

# Increased Resistance of Peptides to Serum Proteases by Modification of their Amino Groups

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The ability of synthetic protein fragments to survive the degradative action of aminopeptidases and serum proteolytic enzymes can be remarkably enhanced by slight modifications at their N-terminal alpha-amino group. This can be achieved by addition of beta-alanine or amino acids of the D-configuration, amino acids which are seldom found in a living organism. These modifications do scarcely modify the chemical and physical properties of the peptides, and should be preferred, especially for *in vivo* tests, to drastic alterations of peptides as produced by dinitrophenylation or dansylation of the amino groups.

**Key words:** Peptides, Proteolytic Degradation, Modifications

## Introduction

With few exceptions, synthetic as well as natural peptides are readily digested by exopeptidases, yielding at the end a mixture of amino acids and eventually a core of undigested material made up by few amino acid residues. As some bonds in the peptide may be more resistant than others (for instance, those involving a proline residue), the step-wise removal of amino residues from both ends of the fragment proceeds rapidly as long as one of these bond is encountered: then the digestion either slows down or even stops. Some fungal or bacterial peptidases, however, are also capable of cleaving such resistant bonds. In contrast, on small peptides comprising 10 to 15 residues, endopeptidases proceed at a relatively low rate.

In recent years, synthetic peptides have been widely used for the *in vivo* treatment of different diseases (Sundaram *et al.*, 2002; Aina *et al.*, 2002). The main problem encountered in such therapeutic trials is the short half life of such peptides in the blood stream, as they are readily digested by circulating serum enzymes. Many of such peptidases have been isolated and characterized: in general, they seem to be more active on the amino

terminus of peptides (Hafkenschied, 1984). Under physiological conditions, serum carboxypeptidases appear to be either less represented or less active, although few of them, notably carboxypeptidases B and N, have been shown to be slightly active on C-terminal lysine or arginine.

In this investigation, we first tested the resistance of a panel of randomly-selected synthetic peptides to a mixture of commercial aminopeptidases. We then investigated whether, by suitable modifications of the amino groups of two of such peptides, it might have been possible to increase their resistance to the action of the enzymes. Finally, in order to simulate as much as possible a physiological situation, we assayed whether such modifications would increase the half life of the fragments when submitted to the action of proteases commonly represented in serum samples.

## Materials and Methods

### Preparations

Synthetic peptides were prepared by either the Boc or the Fmoc strategy (Merrifield, 1984; Atherton *et al.*, 1978). After deprotection, peptides were purified by gel filtration on Aca202 high resolution resin (Spectrum, Los Angeles, CA, USA) in 0.02 M ammonia followed by reverse-phase HPLC on Sephasil C8 columns (Pharmacia, product 17-6000-22), using linear gradients of water-acetonitrile, at

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**Abbreviations:** ACN, acetonitrile; DNP, dinitrophenyl; R. Ph, reverse-phase; HPLC, high-performance liquid chromatography;  $\beta$ , beta-alanine; TEA, triethylamine; zt, zero-time.

pH 3 (Chersi *et al.*, 2000). Eluates were monitored at 235 nm.

Collected fractions were freeze-dried. An aliquot of each sample (0.1 mg) was then hydrolysed at 108 °C for 24 to 48 h with 6 N HCl, then analyzed by fluorescence-HPLC for correct amino acid composition. The sequences of the peptides used for the assays are reported in Table I and Table II.

The alpha-amino groups of peptides to be tested were modified as follows:

- 1) Addition of an amino acid in the L configuration (Ser for peptide GP, Leu for peptide IG).
- 2) Addition of an amino acid in the D configuration.
- 3) Addition of a beta-alanine.
- 4) Addition of a proline, or dinitrophenylation (Sanger, 1945) (only for peptide IG).

Enzymes

Leucine aminopeptidase (LAP) and microsomal aminopeptidase (MAP) were purchased from Sigma, products L5658 and L5006. Digestions were performed on aliquots of 100 µg of peptide, in 0.5 ml of 50 mM ammonium bicarbonate buffer, pH 7.8. The enzyme-to-substrate ratio was 2:100.

Digestions were allowed to proceed for 4 and 18 h, at 37 °C. Then the samples were adjusted to pH 3 with 10 ml of trifluoroacetic acid and centrifuged with a Centricon 3000 (Millipore, product 42403). An aliquot of the centrifugate was then loaded on a 4.6 × 250 mm Sephasil C8 column (Pharmacia, product 17-6000-22). The column was developed with a linear gradient water/80% ACN, pH 3.4. The eluate was monitored at 235 nm. A *zero time* (zt) sample of each peptide was included in each set.

For each run, the following parameters were measured: % ACN of elution of all main peaks and their area.

The error of the method, as determined by several repeated HPLC chromatographies of the same sample, is around ± 10%.

Digestion by serum proteases

The assay was carried on under similar conditions. 100 ml of fresh rabbit serum was used for each peptide sample (100 µg) in 2.5 ml of 0.05 M triethylamine-HCl buffer, pH 7.8. At the end of

the digestions, 4 and in most cases also 18 h, the samples were acidified and centrifuged for 30 min in Centricon 3000. A predetermined aliquot was then analyzed by reverse-phase HPLC. A *zero time* sample (peptide, and serum, at pH 3) was used as a control.

Results and Discussion

In preliminary experiments, using 5 randomly-selected peptides and a mixture of the two commercial aminopeptidases, no one of the fragments survived the 18 h treatment with the enzymes for an extent higher than 5%. There was no substantial difference in resistance whether the amino terminus was an acidic, a basic, or a hydrophobic residue (data not shown).

One of the fragments of this first group, GP, and a sixth peptide named IG, whose sequence was His Gln Gln Asp Ile Asp Asp Leu Lys were selected for further investigations. The two peptides were suitably modified at their amino termini in order to test their resistance to commercial and well as to serum exopeptidases (Table I).

For the *zero time* (zt) sample, an unique peak was recorded in analytical HPLC, in accordance with the preliminary purification step previously carried on. For digested samples, different situations were recorded: in most cases, the zt peak

Table I. Sequence of peptides derived from peptides **GP** and **IG** by modification of the amino terminus. Details for the enzymatic digestions are given in the text.

<b>Original peptide:</b>											
GP		N	T	I	T	L	P	C	R	I	
<b>Related peptides:</b>											
GP Ser	S	N	T	I	T	L	P	C	R	I	
GP β	β	N	T	I	T	L	P	C	R	I	
GP-D-Ala	A°	N	T	I	T	L	P	C	R	I	
<b>Original peptide:</b>											
IG		H	Q	Q	D	I	D	D	L	K	
<b>Related peptides:</b>											
IG Leu	L	H	Q	Q	D	I	D	D	L	K	
IG β	β	H	Q	Q	D	I	D	D	L	K	
IG-D-Leu	L°	H	Q	Q	D	I	D	D	L	K	

A° Indicates alanine in the D-configuration.  
L° Indicated leucine in the D-configuration.  
β is beta-alanine.  
Few other derivatives of peptide IG, as a dinitrophenyl (DNP) derivative, and a fragment obtained by addition of a proline at the N-terminal position, were also occasionally tested or used as controls.

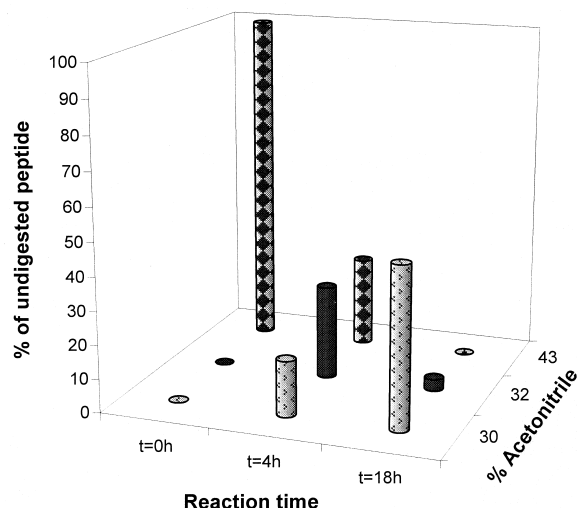


Fig. 1. Degradation pattern of peptide IG-Leu by commercial aminopeptidases at 4 and 18 h, as revealed by analytical HPLC. Fig. A (time 0) shows a single peak, corresponding to the original, undigested peptide, which is eluted at 43% acetonitrile. An arbitrary amount value of 100 is given. Fig. B (4 h digestion) shows two new peaks, both eluted at lower acetonitrile concentrations, representing smaller fragments generated by the removal of N-terminal amino acids from the original peptide, whose peak (at 43% acetonitrile) is now reduced to less than 30% of the original amount. In Fig. C (18 h), peptide IG-Leu, as well as the intermediate fragment eluted at 32% acetonitrile, have been completely digested, as they disappear from the chromatogram. The peak eluted at 30% acetonitrile, which doubles its amount, is likely the final, highly resistant product of the digestion.

either was drastically reduced in its area, or was eluted at different ACN concentrations, this depending on the course of digestion, and on the number of amino acids effectively removed from the amino terminus. In few cases, three or more peaks were generated (Fig. 1). Obviously, the extent of degradation of the peptides was much higher after 18 h.

In general, peptides of the GP family were not very resistant, with only two fragments (GP- $\beta$ -Ala and GP-D-Ala) surviving, but at a low extent, the 18 h treatment. In these peptides, the digestion from the amino terminus probably rapidly proceeds until the L-P bond is encountered: the residual core of five residues LPCRI, on the contrary, might survive in the medium for a long time. The addition of Ser at the amino terminus does not obviously prevent degradation, but both  $\beta$ -Ala as

well as D-Ala slow down the stepwise release of amino acids.

Peptides of the IG family were more resistant, probably because of the sequence of non-hydrophobic amino acid residues at the N-terminus: after 4 h, several intact fragments were still represented in appreciable amounts. After 18 h, the original fragment and IG-Leu disappeared, while IG- $\beta$ -Ala and IG-D-Leu, on the contrary, were still present in remarkable amounts (59 to 75%) (Table II). Obviously, N-terminal Leu did not prevent or slow down the degradation process, as this amino acid is a good substrate for both amino peptidases.

Next, the same panel of peptides derived from IG and GP was submitted to the action of serum peptidases, in order to mimic and possibly approximate physiological conditions of *in vivo* trials. In this medium, the degradation of peptides by peptidases proceeded at much lower rate: after 4 h, all peptides related to GP were still present in the medium, in percentages varying from 37% (GP) to 81% (GP-D-Ala), with intermediate values for GP-Ser and GP- $\beta$ -Ala (Table II). Also peptides of the IG family were only partially degraded: after 4 h, all peptides were still present in percentages varying from 28 to 88% (however, the dinitrophenylated derivative: 95%). After 18 h, apart from the unmodified peptide IG and the closely related fragment IG-Leu, all others survived in percentages over 50%, exhibiting thus remarkable resistance to serum peptidases.

Obviously, preliminary experiments carried on with the two commercial amino peptidases represent only a far approximation of the complexity of

Table II. Resistance of peptides to commercial amino peptidases (A) and to serum proteases (B), at different times.

	% of undigested peptide			
	A		B	
	4 hours	18 hours	4 hours	18 hours
<b>GP</b>	0	0	27	–
<b>GP-Ser</b>	9	0	51	–
<b>GP-<math>\beta</math></b>	17	11	59	–
<b>GP-D-Ala</b>	47	15	61	–
<b>IG</b>	6	5	28	3
<b>IG-Leu</b>	27	0	60	10
<b>IG-<math>\beta</math></b>	73	59	78	66
<b>IG-D-Leu</b>	81	75	74	70
<b>IG-DNP</b>			95	73

the different enzymatic reactions occurring in sera, where the number of aminopeptidases, with different substrate specificities, is likely very high, and where the action of carboxypeptidases and of endopeptidases could not be *a priori* excluded. However, data on the degradation of the peptides by serum enzymes seem to correlate quite well with those obtained with commercial amino peptidases:

1) Peptide IG and related fragments are substantially more resistant to degradation by serum peptidases than peptides from the GP family.

2) In both experiments, the best protection against degradation is achieved by a N-terminal amino acid in the D conformation. It is known that only very few enzymes are able to slowly hydrolyse peptide bonds involving D-amino acids (Sela and Zisman, 1997; Yamada and Kera, 1998). Surprisingly, beta-alanine, although not an  $\alpha$ -amino acid, does not provide such a protection: however the survival time of  $\beta$ -Ala-modified peptides remarkably increased as compared to controls.

3) The degradation of peptides by serum carboxypeptidases of the B-type, although possible, appears to be negligible, as compared to that of aminopeptidases: in fact, peptide IG and related fragments (including the DNP derivative), possessing a C-terminal lysine, would have been degraded at both ends, thus much faster than the peptide GP. The removal of the lysine residue from all peptides would be easily detectable by HPLC analysis: on the contrary, data show that the percentages of IG peptides surviving in the medium after an 18 h digestion remain higher than those of the intact GP fragments.

4) It is conceivable that drastic chemical modifications of the N-terminal amino group of a peptide, as for instance, dinitrophenylation (Sanger, 1945), although providing good resistance of peptides to enzymatic degradation would too drastically change the chemical and physical properties of the fragments, by increasing their aromatic and hydrophobic character. In addition, they might not be compatible with life in *in vivo* experiments.

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