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Mass Spectrometric and Chemical Stability of the Asp-Pro Bond in Herpes Simplex Virus Epitope Peptides Compared with X-Pro Bonds of Related Sequences

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Abstract: The mass spectrometric analysis of the immunodominant epitope region (273–284) of herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) showed a favoured fission at the Asp-Pro peptide bond. The fast atom bombardment collision induced dissociation (FAB-CID) study of closely related X-Pro peptides documented that neither the length nor the amino acid composition of the peptide has a significant influence on this preferential cleavage. At the same time the DP bond proved to be sensitive to acidic conditions in the course of peptide synthesis. These observations prompted us to compare the chemical and mass spectrometric stability of a new set of nonapeptides related to the 273–284 epitope region of gD, i.e. SALLEDPVG and SALLEXPVG peptides, where X = A, K, I, S, F, E or D, respectively. The chemical stability of these peptides during acidic hydrolysis was investigated by electrospray ionization mass spectrometry (ESI-MS) and the products were identified by ESI-MS and on-line high performance liquid chromatography — mass spectrometry (HPLC-MS). The mass spectrometric fragmentation and bond stability of the untreated peptide samples were also studied using ESI-MS and liquid secondary ion mass spectrometry (LSIMS). Both the chemical hydrolysis and the mass spectrometric fragmentation showed that the Asp-Pro bond could easily be cleaved, while the KP bond proved to be stable under both circumstances. On the other hand, the XP bond (X = A, I, S, F or E) fragmented easily under the mass spectrometric conditions, but was not sensitive to the acidolysis.

Keywords: HSV-1 epitope peptide; Asp-Pro bond; chemical stability; mass spectrometry; electrospray ionization

INTRODUCTION

It was observed earlier that the peptide bond on the *C*-terminal of the aspartyl residue is cleavable in diluted acid solution at least 100 times better than the other amide bonds in the same proteins [1]. The selective hydrolysis could be achieved by heating the peptide sample in either 0.03 M HCl or 0.25 M acetic acid for 5–18 h at 110 °C [2]. The aspartyl-prolyl peptide bond, especially, was found to be exceptionally labile, therefore it could be hydrolysed under conditions in which other aspartyl peptide bonds were stable [3]. The cleavage of Asp-Pro bonds in proteins under acidic conditions was first reported in the 1960s. The splitting of this bond in rabbit antibody light-chain, carboxypeptidase inhibitor, lysozyme and tobacco mosaic virus protein was

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observed in 10% acetic acid or in 70%–90% formic acid solutions at 37°–40 °C. The treatment resulted in 12%–70% cleavage depending on the pH, temperature and/or reaction time. In the case of rabbit antibody light-chain, the sample was dissolved in 10% acetic acid in 7 M guanidinium chloride, adjusted to pH 2.5 with pyridine and the reaction allowed to proceed at 40 °C for 96 h. Under these conditions the cleavage yield of the single sensitive Asp-Pro bond was 90%. In the case of the carboxypeptidase inhibitor from potato, splitting was obtained in a yield of 84% using 10% acetic acid solution in water (pH 2.5). Quantitative cleavage of the single Asp-Pro bond was reported in the case of mare milk lysozyme after incubation with 75% formic acid solution with 7 M guanidinium chloride, at 37 °C for 48 h. Two Asp-Pro bonds in tobacco mosaic virus protein could be split with only a 38% yield in 90% formic acid, at 37 °C after 24 h. The protein concentrations in the above experiments were in the range 2–5 mg/ml [4,5]. Piszkiewicz *et al.* proposed a mechanism to describe the hydrolytic reaction. According to this an intramolecular catalysis initiated by the carboxylate anion in the side chain of Asp residue occurs and leads to the displacement of the protonated nitrogen of the peptide bond. Consequently the increased cleavage rate with proline compared with other amino acids is undoubtedly due to the higher basicity of the proline nitrogen [3].

One of the major immunogenetic components of the envelope of mature herpes simplex virions is glycoprotein D (gD). This glycoprotein is able to elicit a high level of virus neutralizing antibody response and protection in mice against a lethal challenge with either HSV-1 or HSV-2. Consequently this protein is a logical target for developing a synthetic antigen for vaccination. Based on the primary structure analysis, different immunodominant regions were localized and studied by several laboratories [6,7]. Peptides corresponding to two epitope regions (^{9}LKMADPNRFRGKD^{21} and ^{268}LAPEDPEDSALLEDPVG^{284}) of HSV-1 glycoprotein D studied in our laboratory contain Asp-Pro bond [8,9]. Interestingly the Asp-Pro sequence is often a part of turn structures and located in several immunodominant regions of surface proteins of pathogens. For example this motif can be found in many epitope regions, for example in adenovirus AD-2 protein (NQAVDSYDPD) and in the HIV virus gp-120 protein (VLEDPFILLV) [10].

The acid sensitivity of Asp-Pro bond was first observed during the synthesis of gD-peptides of various lengths (H-DPVG-NH_2, H-EDPVG-NH_2, H-LEDPVG-NH_2, H-LLEDPVG-NH_2, H-ALLEDPVG-NH_2, H-SALLQDPVG-NH_2, H-SALLQDPVG, SALLENPVG-NH_2, H-SALLQDPVGSALLQDPVG-NH_2) [12]. The crude product of peptide 270–284 contained approximately 25% byproducts as a result of splitting the Asp-Pro bonds after cleavage from the resin with hydrogen fluoride (HF) and in the following work-up procedure in acetic acid solution [11–13]. The fast atom bombardment collision induced dissociation (FAB-CID) study of the same set of peptides showed that neither the length nor the amino acid composition of the peptide had a significant influence on this preferential cleavage [14]. At the same time we observed different stability of the closely related NP and DP bonds. Mák *et al.* suggested that the analysis of the mass spectrometric fragmentation behaviour of the peptides might provide useful information for understanding the chemical sensitivity of Asp-Pro peptide bonds [14].

In this study we describe our findings on the chemical and mass spectrometric stability of peptides of the same length containing a single X-Pro bond. For this systematic study seven peptides were prepared with the sequence of Ac-SALLEXPVG-NH_2 (X = A, D, E, F, I, K and S) where the amino acid residue X has an acidic (D, E), basic (K) or apolar (A, I, F) character. The stability of these peptides under acidic hydrolysis conditions was characterized and compared with that observed under the various conditions used for mass spectrometric analysis.

MATERIALS AND METHODS

Solvents and Reagents

Solvents [acetonitrile, methanol (MeOH), acetic acid (AcOH), dichloromethane (DCM), *N*,*N*-dimethyl formamide (DMF), diethyl ether] were obtained from Reanal (Budapest, Hungary). Other chemicals [trifluoroacetic acid (TFA), thioanisole, 1-hydroxybenzotriazole (HOBt), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*,*N*-diisopropyl ethylamine (DIEA), guanidine hydrochloride, hydrogen fluoride (HF)] were purchased from Fluka (Buchs, Switzerland). Boc-amino acid derivatives and 4-methyl-benzhydrylamine (MBHA) resin (1.1 mequiv/g) were purchased from Bachem (Bubendorf, Switzerland).

Peptide Synthesis

Seven Ac-SALLEXPVG-NH_2 peptides with X = A, D, E, F, I, K or S were synthetized by solid phase

methodology using 4-methyl-benzhydrylamine resin (1.1 mequiv/g). Amino acids were coupled as Boc derivatives by the DCC/HOBt coupling method using a 3 molar excess over the resin capacity in DMF/DCM (1 : 3, v/v). The side chains of Asp and Glu residues were blocked with a cyclohexyl ester protecting group. Benzyl ether was applied for the protection of the OH group of Ser, while the 2-chloro-benzyloxycarbonyl group was used for the ε-amino group of Lys. The Boc group was removed by treatment of the resin-bound peptide with TFA/DCM (33 : 67, v/v) mixture for 2 + 20 min. Neutralization was carried out by DIEA/DCM (1 : 9, v/v). The success of the coupling was controlled by the ninhydrin reaction [15] or the bromophenol blue assay in the case of the coupling to the Pro residue [16]. After removal of the last Boc group, the *N*-terminus was acetylated with acetic anhydride using a 5 molar excess over the resin capacity in the presence of DIEA in dimethyl formamide. Finally peptides were deprotected and cleaved from the resin with anhydrous liquid HF at 0 °C for 1.5 h in the presence of *p*-cresol as a scavenger (approximately 5–10 equiv). The crude product was precipitated by dry ether. After filtration off the resin the peptide samples were dissolved in diluted AcOH and freeze dried.

High-performance Liquid Chromatography

The crude and purified products were analysed by reverse phase high performance liquid chromatography (RP-HPLC) using a Waters HPLC system with Vydac C_4 column (4.6 mm × 250 mm) packed with spherical 5 μm silica of 300 Å pore size. The gradient elution system consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water = 80 : 20 v/v (eluent B). The gradient was from 15% to 75% of eluent B in 30 min. The flow rate was 1 ml/min at ambient temperature. Peaks were detected at $\lambda = 214$ nm. The peptide samples were dissolved in eluent A (1 mg/ml) and 20 μl of solution was injected. Crude products were purified on a semipreparative column (10 mm × 250 mm) containing the same packing material (Vydac C_4). The following conditions were applied: flow rate 4 ml/min, sample concentration 20 mg/ml, injection volume 2 ml.

Mass Spectrometry

Electrospray mass spectrometry (ESI-MS) and liquid secondary ion mass spectrometry (LSIMS) measurements were performed using a Finnigan MAT 95SQ tandem mass spectrometer (Finnigan MAT, Bremen, Germany).

ESI measurements. Nitrogen was used as a drying and nebulizing gas, the capillary potential was set to 3 kV and the potential difference between the skimmer and the skimmer lens was kept under 10 V, to maintain mild ionization conditions. Samples dissolved in MeOH/H_2O/AcOH (49.5 : 49.5 : 1, v/v) solvent mixture were injected in the carrier stream of the same eluent at a flow rate of 50 ml/min.

LSIMS measurements. The ions studied were produced using a caesium ion gun operated at 20 kV. The liquid matrix used was glycerol. The analyses were performed at an accelerating voltage of 5 kV. The peptide samples were dissolved in MeOH and mixed with glycerol matrix for LSIMS measurements.

On line HPLC-MS. The reaction mixture of cleavage products obtained during acidolysis was analysed by on-line HPLC-MS measurements in the case of peptide Ac-SALLEDPVG-NH_2. The MS circumstances were the same as described for the ESI-MS measurement. The applied HPLC column was a Phenomenex Jupiter C_{18} (4.6 mm × 250 mm) packed with spherical 5 μm silica of 300 Å pore size. The gradient elution system consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water (80 : 20, v/v) (eluent B). The gradient was from 25% to 90% of eluent B in 40 min. The flow rate was 0.5 ml/min at ambient temperature. Peaks were detected at $\lambda = 214$ nm. The injected volume was 20 μl.

Analysis of Chemical Stability

Acidolysis of Ac-SALLEXPVG-NH_2 peptides was performed either in formic acid (protocol A) or in acetic acid (protocol B) solutions. The extent of the acidolysis was determined by ESI-MS and on-line HPLC-MS analysis as described above.

Protocol A. Freeze-dried peptide samples (5 mg) (5.14–5.18 μmol) were dissolved in 2.5 ml 75% v/v formic acid (c = 2 mg/ml) containing 7 mol/dm^3 guanidine hydrochloride. The peptides were incubated for 24 or 72 h at 37 °C. The reaction was terminated by adding 0.6 ml of ice cold distilled water and the solvent was evaporated *in vacuo* to half of the original volume. The pH value of the samples was adjusted to pH 7 with NH_3 solution (25% v/v) and diluted with distilled water.

Table 1 Molecular Mass and Retention Time Values of the Ac-SALLEXPVG-NH_2 Peptides

Peptide	Molecular mass		R_t[min][b]
	MH^+ calculated	MH^+ measured[a]	
Ac-SALLEAPVG-NH_2	898.0	897.5	17.8
Ac-SALLEDPVG-NH_2	942.0	941.4	17.7
Ac-SALLEEPVG-NH_2	956.1	955.6	17.2
Ac-SALLEFPVG-NH_2	974.1	973.5	22.0
Ac-SALLEIPVG-NH_2	940.1	939.5	21.3
Ac-SALLEKPVG-NH_2	955.1	954.5	15.0
Ac-SALLESPVG-NH_2	914.0	913.3	17.4

[a] Determined by ESI-MS.
[b] Conditions: Vydac C_4 column (4.6 × 250 mm) with 5 μm silica (300 Å pore size); linear gradient elution 15%–75% B in 30 min; eluents: 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water (80 : 20 v/v) (eluent B); flow rate: 1 ml/min; detection: $\lambda = 214$ nm.

Protocol B. The peptide samples (5 mg) (5.14–5.18 μmol) were dissolved in 2.5 ml (20% v/v or 40% v/v, c = 2 mg/ml) acetic acid and the reaction proceeded for 14 days at 0 °C.

Analysis of Mass Spectrometric Stability

The splitting of the X-Pro bond and its rate in Ac-SALLEXPVG-NH_2 peptides were studied by using ESI-CID and LSIMS-daughter techniques. The mass spectrometer and the circumstances used are described above.

The high energy (CID) spectra of the $[M + H]^+$ ions were obtained by ESI-CID measurements when the ionization conditions were varied by changing the potential difference between the heated capillary and the source house (cone voltage 40–140 V). For LSIMS-daughter measurements the $[M + H]^+$ ions were selected by the BE double-focusing mass spectrometer and its daughter ions were detected.

RESULTS AND DISCUSSION

Synthesis of Ac-SALLEXPVG-NH_2 Peptides

In order to analyse the effect of the side chain of amino acid X in SALLEXPVG-amide on the stability of X-P bond, seven peptides were prepared containing the non-charged apolar aliphatic alanine (SALLEAPVG-amide) or isoleucine (SALLEIPVG-amide), non-charged polar serine (SALLESPVG-amide), acidic amino acid (aspartic acid, SALLEDPVG-amide; glutamic acid, SALLEEPVG-amide) or a basic amino acid (lysine, SALLEKPVG-amide) residue in X position. An acetyl group at the *N*-terminal was introduced to detect mass differences between the *C*- and *N*-terminal fragments (SAL and PVG), respectively, in MS spectra.

For solid phase synthesis the Boc strategy was used with an *in situ* active ester (HOBt/DCC) coupling. The peptides were purified by semipreparative HPLC and they were characterized by analytical HPLC, amino acid analysis (data not shown) and mass spectrometry. The yields of the purified peptides were over 70% calculated for the resin capacity. The characteristic data of peptides are shown in Table 1. These highly pure compounds (>99%) were used for analysis of the stability of the X-P bond under acidic conditions in solution as well as in circumstances present in mass spectrometry.

Chemical Stability

Several cleavage mixtures developed for protein samples are described in the literature. For the present studies the most effective and selective solution was used which consisted of 75% v/v formic acid and 7 mol/dm^3 guanidine hydrochloride. After 24 h incubation the reaction mixture was analysed for the products of cleavage during acidolysis by off-line HPLC-MS measurements. There was no

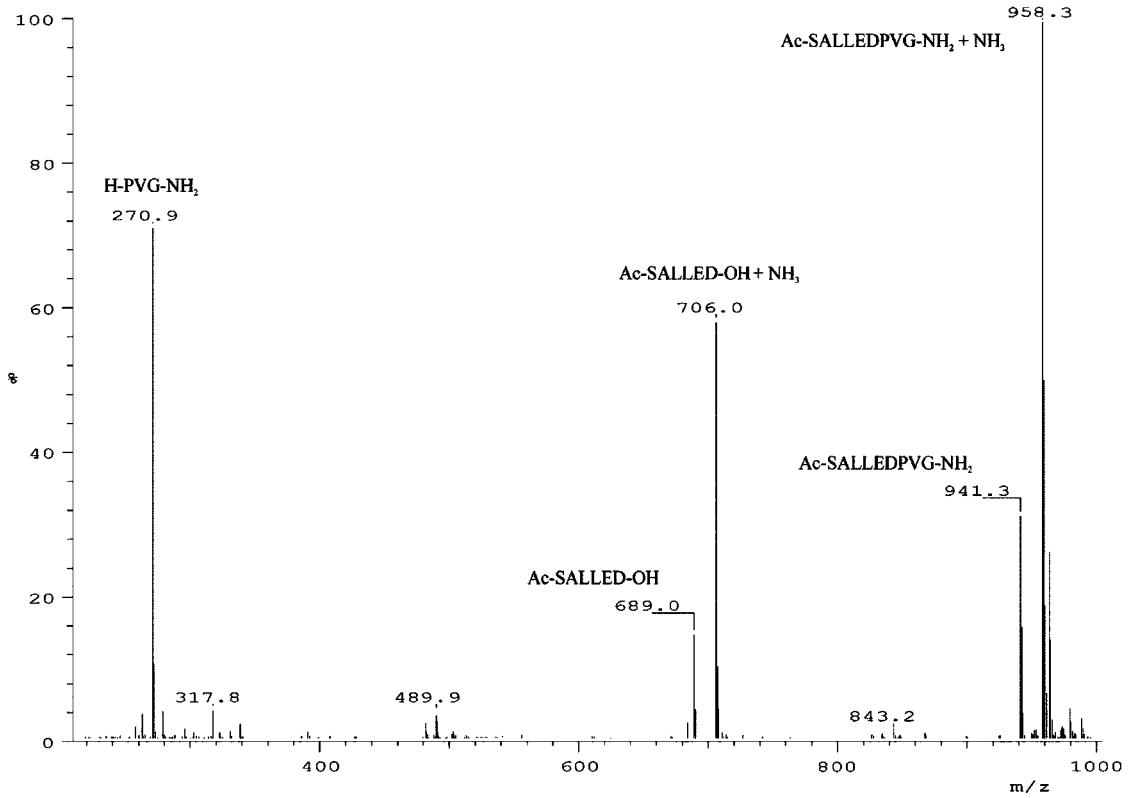

Figure 1 The ESI-MS spectrum of peptide Ac-SALLEDPVG-NH_2 after 72 h incubation in 75% v/v formic acid containing 7 mol/dm^3 guanidine hydrochloride.

amide bond splitting observed in either of the peptides studied. However, after 72 h incubation the presence of two components was detected in the solution of peptide Ac-SALLEDPVG-NH_2. ESI-MS measurements demonstrated peptides Ac-SALLED-OH and H-PVG-NH_2 in the solution (Figure 1). These peptides originated from peptide Ac-SALLEDPVG-NH_2 by amide bond splitting between Asp and Pro during the incubation under the acidic condition described above. This observation was confirmed by on-line HPLC-MS measurement (Figure 2), which makes it possible to analyse samples without the need to collect fractions or to freeze-dry. In case of the other six peptides only the presence of full-size molecules with no fragmentation could be detected both by off- and on-line HPLC-MS measurements even under an elongated period of acidolysis.

Results similar to the above after incubation of the octapeptides in 20% v/v and 40% v/v acetic acid solutions were observed (data not shown). The amide bond between Asp-Pro split only in peptide Ac-SALLEDPVG-NH_2 and fragments Ac-SALLED-OH and H-PVG-NH_2 were clearly seen by ESI-MS measurements after the incubation in 40% v/v acetic acid solutions for 2 weeks. Similar findings were observed using 20% v/v acetic acid solution. In contrast no decomposition of the other octapeptides was observed even in the more concentrated (40% v/v) acetic acid solutions (data not shown).

Mass Spectrometric Stability

The split of the X-Pro bond was also investigated in the Ac-SALLEXPVG-NH_2 peptides by two MS techniques, ESI-CID and LSIMS-daughter measurements. In the case of the cleavage of the X-Pro bond two fragment ions (B_6 and Y_3) were produced (Ac-SALLE$X^{B6}/_{Y3}$PVG-NH_2, respectively) [17].

ESI-CID measurements. The B_6 and Y_3 fragment ions were detected with different intensities in six octapeptides when X = A, D, E, F, I or S. The fragmentation of the X-Pro bond was the most abundant amide bond cleavage of the six cases. No B_6 and Y_3 ions were detected in peptide Ac-SALLEKPVG-NH_2, indicating that the K-P bond was stable under these conditions.

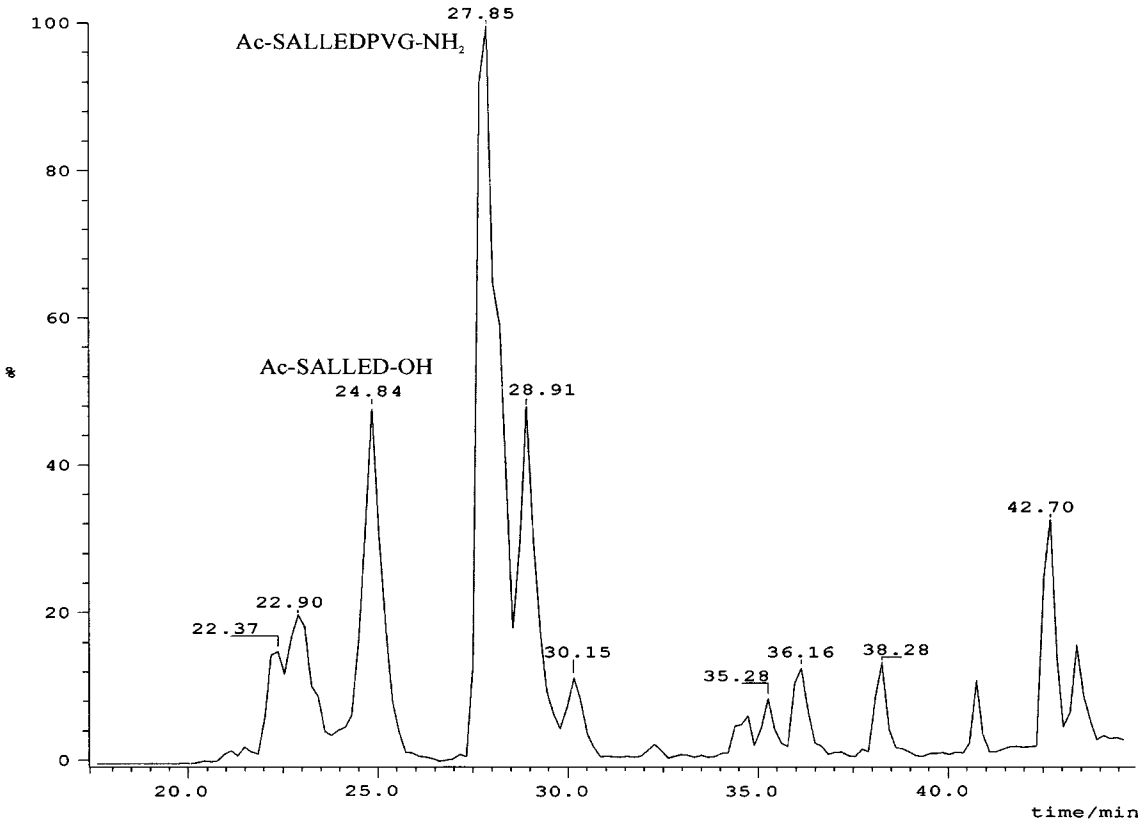

Figure 2 The total ion chromatogram of peptide Ac-SALLEDPVG-NH_2 after 72 h incubation in 75% v/v formic acid containing 7 mol/dm^3 guanidine hydrochloride produced by on-line HPLC-MS measurement.

In another set of experiments the voltage ratio was increased between the heated capillary and the source house (cone voltage). Under these conditions the splitting rate of X-Pro bond was increased. At the same time the changes in the mass spectrum of Ac-SALLEXPVG-NH_2 could be documented. Interestingly, the K-P bond did not split even during application of an increased voltage ratio (Figure 3).

LSIMS-daughter measurements. In the LSIMS-daughter experiments the splitting of the X-Pro bond was the most abundant cleavage process compared with the fission of the other amide bonds (Figure 4), except when X = Lys (Table 2). The order of the splitting capability for X-Pro bond considering the nature of the side chain of amino acid X was: apolar amino acids > polar amino acids > acidic amino acid > basic amino acid.

Table 2 The Intensity of the Fragment Ions ($B_6 + Y_3$) Produced by Splitting of the X-Pro bond Relative to the Molecular Ion Intensity as Determined in LSIMS-daughter Measurements

Amino acid at position X	Intensity of fragment ions $B_6 + Y_3$ (%)	Intensity of protonated molecular ion MH^+ (%)
A	18.2[a]	100
F	16.9	100
I	15.9	100
E	10.5	100
S	2.1	100
D	1.2	100
K	0.0	100

[a] Intensity of fragment ions ($B_6 + Y_3$) related to the protonated molecular ion.

CONCLUSION

The X-Pro bond in peptides Ac-SALLEXPVG-NH_2 was split under acidic conditions (pH 1.5 or 4, 72 h incubation) only when Asp was at position X. From these data one can conclude that the length of the acidic hydrolysis and the character of the amino

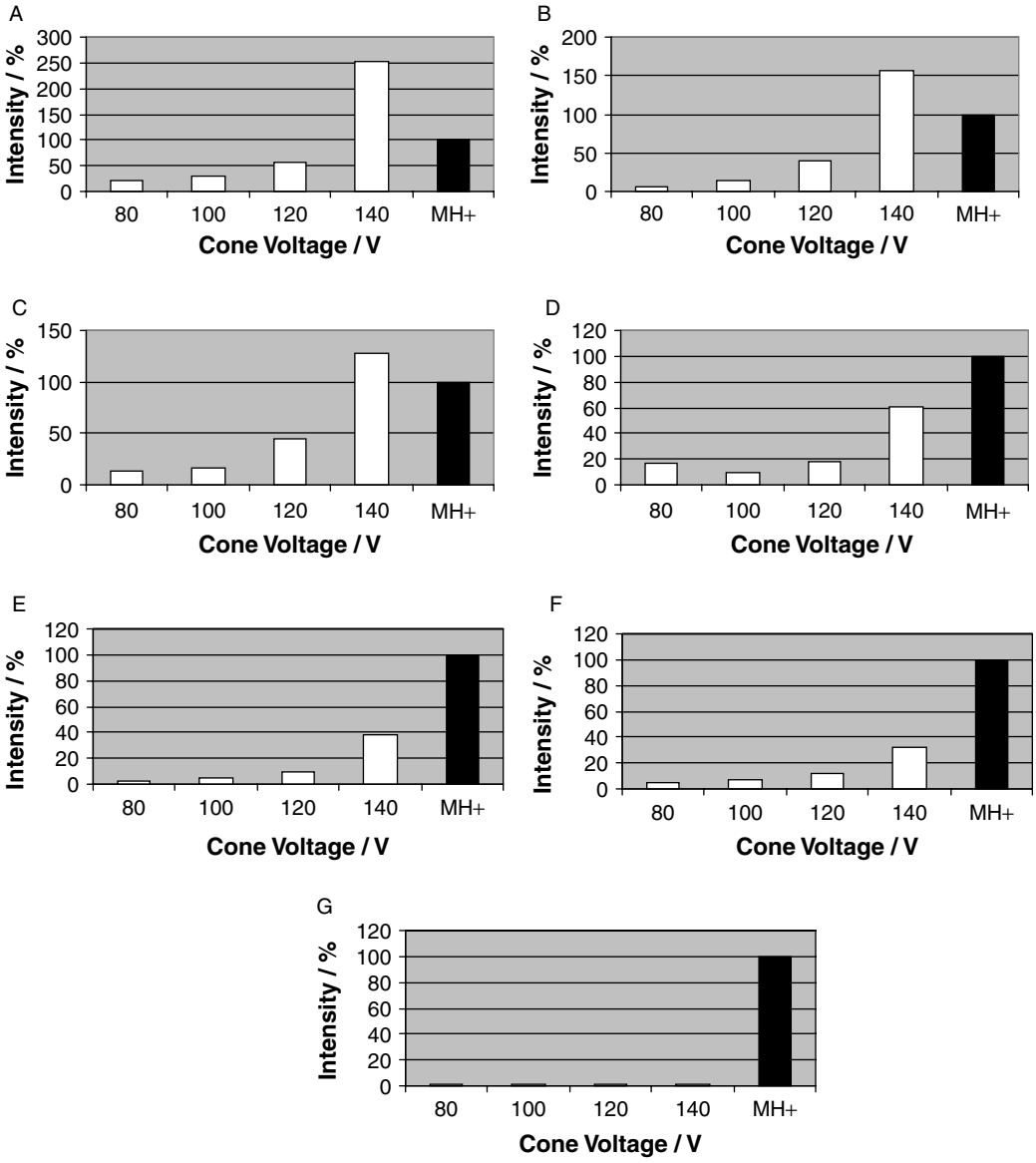

Figure 3 The intensity of the fragment ions (B_6 and Y_3) produced by splitting of the X-Pro bond relative to the molecular ion (MH^+) intensity at different cone voltage values by ESI-CID measurements. X = I (A), A (B), F (C), E (D), S (E), D (F), K (G). In each diagram a bar corresponds to a single ESI-CID mass spectrum.

acid side chain at position X plays a significant role in the X-Pro bond fission. The split of the Asp-Pro bond under the conditions studied indicates the acid sensitivity of this amide linkage, not only in the proteins described in the literature but even in small octapeptides. The mass spectrometric stability of the X-Pro bond differs from its chemical one. It was found that peptides, except peptide Ac-SALLEKPVG-NH_2, are highly fragmented at the X-Pro bond. The stability order was as follows: apolar amino acids > polar amino acids > acidic amino acid > basic amino acid. The X-Pro bond under the conditions for MS was stable with Lys at position X. In the ESI-MS experiment by increasing the voltage between the heated capillary and the source house a more intensive amide bond fragmentation was achieved. The ratio of the individual amide bond fragmentation of peptides compared with each other, however, did not change. It is important to note that this technique might be an appropriate tool

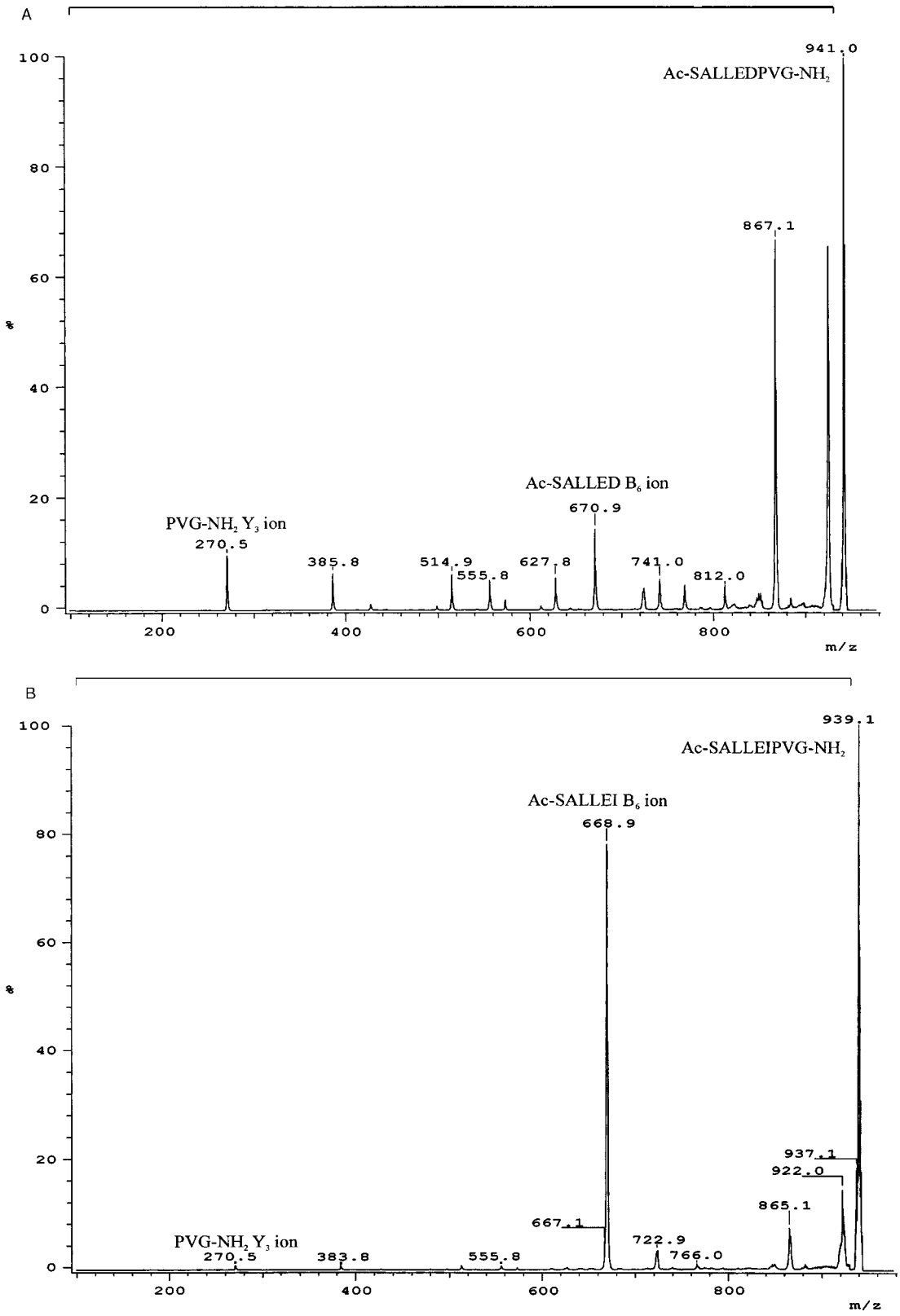

Figure 4 The LSIMS-daughter ion spectrum of peptide Ac-SALLEDPVG-NH_2 (A) and of peptide Ac-SALLEIPVG-NH_2 (B).

for the sophisticated investigation of differential amide bond stability. Comparison of the chemical and mass spectrometric stability of the Asp-Pro bond under the conditions studied indicated that the MS dissociation and the chemical acidolysis proceed by possibly a different mechanism. This study also shows that the Asp-Pro peptide bond in short synthetic oligopeptides is particularly labile and hydrolyses under conditions in which other aspartyl peptide bonds are stable. This phenomenon has to be taken into consideration mainly during the cleavage of peptides from the resin with HF and also during the work-up procedure of the cleavage mixture, as well as during the purification of the crude products.

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