, the overall cleavage rate of peptide **2** at pH 7.10 remained unchanged, arguing in favour of the influence of the protonation level of the side chain, which compensates for the diminished activity of the enzyme.

Finally, we checked for the absence of inhibiting activity of peptide **2** or of the tripeptide H-YGK($NH_2$)-OH generated during the proteolysis by demonstrating their inability to modify the progress curve of the enzyme-catalysed proteolysis of peptide **1** at pH 8.48 (data not shown).

To further explore the behaviour of Lys($NH_2$), we examined the ability of API to cleave peptides **1** and **2** (Figure 4). API (E.C. 3.4.21.50) is a serine protease that cleaves peptide bonds specifically after lysyl residues [21]. As expected, peptide **1** was rapidly cleaved by API, while peptide **2** proved to be very resistant to proteolysis, since the decrease in signal intensity was limited to the amount of contaminating peptide **1**. Modification of the pH or of the substrate/enzyme ratio had no detectable influence on the stability peptide **2** toward API.

We finally examined the proteolysis of peptides **1**–**3** by clostripain (E.C. 3.4.22.8), a cysteine protease that cleaves peptide bonds specifically after arginyl residues (Figure 5) [22,23]. The optimal pH for clostripain is in the range 7.4–7.8. The experiments were thus performed at the lowest pH value 7.4 to allow a better protonation of the Lys($NH_2$) side chain. As expected, peptide **3** was rapidly cleaved by clostripain ($t_{1/2}$ 1.7 min). Using the same experimental conditions, peptide **2** was cleaved to an appreciable rate ($t_{1/2}$ 25.6 min) whereas peptide **1** was much more resistant to proteolysis. Clostripain is known to have a small lysyl-endopeptidase activity, evaluated at about 1% of the arginyl-endopeptidase activity [24].

### Enzymatic Studies: Cleavage of Peptides 4–8 by Trypsin, API, Clostripain and Human α-thrombin

All the assays were conducted at pH 7.36 to allow both the protonation of the Lys($NH_2$) or Orn($NH_2$) side

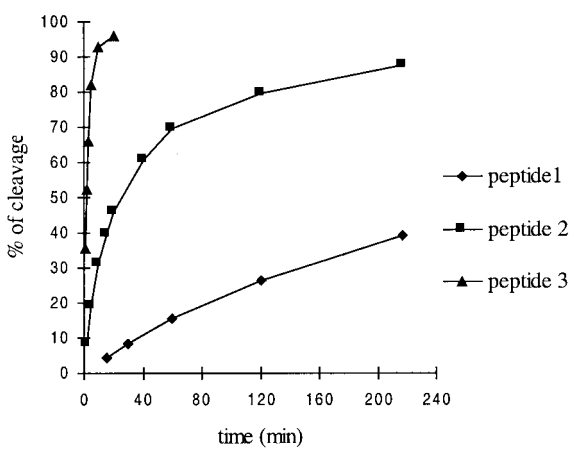


Figure 5 Clostripain-catalysed proteolysis of peptides **1**–**3** at 22°C (pH 7.40, subs/enz ratio 1588 in mol/mol).

 

Table 2 Initial Hydrolysis Rates for the Proteolysis of Peptides **4–8** by Trypsin, Thrombin, API and Clostripain at 24°C

| Enzyme | Units | H-YLVPXGSY-$NH_2$ initial hydrolysis rates (nmol/min) | | | | |
|---|---|---|---|---|---|---|
| | | 4, X = R | 5, X = K | 6, X = K($NH_2$) | 7, X = Orn | 8, X = Orn($NH_2$) |
| Trypsin | 5.7 | 23 ± 2 | 19.5 ± 0.3 | 0.43 ± 0.03 | nc | 0.18 ± 0.03 |
| | 57 | | | | nc | 0.9 ± 0.03 |
| Thrombin | 3.58 | 31 ± 2 | 4.3 ± 0.3 | 0.27 ± 0.06 | nc | nc |
| | 35.8 | | | | 0.31 ± 0.05 | 0.29 ± 0.06 |
| API | 0.22 | nc | 7.0 ± 0.3 | 0.12 ± 0.06 | nc | 0.21 ± 0.03 |
| | 1.42 | | | 0.3 ± 0.1 | nc | 1.52 ± 0.04 |
| Clostripain | 0.57 | 49 ± 3 | 0.21 ± 0.03 | 0.8 ± 0.1 | nc | nc |

nc, not calculated.

chains and an efficient endoprotease activity for all the enzymes studied. The initial hydrolysis rates obtained for the cleavage of peptides **4–8** by trypsin, human α-thrombin, API and clostripain are compiled in Table 2. For peptides **7** and **8**, the cleavage rate was sometimes very low or below the detection limit, so that additional experiments were performed with more enzyme. The initial hydrolysis rates displayed by peptides **4–6** confirmed the preliminary results obtained with peptides **1–3**. In particular, peptide **6** was found to be more stable toward trypsin, thrombin, API and clostripain than the corresponding natural subtrates.

### Stability of Peptides 4–6 in Diluted Human Serum

There is a constant interest in the design and synthesis of minimally modified peptides possessing peptidase-resistant properties. Indeed, most endogenous and exogenous biologically active peptides are short-lived molecules that are rapidly degraded *in vivo* by proteases. One way to improve the *in vivo* stability of peptides is to introduce isosteres of the amide bond in the peptide backbone. Another possibility is to use unnatural α-amino acids, whose side chains are little recognized by proteases. In this context, we studied the stability of peptides **4–6** in PBS diluted human serum to see if the replacement of Arg or Lys by Lys($NH_2$) would increase the half-life of the peptide *in vivo*.

The behaviour of peptides **4–6** in a human serum/PBS: 1/10 mixture are presented in Figure 6. Peptides **4** and **5** were rapidly cleaved, with half-lives of 26.7 and 35.0, min respectively. After 120 min of incubation, peptides **4** and **5** were completely digested. On the other hand, peptide **6** was still present in a significant amount after the same period of incubation ($t_{1/2}$ 55.8 min). These data suggest that Arg or Lys residues could be replaced by Lys($NH_2$) to increase the half-life of a peptide *in vivo* without changing significantly the physicochemical properties of the compound.

## DISCUSSION

Lys($NH_2$) (Figure 1) is an unnatural α-amino-acid which can be easily introduced into peptides by using automated solid phase methodologies. We were interested to see if Lys($NH_2$) could have some resemblance with Lys or not. On the other hand, we could not exclude similarities between Lys($NH_2$) and arginyl residues. Indeed, Figure 1 highlights the side chain length similarity between Lys($NH_2$) and Arg (or Orn($NH_2$) and Lys). For example, the terminal amino group of Lys($NH_2$) could interact with the carboxylate ion of $Asp^{182}$ or $Asp^{189}$ present in the

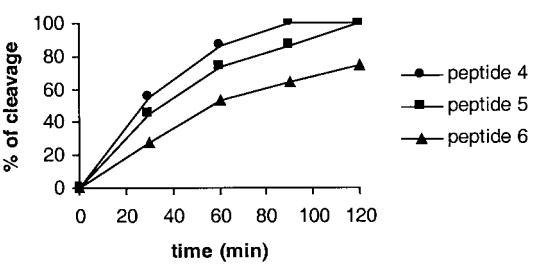


Figure 6 Stability of peptides **4–6** in diluted human serum at 37°C.

primary specificity pocket of trypsin or thrombin, respectively. Moreover, hydrazinium compounds are known to interact with carboxylates in a manner similar to the mode of interaction of guanidinium ions with carboxylates. Indeed, Hady *et al.* [25] have studied the crystal structure of hydrazinium acetate. The NH bonds of the hydrazinium ion are in an eclipsed conformation. Two adjacent NH bonds make efficient hydrogen bonds with the two oxygen atoms of the acetate ion.

The data obtained for peptides **1**–**3** give a profile of the proteolytic susceptibility associated with the Lys($NH_2$) side chain. Peptide **2** was completely resistant to proteolysis by API. The substitution of the $\varepsilon$ amino group of Lys by a hydrazino moiety seems to preclude the recognition of the unnatural amino acid by an endoprotease specific for lysyl residues. With trypsin, peptide **2** was hydrolysed more slowly than the corresponding arginyl and lysyl analogues. On the other hand, peptide **2** was cleaved much more rapidly by clostripain than the lysyl counterpart **1**. Clostripain is a sulphydryl protease obtained from the culture filtrate of *Clostridium histolyticum*. Its substrate specificity has been demonstrated to be similar to that of trypsin in that hydrolysis is restricted to substrates containing arginyl or lysyl residues. However, clostripain is much more specific for arginyl bonds in synthetic peptides and proteins. It was proposed that the anionic binding site in clostripain distinguishes the delocalized positive charge of the planar guanidinium moiety from the static charge of the tetrahedral ammonium ion. In the case of peptide **2**, the substitution of the $\varepsilon$ amino group by a hydrazino group enhances significantly the sensitivity to hydrolysis. *N*-alkyl hydrazines such as Lys($NH_2$) are protonated exclusively on the alkylated nitrogen atom [20]. The positive charge is not delocalized and other factors must contribute to explain the experimental data. Unfortunately, the complete amino acid sequence of clostripain has not been reported to date and the lack of structural data discourages further analysis.

The initial hydrolysis rates obtained for peptides **4**–**6** corroborate the previous findings. Peptide **6** was slowly hydrolysed by trypsin when compared to the arginyl and lysyl analogues (Table 2). Peptide **7** was stable even with more enzyme, whereas peptide **8** containing the Orn($NH_2$) residue was cleaved in twice the time of the Lys($NH_2$) analogue.

Peptide **6** was cleaved slowly by thrombin (Table 2), but as for trypsin, at a rate well below those found for the arginyl and lysyl analogues. Peptides **7** and **8** were not digested using the same experimental conditions. By using more enzyme, thrombin did not distinguish Orn and Orn($NH_2$). The comparison of the stability of peptides **6** and **8** in the presence of trypsin or thrombin indicates that thrombin is more sensitive than trypsin to the side chain length of the unnatural amino acid.

The Lys($NH_2$) containing peptide **6** was cleaved four times more rapidly by clostripain than the lysyl counterpart **5** (Table 2). The hierarchy found for the cleavage of peptides **1**–**3** by clostripain was respected. Alternately, peptides **7** and **8** proved stable under the same experimental conditions, revealing again the importance of the side chain length.

API was able to cleave peptide **6** but only at a rate just above the detection limit. This result is in accord with the resistance to proteolysis observed for peptide **2**. Thus, the substitution of the amino group of Lys by a hydrazino moiety prevented the cleavage of the corresponding amide bond. Alternately, the same modification applied to Orn led to the opposite effect. Indeed, peptide **7** was completely resistant to API hydrolysis, whereas the hydrazino analogue **8** was cleaved significantly by the enzyme. Moreover, peptide **8** with an Orn($NH_2$) residue was digested more efficiently than the Lys($NH_2$) analogue. Orn($NH_2$) and Lys have similar side chain length, but present the charge differently, while Lys($NH_2$) situates the charge correctly yet the bulkiness of the additional $NH_2$ moiety may sterically hinder the positioning of the side chain in the primary specificity pocket of API.

The initial hydrolysis rates obtained with thrombin led us to study the stability of peptides **4**–**6** in PBS diluted human serum. Indeed, the protection from proteolytic degradation of biological active peptides through the modification of either backbone or side chains represents an important task. The data presented in Figure 6 indicate that the replacement of Lys by Lys($NH_2$) is a way to enhance the stability of peptides *in vivo*. This result is all the more interesting since the substitution of Lys by Lys($NH_2$) is accompanied by minor changes of important physico-chemical properties such as solubility, hydrophobicity, charge and size.

## CONCLUSION

Lys($NH_2$)-containing peptides were synthesized using our optimized solid phase *N*-amination protocol

 

to study the proteolytic susceptibility associated with this unnatural amino acid. The enzymes were selected for their well-documented specificity for arginyl and/or lysyl peptide bonds. Lys($NH_2$)-containing peptides are cleaved more rapidly by clostripain than the corresponding lysyl peptides, while proving resistant to API hydrolysis. The modified peptides synthesized in this study were more stable than the arginyl and lysyl analogues when incubated with trypsin or thrombin. The same tendency was obtained when Lys($NH_2$)-containing peptides were incubated in diluted human serum.

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