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Analysis of aspartyl peptide degradation products by high-performance liquid chromatography and high-performance liquid chromatography-mass spectrometry

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Abstract

A reversed-phase HPLC method for the analysis of degradation products of the model aspartyl tripeptides Phe-Asp-GlyNH_2 and Gly-Asp-PheNH_2 after incubation at pH 2 and 10 was developed. Most of the compounds could be separated with a gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. Resolution of the isomeric pairs L-Phe-α-L-Asp-GlyNH_2/L-Phe-β-L-Asp-GlyNH_2 and L-Phe-α-D-Asp-GlyOH/L-Phe-β-D-Asp-GlyOH was achieved with a gradient of acetonitrile in phosphate buffer, pH 5.0. Under acidic conditions the major degradation pathway was cleavage of the peptide backbone amide bonds yielding dipeptides and amino acids, C-terminal deamidation as well as formation of succidinimyl peptides. At alkaline pH both deamidation of the C-terminal amide as well as isomerization and concomitant enantiomerization of Asp were observed. The peaks were identified both by reference substances and by online electrospray mass spectrometry. The results were compared to a previous developed capillary electrophoresis method. Diastereomeric pairs of peptides that could not be separated by capillary electrophoresis were resolved by HPLC while the separation of corresponding pairs of α- and β-Asp peptides was not always achieved by HPLC in contrast to capillary electrophoresis illustrating that both techniques can be complimentary in peptide analysis.

Keywords: Aspartimide formation; Peptides; Aspartyl peptides

1. Introduction

Recent advances in biotechnology have dramatically increased the number of biologically active peptides, proteins and peptidomimetics used as pharmaceutical drugs. In 2001, over 50 recombinant proteins were on the pharmaceutical market and more than 40 products were estimated in pre-registration and phase III, with other 60 products in phase II [1]. During processing, storage and delivery peptides and proteins can undergo both chemical and physical degradation such as oxidation, hydrolysis, isomerization, enantiomerization, deamidation, aggregation and surface adsorption [2,3]. These processes can lead to loss of structure and function as well as to the formation of toxic degradation products.

Asparagine (Asn) and aspartic acid (Asp) are among the most unstable amino acids in peptides and proteins, undergoing deamidation, isomerization and enantiomerization [4,5]. Intramolecular formation of an aminosuccinimidyl (Asu) peptide intermediate is the first step in these reactions (Fig. 1). The mechanism of succinimide formation involves deprotonation of the carboxyl side backbone amide followed by attack of the anionic nitrogen on the side chain carbonyl group. The succinimide is subject to spontaneous hydrolysis generating either the native L-aspartyl residue (α-Asp) or L-isoaspartyl residue (iso-Asp or β-Asp). In addition, because of the increased acidity of the succinimidyl α-carbon [6] enantiomerization of the L-succinimide to D-succinimide may occur. Successive hydrolysis leads to the formation of D-Asp and D-iso-Asp (D-β-Asp) residues (Fig. 1). The rate of the degradation process depends on the primary sequence of the peptide as well as temperature, buffer pH and concentration [3–5,7–9]. Neighboring amino acid residues that allow for chain flexibility and hydrogen-bonding interactions such as Gly or Ser facilitate the formation of the

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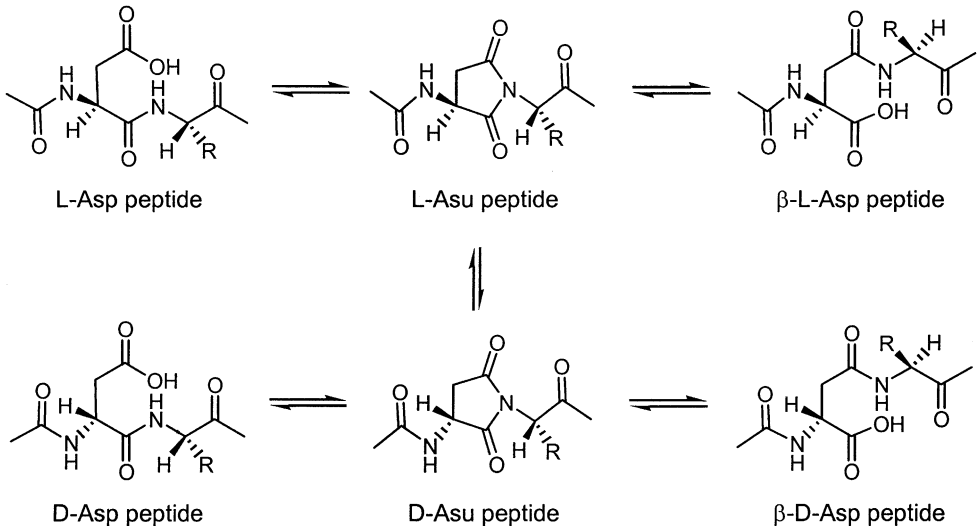

Fig. 1. Spontaneous isomerization and enantiomerization of L-Asp in peptides.

succinimidyl intermediate. Besides being side reactions in peptide synthesis, isomerization and deamidation are degradation reactions of natural proteins and peptides as well as peptide drugs [3,5,10]. Spontaneous deamidation and isomerization of Asn was found in prion proteins [11] and fibrillar deposits of β-amyloid proteins in Alzheimers disease [12,13]. β-Asp proteins can also be immunogenic [14]. Accumulation of D-Asp residues is an aging process of proteins in vivo and in vitro [15,16] and has been used to date paleontological material [17].

Chemical instability of peptides and proteins can be investigated by different analytical techniques [18]. Hydrolysis can be monitored by changes in mass, size, charge, hydrophobicity as well as in UV absorption and fluorescence. Deamidation and isomerization of Asn and Asp can be studied by chromatographic methods, such as ion-exchange chromatography, RP-HPLC, hydrophobic and affinity chromatography as well as by electrophoretic techniques, such as isoelectrofocusing, gel electrophoresis and capillary electrophoresis [19]. To determine the level of isomerization of Asp residues in proteins, immunoassays [16], isotopic labeling [20] and non-isotopic detection [21,22] with L-isoaspartyl methyltransferase were developed.

We have recently investigated the isomerization and enantiomerization of Asp in the model tripeptides Phe-Asp-$GlyNH_2$ and Gly-Asp-$PheNH_2$ by capillary electrophoresis (CE) including identification of the degradation products by electrospray tandem mass spectrometry [23]. The assay allowed the simultaneous analysis of most D/L-stereoisomers of Asp and β-Asp containing degradation products. As CE is not yet established in all laboratories the aim of the present study was the development of an HPLC method for the analysis of the isomerization and enantiomerization of the Asp tripeptides and to compare both methods. In addition, the peptide degradation products were identified by HPLC-mass spectrometry.

2. Experimental

2.1. Chemicals

Commercially available amino acids, dipeptides and protected amino acid derivatives were purchased from Bachem (Heidelberg, Germany). HPLC-grade acetonitrile, isopropanol and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Buffers and solutions were prepared in double distilled, deionized water, filtered (0.47 μm) and degassed with helium. L-Phe-α-L/D-Asp-$GlyNH_2$, L-Phe-β-L/D-Asp-$GlyNH_2$, Gly-α-L/D-Asp-L-$PheNH_2$, Gly-β-L/D-Asp-L-$PheNH_2$ were synthesized in solution using *N*-*tert*-butyloxycarbonyl- or *N*-benzyloxycarbonyl-protected amino acid derivatives and *N*-(3-dimethylaminopropyl)-*N*-carbodiimide as coupling reagent according to [24]. Deprotection was performed by hydrogenolysis or by treatment with 6 M hydrochloric acid in dioxane. L-Phe-α-L/D-Asp-GlyOH, L-Phe-β-L/D-Asp-GlyOH, Gly-α-L/D-Asp-L-PheOH and Gly-β-L/D-Asp-L-PheOH were prepared by solid phase synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acid and *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) as coupling reagent [25]. Cyclo(Phe-Asp) was synthesized according to the literature [26]. The crude peptides were purified by preparative RP-HPLC and their identity was confirmed by mass spectrometry.

2.2. Reversed-phase HPLC

Reversed-phase HPLC with UV detection was performed on a Shimadzu system consisting of two LC-10AD VP pumps, a SPD-10A VP detector, a SCL-10A VP system controller and Class VP software version 5.0 (Shimadzu,

Kyoto, Japan). The compounds were separated on a Grom-sil 120 ODS-4 HE column (125 mm × 2 mm i.d.) (Grom Analytik, Rottenburg-Hailfingen, Germany). Analyses were performed at a flow rate of 0.2 ml/min, detection was carried out at 215 nm. Either 0.1% TFA in water or phosphate buffer were used as eluent. When 0.1% TFA in water was used, a gradient of two different eluents was applied. Eluent A consisted of 0.1% TFA in water and eluent B of 0.1% TFA in water–acetonitrile (5:95, v/v). The gradient was optimized for every sample. Separations in phosphate buffer were carried out with a gradient of eluent A consisting of 15 mM aqueous sodium dihydrogen phosphate and eluent B consisting of water–acetonitrile (5:95, v/v). The pH of the aqueous buffer was adjusted using 100 mM phosphoric acid or 100 mM sodium hydroxide. Gradient and the pH of eluent A were optimized for every sample.

Reversed-phase HPLC with MS detection was performed using a HP 1100 series HPLC system (Agilent, Waldbronn, Germany) with autosampler, high-pressure mixing pump, column oven, diode array detection (DAD) system and HP workstation connected to an API 165 single quadrupole MS system with turbo ion spray interface (Applied Biosystems, Langen, Germany). Analyses were performed using a LiChroCART 125-4 LiChrospher 100 RP-18 (5 μm) cartridge (Merck). The column temperature was set to 25 °C and the flow rate to 1.0 ml/min, with a split of 1:4 for MS detection. The injection volume was 20 μl. UV detection was carried out at 215 nm (DAD spectra 190–700 nm). MS spectra were obtained in the positive ion mode in the scan range between 100 and 400 amu with a ion spray voltage of 5.2 kV. Source temperature was 350 °C, focusing potential 230 V, declustering potential 50 V and entrance potential −10 V.

2.3. Capillary electrophoresis

CE was performed on a Beckman P/ACE 5510 instrument (Beckman Coulter, Unterschleissheim, Germany) equipped with a diode array detector at 20 °C using 50 μm i.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an effective length of 40 cm and a total length of 47 cm. UV detection was carried out at 215 nm at the cathodic end of the capillary. Sample solutions were introduced at the anodic end by hydrodynamic injections at a pressure of 0.5 psi for 3 s (1 psi = 6894.76 Pa). Separations were performed in 50 mM sodium phosphate buffer adjusted to pH 3.0 by the addition of 100 mM phosphoric acid. The applied voltage was 23 kV. Between the analyses the capillary was washed for 1 min with 100 mM phosphoric acid and 3 min with the run buffer.

2.4. Incubations

Solutions of the tripeptides Phe-Asp-GlyNH_2 and Gly-Asp-PheNH_2 with an initial concentration of 2 mg/ml were prepared in 50 mM phosphate buffer adjusted to pH 2.0 with 100 mM phosphoric acid or in 50 mM borate buffer adjusted to pH 10.0 with 100 mM sodium hydroxide. The solutions were incubated at 80 °C. At selected time intervals 100 μl aliquots were withdrawn and added to 200 μl of ice-cold water in the case of the incubations at pH 2 and to 200 μl of ice-cold 100 mM phosphoric acid in case of the incubations at pH 10. The samples were thoroughly mixed and stored at −20 °C until analyzed.

3. Results and discussion

A gradient reversed-phase HPLC method for the separation of the model aspartyl peptides Phe-Asp-GlyNH_2 and Gly-Asp-PheNH_2 and their degradation products after acidic and alkaline incubation has been developed. The gradient of eluent A consisting of 0.1% TFA in water and eluent B consisting of 0.1% TFA in water–acetonitrile (5:95, v/v) was optimized for each sample. Under these conditions all compounds were separated except for two pairs of isomers whose resolution could be achieved in phosphate buffer. The peaks were identified by injection of mixtures of incubated samples with reference substances and confirmed by electrospray mass spectrometry.

3.1. Incubations at pH 2

A typical chromatogram of a Phe-Asp-GlyNH_2 sample incubated at pH 2 for 24 h is shown in Fig. 2A. Separation of all degradation products was achieved with a gradient of 7–20% of eluent B within 15 min. Deamidation of the C-terminal amide and hydrolysis of the peptide backbone were observed. Generally, the amidated peptides had shorter retention times compared to the corresponding peptides with free carboxyl groups. The hydrolysis products Phe-Asp and Phe were observed while GlyNH_2 and Gly could not be detected due to their poor absorbance at 215 nm. Moreover, the succinimidyl intermediates of both the native peptide and its deamidated form could be detected. As expected, the Asu peptides eluted after the corresponding aspartyl peptides because of the higher hydrophobicity of the succinimide moiety compared to Asp with a side chain carboxyl group. The diketopiperazine cyclo(Phe-Asp) resulting from attack of the N-terminal nitrogen on the Asp-Gly amide carbonyl had the longest retention time. Clearly, backbone hydrolysis resulting in Phe-Asp and succinimide formation yielding Phe-Asu-GlyNH_2 were the major degradation routes of Phe-Asp-GlyNH_2 at pH 2. These results are in accordance with previous studies on recombinant human parathyroid hormone [9] and aspartyl hexapeptides [7,27] in which the major degradation pathway after incubation in acidic solution was cleavage of the backbone amide bond with consequent formation of smaller fragments.

Fig. 2C shows a chromatogram of an incubation of the peptide with the inverse amino acid sequence, Gly-Asp-PheNH_2, after 24 h at pH 2. The deamidation product Gly-α-L-Asp-L-PheOH and the succinimidyl derivative

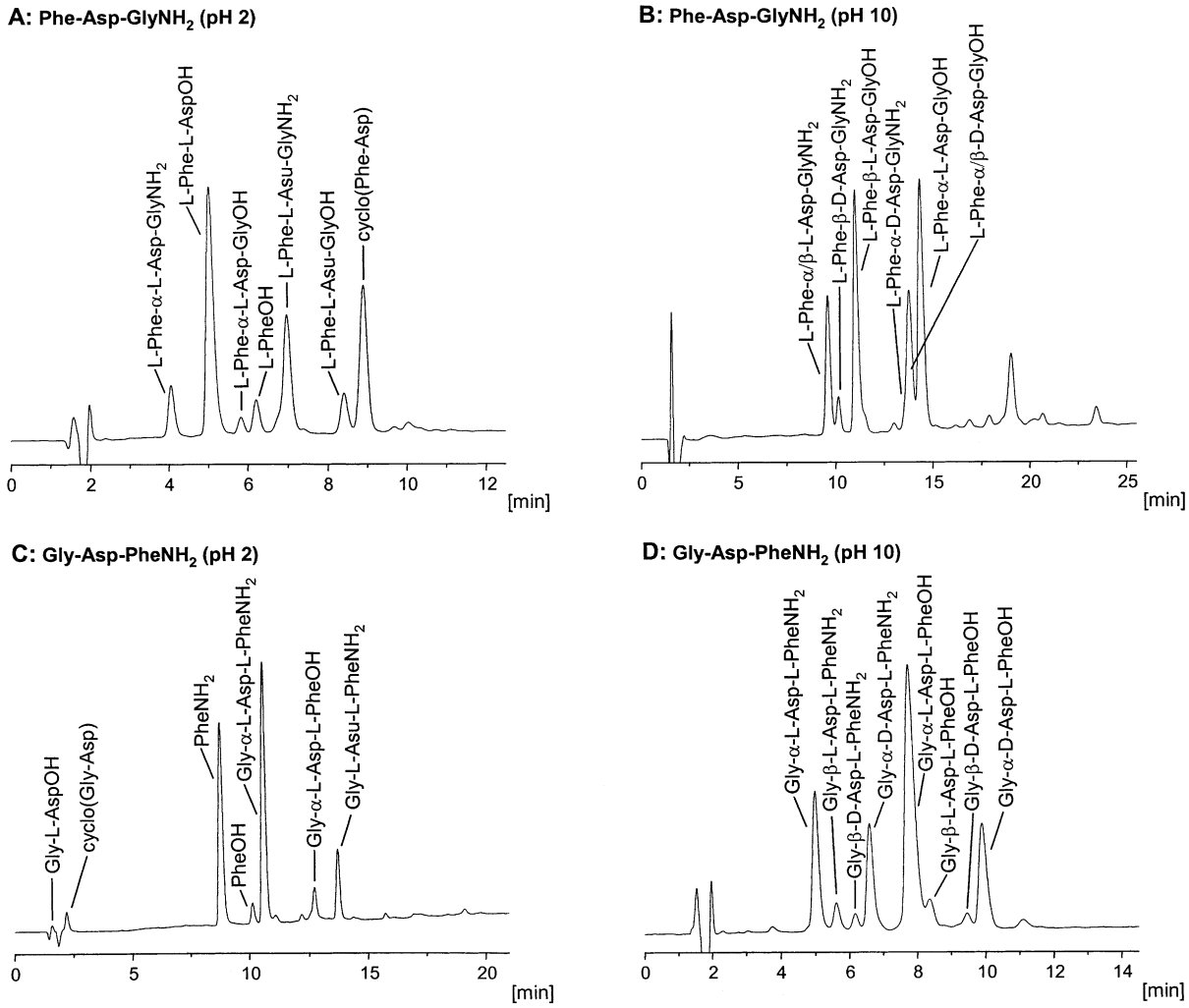

Fig. 2. Chromatograms of Phe-Asp-$GlyNH_2$ incubated at pH 2 for 24 h (A) and at pH 10 for 48 h (B) and of Gly-Asp-$PheNH_2$ incubated at pH 2 for 24 h (C) and at pH 10 for 48 h (D). Conditions: mobile phase gradient 7–20% B within 15 min (A), 2–13% B within 30 min (B), 1–20% B within 10 min (C) and 10–20% B within 15 min (D). Eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in water–acetonitrile (5:95, v/v). Flow 0.2 ml/min. Detection at 215 nm.

Gly-L-Asu-L-$PheNH_2$ along with $PheNH_2$ and Phe could be detected. The best separation of the degradation products was achieved with a gradient of 1–20% of eluent B within 10 min. As already observed for Phe-Asp-$GlyNH_2$, the amide eluted before the deamidated peptide and the succinimide had the longest retention time. Because of their high polarity, Gly-Asp and cyclo(Gly-Asp) did not interact with the stationary phase and eluted in the solvent front. Hydrolysis of the peptide backbone was the major degradation pathway after incubation in acidic medium. In accordance with previous studies on hexapeptides [3,5,7], Gly-Asp-$PheNH_2$ was less susceptible to degradation because of the presence of a sterically hindered amino acid at the Asp-X position compared to Gly in Phe-Asp-$GlyNH_2$.

Peak assignment was made by injection of mixtures of incubated samples with reference substances and confirmed by online electrospray mass spectrometry. All compounds could be identified by their $[M + H]^+$-ions summarized in Table 1. The aspartyl succinimide peptides Phe-Asu-$GlyNH_2$, Gly-Asu-$PheNH_2$ and Phe-Asu-GlyOH were identified solely based on their mass spectra. In addition to the $[M + H]^+$-ions at *m/z* 319 (amides) and *m/z* 320 (acid), respectively, the spectra showed a rather simple fragmentation pathway with the ion a_1 at *m/z* 120 as the principal fragment. Cyclo(Phe-Asp) showed the molecular ion at *m/z* 263 and a high abundant peak at *m/z* 245, corresponding to the loss of water ($[M-H_2O]^+$-ion). The spectra of the tripeptides are discussed in more detail in Section 3.2.

3.2. Incubations at pH 10

Incubations of Phe-Asp-$GlyNH_2$ and Gly-Asp-$PheNH_2$ at pH 10 resulted in different degradation patterns compared to pH 2. Cleavage of the backbone amide bonds was not observed to a significant extent. Instead, isomerization and enantiomerization via the cyclic imide dominated. A

Table 1
$[M + H]^+$-ions detected by HPLC-MS experiments

Sequence	Peptide/amino acid	$[M + H]^+$
Phe-Asp-Gly	Phe-Asp-$GlyNH_2$	337
	Phe-β-Asp-$GlyNH_2$	337
	Phe-Asp-GlyOH	338
	Phe-β-Asp-GlyOH	338
	Phe-Asu-$GlyNH_2$	319
	Phe-Asu-GlyOH	320
	Phe-AspOH	281
	Cyclo(Phe-Asp)	263
	PheOH	166
Gly-Asp-Phe	Gly-Asp-$PheNH_2$	337
	Gly-β-Asp-$PheNH_2$	337
	Gly-Asp-PheOH	338
	Gly-β-Asp-PheOH	338
	Gly-Asu-$PheNH_2$	319
	Gly-AspOH	191
	Cyclo(Gly-Asp)	173
	PheOH	166
	$PheNH_2$	165

The compounds were observed either from incubations at pH 2 or from incubations at pH 10.

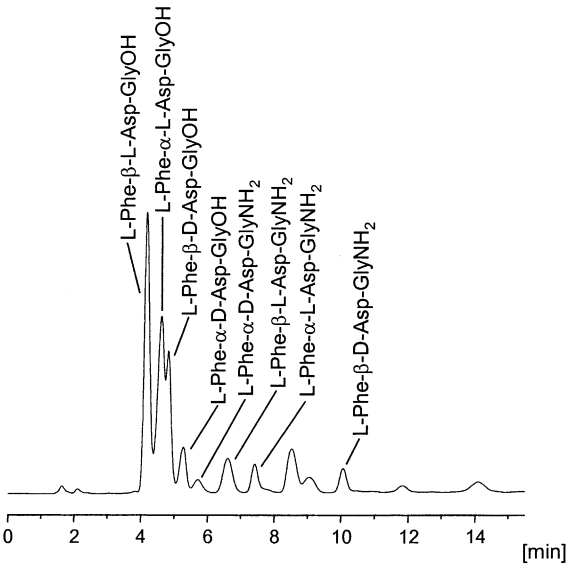

Fig. 3. Chromatogram of Phe-Asp-$GlyNH_2$ incubated at pH 10 for 48 h analyzed in phosphate buffer with a gradient of 2–13% of eluent B in 30 min. Eluent A was 15 mM aqueous phosphate, pH 5.0, and eluent B was water–acetonitrile (5:95, v/v). Flow 0.2 ml/min. Detection at 215 nm.

representative chromatogram of the degradation products of Phe-Asp-$GlyNH_2$ after incubation at pH 10 for 48 h is depicted in Fig. 2B. The best separation was achieved with a gradient of 2–13% of eluent B within 30 min. Deamidation of the C-terminal amide as well as isomerization and concomitant enantiomerization of Asp were observed in the sample. Amidated peptides displayed shorter retention times compared to the corresponding compounds with free terminal carboxyl group. Except for the diastereomeric pair Phe-α-L-Asp-GlyOH/Phe-α-D-Asp-GlyOH the L-Asp containing isomers eluted faster than the corresponding D-Asp peptides.

The isomeric pairs L-Phe-α-L-Asp-$GlyNH_2$/L-Phe-β-L-Asp-$GlyNH_2$ and L-Phe-α-D-Asp-GlyOH/L-Phe-β-D-Asp-GlyOH could not be resolved in water–acetonitrile mixtures containing TFA. Kaneda et al. reported on the separation of isomeric peptides of αA-crystallin using 15 mM phosphate buffer instead of aqueous TFA [28]. Subsequently, various isocratic acetonitrile–phosphate buffer mixtures were investigated as mobile phases. Partial resolution of L-Phe-α-L-Asp-$GlyNH_2$ and L-Phe-β-L-Asp-$GlyNH_2$ could be achieved at pH 5.0 with 85:15 (v/v) phosphate buffer–acetonitrile, the retention times were 3.7 and 3.2 min, respectively (data not shown). Baseline resolution of L-Phe-α-D-Asp-GlyOH and L-Phe-β-D-Asp-GlyOH was achieved at pH 4.0 under otherwise identical conditions (retention times: 4.6 and 3.5 min, respectively, data not shown). Fig. 3 shows the chromatogram of a Phe-Asp-$GlyNH_2$ sample incubated at pH 10 for 48 h and analyzed with an acetonitrile–phosphate buffer, pH 5.0, gradient (2–13% eluent B within 30 min). Although only partial resolution of L-Phe-α-L-Asp-GlyOH and L-Phe-β-D-Asp-GlyOH was achieved, all other compounds were separated, including those whose separation could not be achieved with the TFA in water–acetonitrile gradient. At pH 5.0 all peptides with free carboxyl groups eluted faster than the amidated peptides, because of the higher polarity of the deprotonated terminal carboxyl group.

Fig. 2D shows a chromatogram of a sample of Gly-Asp-$PheNH_2$ after incubation at pH 10 for 48 h under optimized conditions with a gradient of 10–20% of eluent B in 15 min. In contrast to Phe-Asp-$GlyNH_2$, all isomers of Gly-Asp-$PheNH_2$ and its deamidated form could be separated by TFA in water–acetonitrile. The amide peptides eluted before the deamidated compounds and L-Asp containing peptides eluted faster than the corresponding D-Asp peptides.

Online mass spectrometry detection confirmed the peak assignment in TFA in water–acetonitrile mobile phases. The native peptides L-Phe-α-L-Asp-$GlyNH_2$ and Gly-α-L-Asp-L-$PheNH_2$ can be easily discriminated from their deamidated forms by the molecular ions at *m/z* 337 (amides) and *m/z* 338 (carboxylic acid). In addition, α- and β-Asp peptides can be distinguished by careful analysis of the fragmentation pattern as shown for L-Phe-α-L-Asp-$GlyNH_2$ (Fig. 4A) and L-Phe-β-L-Asp-$GlyNH_2$ (Fig. 4B). Besides the $[M + H]^+$- and $[M + Na]^+$-ions the spectra displayed the a_1 ion at *m/z* 120, the y_2 ion at *m/z* 190 and the b_2 ion at *m/z* 263. Differences between peptides with an α- and β-linkage can be found in the b/y ratio of complementary b- and y-ions generated by cleavage of the α/β-Asp-X bond. The α-Asp peptide exhibited a more intense b_2 fragment ion and a low abundant y_2 fragment ion, while in the case of the β-Asp peptide the b_2 ion had lower intensity compared to the y_2 fragment ion (Fig. 4A and B). This is in accordance with previous studies on the peptides by CE-electrospray

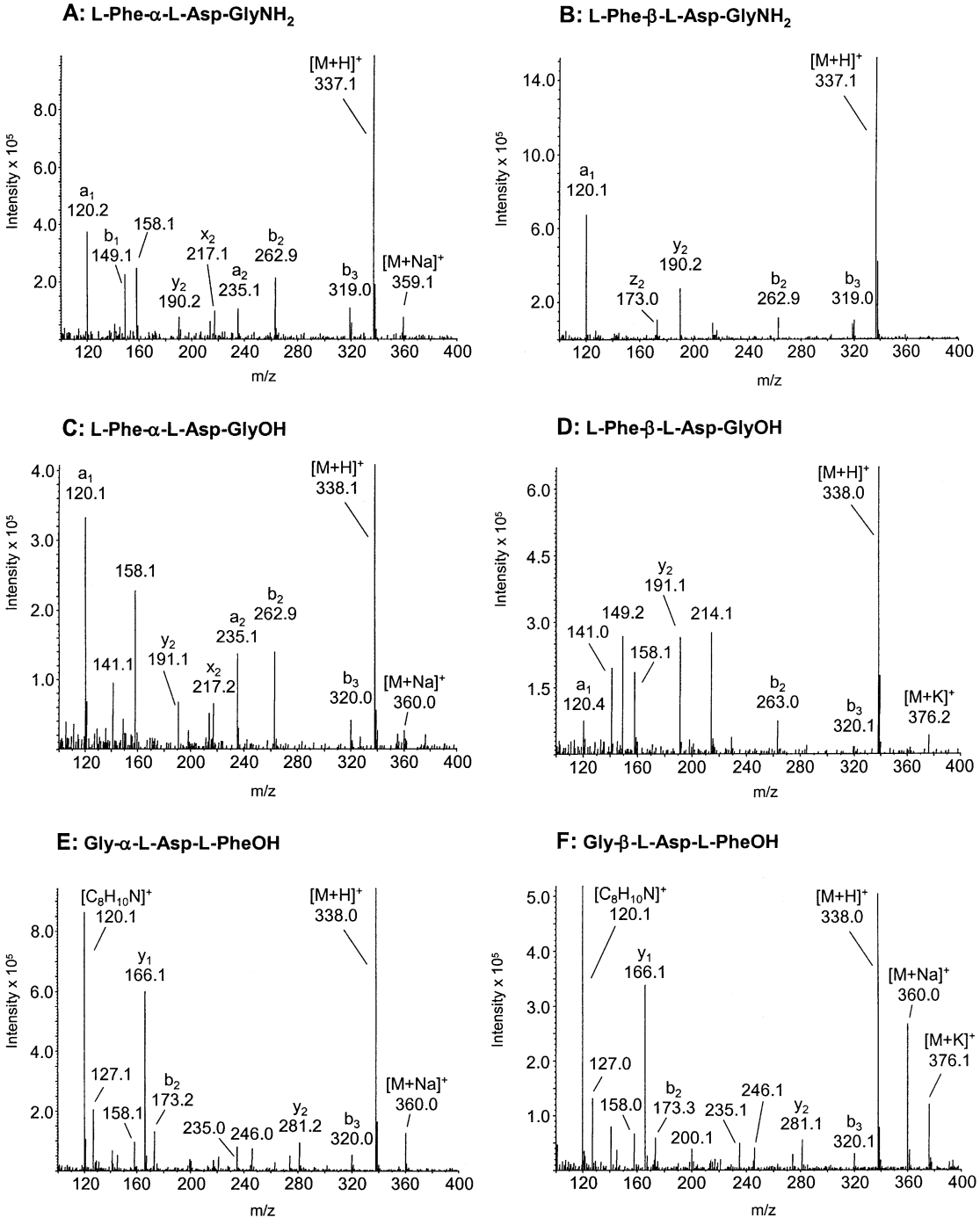

Fig. 4. Mass spectra of L-Phe-α-L-Asp-GlyNH$_2$ (A), L-Phe-β-L-Asp-GlyNH$_2$ (B), L-Phe-α-L-Asp-GlyOH (C), L-Phe-β-L-Asp-GlyOH (D), Gly-α-L-Asp-L-PheOH (E) and Gly-β-L-Asp-L-PheOH (F). (A) and (B) were analyzed by injection of reference substances, (C)–(F) were recorded from samples of Phe-Asp-GlyNH$_2$ and Gly-Asp-PheNH$_2$ after incubation at pH 10.

tandem mass spectrometry [23] as well as publications on Asp peptides [29–31]. L-Phe-α-L-Asp-GlyOH and L-Phe-β-L-Asp-GlyOH (Fig. 4C and D) displayed a similar fragmentation pattern, with the a_1 ion at *m/z* 120, the y_2 ion at *m/z* 191 and the b_2 ion at *m/z* 263. Both the amidated and the deamidated form of the α-Asp peptides showed the x_2 ion at *m/z* 217 and the a_2 ion at *m/z* 235, which were not present in the spectra of the corresponding β-Asp peptides. Reliable differences in the spectra of D-Asp and L-Asp containing peptides could not be observed. Thus, identification was performed by comparison with synthetic peptides containing Asp with known configuration. Differences in the mass spectra of α- and β-Asp peptides derived from Phe-Asp-GlyNH$_2$ were obvious but less pronounced. All

spectra showed an intense a_1 fragment at *m/z* 120, an intense y_1 fragment at *m/z* 166, and a b_3 fragment at *m/z* 319 or 320 for the native peptide and its deamidated form, respectively (Fig. 4E and F). Less intense ions at *m/z* 173 and 281 corresponding to the fragments b_2 and y_2, respectively, could be detected in both α- and β-Asp peptides, the b_2 fragment having a higher intensity in case of the α-linkage. Apparently, the tendency to form ions with charge retention at the N terminus is low probably due to the presence of Gly as terminal amino acid.

3.3. Comparison of HPLC and CE for the analysis of tripeptide degradation

HPLC and CE are complementary techniques due to the different separation mechanisms. While polarity of the analytes is primarily responsible for the separation of compounds in reversed-phase HPLC, analytes are separated in CE based on their charge density (charge-to-mass ratio). Consequently, differences in the elution and migration order as well as in the resolution of the compounds can be expected.

Fig. 5 shows electropherograms of the samples of the tripeptides incubated at pH 2 and 10. The separation was performed in 50 mM phosphate buffer, pH 3.0, as developed in the previous CE study [23]. In incubations at pH 2, all compounds analyzed by HPLC can also be detected by CE except for the diketopiperazines cyclo(Phe-Asp) and cyclo(Gly-Asp) which migrate as a anionic compounds behind the electroosmotic flow (EOF) at pH 3.0. However, the compounds can be analyzed by switching to an ammonium formate buffer, pH 2.9, containing 10% acetonitrile [23]. On the other hand, Gly-Asp and cyclo(Gly-Asp) eluted in the solvent front in HPLC and can, therefore, not be reliably analyzed in this system. $GlyNH_2$ could not be detected in HPLC while the compound was easily analyzed by CE. As pointed out above, the compounds have different migration orders compared to the elution orders in HPLC due to the different separation mechanisms of both techniques.

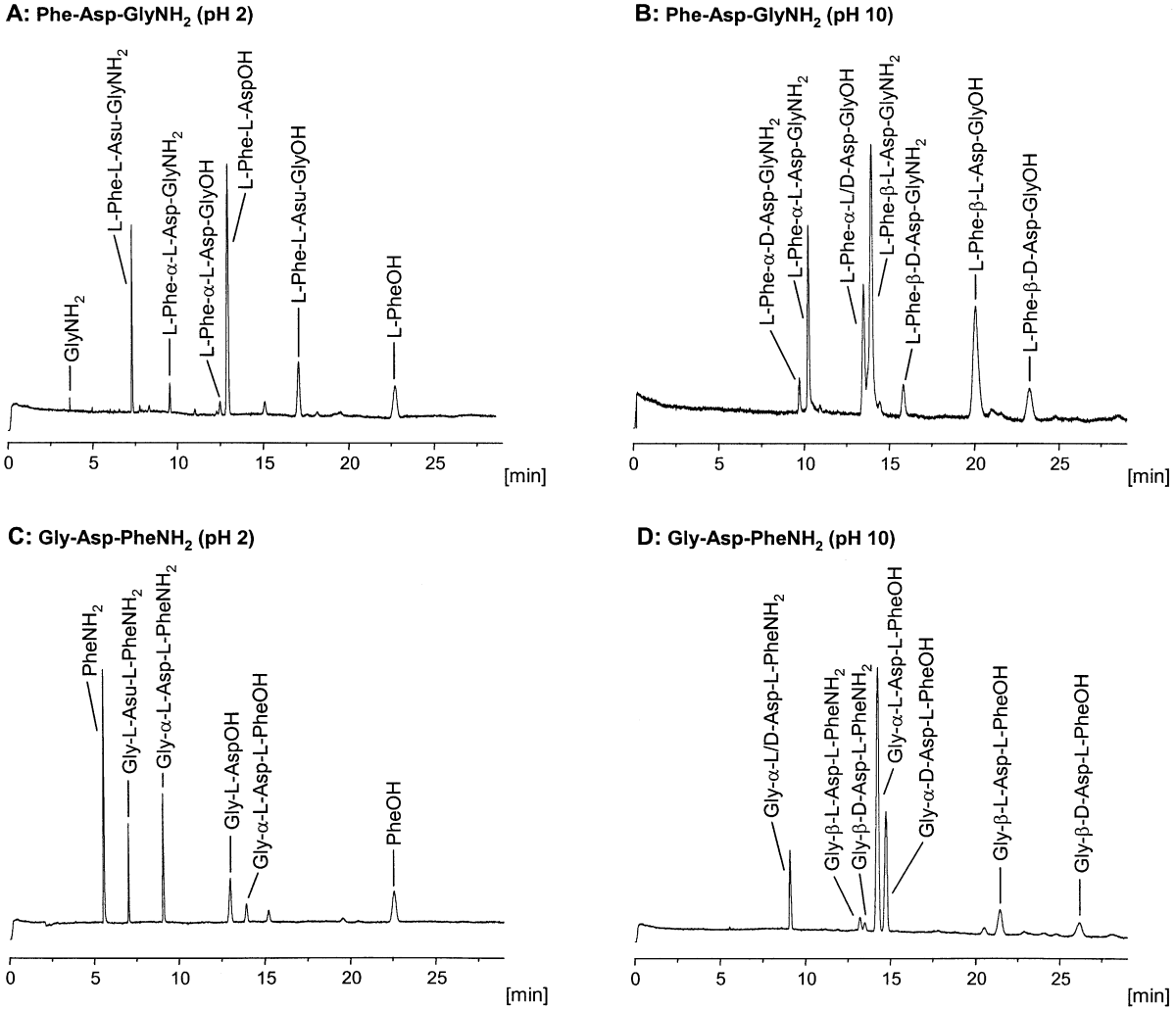

Fig. 5. Electropherograms of Phe-Asp-$GlyNH_2$ incubated at pH 2 for 31 h (A) and at pH 10 for 24 h (B) and of Gly-Asp-$PheNH_2$ incubated at pH 2 for 48 h (C) and at pH 10 for 72 h (D). Conditions: 50 mM phosphate buffer, pH 3.0, 47/40 cm capillary, 50 mM i.d., UV detection at 215 nm, 23 kV.

CE analysis of the pH 10 incubations revealed that the diastereomeric pairs Phe-α-L/D-Asp-GlyOH and Gly-α-L/D-Asp-$PheNH_2$ could not be resolved using a pH 3.0 run buffer. These pairs could be easily resolved by HPLC. However, while all isomers of the Gly-Asp-Phe series could be at least partially separated by HPLC, the α- and β-Asp pairs Phe-α/β-L-Asp-$GlyNH_2$ and Phe-α/β-D-Asp-GlyOH could not be resolved by HPLC using TFA in water–acetonitrile. As pointed out above, resolution of the compounds can be achieved using a phosphate buffer, pH 5.0, instead of aqueous TFA in the mobile phase. However, the use of phosphate buffers does not allow coupling of mass spectrometry to HPLC due to contamination of the ion source. Interestingly, the deamidated peptides displayed in CE longer migration times compared to the C-terminal amidated analogs similarly to HPLC using aqueous TFA in the mobile phase where the amides eluted faster than the corresponding peptides with a free terminal carboxyl group.

4. Conclusions

HPLC methods have been developed for the analysis of the degradation of the aspartyl model peptides Phe-Asp-$GlyNH_2$ and Gly-Asp-$PheNH_2$ at pH 2 and 10 optimizing the water–acetonitrile gradient containing TFA for each sample. The system allowed the detection of most degradation products except for two isomeric pairs of peptides containing α-Asp and β-Asp, Phe-α/β-L-Asp-$GlyNH_2$ and Phe-α/β-D-Asp-GlyOH, which coeluted in the assay. The analytes could be resolved by replacing aqueous TFA by a phosphate buffer, pH 5.0. Peak identification by online mass spectrometry revealed differences in the mass spectra of α- and β-Asp containing peptides.

In addition, HPLC was compared to CE. Both methods allowed the analysis of most compounds. While a pair of diastereomers could not be separated by CE, HPLC was less efficient resolving peptides containing α- and β-Asp linkages. Overall, separations that were difficult with one technique proved to be easily achieved by the other method illustrating that HPLC and CE are complementary techniques in peptide analysis.

Acknowledgements

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