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Capillary electrophoresis analysis of the degradation of the aspartyl tripeptide Phe-Asp-GlyOH at pH 2.0 and 7.4 under forced conditions

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ABSTRACT

The degradation of the tripeptide L-Phe-α-L-Asp-GlyOH was studied at 80 °C and pH 2.0 and 7.4 by capillary electrophoresis. Separation of most known as well as unknown degradation products was achieved in a 50 mM sodium phosphate buffer, pH 3.0. The diastereomers L-Phe-α-L-Asp-GlyOH/L-Phe-α-D-Asp-GlyOH could only be separated upon addition of 16 mg/ml carboxymethyl-β-cyclodextrin and 5% acetonitrile to the background electrolyte. Compound identification was performed by capillary electrophoresis-electrospray ionization-mass spectrometry. In addition to Asp isomerization and epimerization products as well as hydrolysis products four diketopiperazine derivatives were identified. Moreover, two degradation products were observed containing the amino acids Asp, Gly and Phe but the unequivocal assignment could not be accomplished based on the mass spectra. Following validation with regard to linearity, range, limit of detection, limit of quantitation and precision the assay was applied to the analysis of the incubation solutions. While peptide backbone hydrolysis dominated at pH 2.0, isomerization and enantiomerization yielding β-Asp and D-Asp peptides as well as cyclization to diketopiperazine derivatives were observed at pH 7.4. The diketopiperazines were the dominant reaction products amounting to about 85% of the compounds detected after the maximal incubation time of 240 h. A kinetic model was used to fit the concentration versus time data.

1. Introduction

Peptides and proteins are susceptible to chemical and physical degradation, such as deamidation, oxidation, proteolysis, racemization, aggregation and surface absorption [1]. These processes can lead to the loss of activity as well as to the formation of toxic degradation products. Aspartic acid is an unstable amino acid undergoing isomerization and enantiomerization [1–4]. The degradation mechanism of aspartic acid involves the formation of an aminosuccinimide (Asu) through nucleophilic attack of the β-carbonyl carbon by the α-nitrogen of the next amino acid. Subsequently, different products result depending on the pH [1,5–7]. At strongly acidic pH, cleavage of the peptide backbone is predominant while with increasing pH values isomerization yielding iso-aspartyl (β-aspartyl) residues as well as enantiomerization of Asp is primarily observed. Because of the increased acidity of the succinimidyl α-carbon the rate of Asp racemization in peptides is about 10^5 times faster than that of Asp itself under similar conditions [4,8]. Recently, Li et al. provided evidence that enantiomerization of Asn peptides may also occur in the tetrahedral intermediate preceding the succinimide [9]. Hydrolysis of the succinimide produces either the native aspartyl residue (α-Asp) or the iso-aspartyl residue (β-Asp). The reaction rates of aspartyl residues depend on steric hindrance of the amino acid following Asp [2,3,5,10]. Moreover, protein structure and conformation can affect the reaction rates of aspartyl residues [11,12] as can buffer type, pH, temperature, etc. [2,10,13]. Asp isomerization and enantiomerization is a natural ageing process for proteins leading to age-dependent accumulation of β-Asp and D-Asp containing proteins in numerous human tissues, such as tooth dentine, skin, bone and ocular lens which may increase under pathological conditions [14]. For example, β-Asp linkage and/or D-Asp have been detected in neurofibrillary tangles and plaques in Alzheimer disease [15,16]. Furthermore, degradation via succinimide formation was also observed in synthetic peptide drugs such as klerval [6] and pramlintide [17].

Traditionally, enantiomerization of amino acids in peptides and proteins is determined by chiral chromatography upon total hydrolysis of the proteins. However, this causes enantiomerization so that correction factors have to be applied [18]. Moreover, this method is not able to discriminate between α-Asp and β-Asp residues so that the presence of β-Asp has to be indirectly

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confirmed by the resistance of β-Asp bonds to Edman degradation [19]. Separation techniques, especially capillary electrophoresis (CE), have been shown to provide powerful analysis of peptides [20,21] including peptide stereoisomers [22,23]. Recently, CE and HPLC assays for the analysis of the model tripeptides Phe-Asp-GlyNH_2 and Gly-Asp-PheNH_2 including the identification of β-Asp and D-Asp containing degradation products by on-line mass spectrometry have been published [7,14,25].

The present study was conducted in order to determine the degradation of the model tripeptide Phe-Asp-GlyOH at 80 °C and pH 2.0 and 7.4. DeHart and Anderson recently reported the degradation of Phe-Asn-GlyOH and Phe-Asp-GlyOH in alkaline sodium carbonate buffers, pH 8.5–10.5, at 25 °C [26]. While isomerization to Phe-β-Asp-GlyOH was observed, the main degradation product proved to be the diketopiperazine cyclo(Phe-Asp)-GlyOH which was found to epimerize under the experimental conditions. The enantiomerization of Asp in the course of the isomerization via the succinimide was not addressed in detail in this study.

2. Experimental

2.1. Chemicals

Phe, L-Phe-L-AspOH, cyclo(L-Asp-L-Phe), Fmoc-L-Phe-OH, Fmoc-L-Asp(OtBu)-OH, Fmoc-D-Asp(OtBu)-OH, Fmoc-L-AspOtBu, Fmoc-D-AspOtBu and Fmoc-Gly-Wang resin were purchased from Bachem AG (Heidelberg, Germany). The peptides L-Phe-α-L-Asp-GlyOH, L-Phe-α-D-Asp-GlyOH, L-Phe-β-L-Asp-GlyOH and L-Phe-β-D-Asp-GlyOH were synthesized by standard solid phase synthesis using 2-(1H-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as coupling reagent and 1-hydroxybenzotriazole (HOBt) as additive. L-Phe-L-Asu-GlyOH was prepared from L-Phe-L-α-Asp-GlyOH by treatment with 1.8 M HCl in glacial acetic acid according to [27]. The crude peptides were purified by preparative RP-HPLC using an acetonitrile–0.1% (v/v) aqueous trifluoroacetic acid (TFA) gradient. Collected fractions were combined and lyophilized. The peptides were characterized by capillary electrophoresis (CE), HPLC and mass spectrometry. The purity was at least 99% by HPLC and CE analysis.

HPLC quality isopropanol, acetonitrile and TFA were purchased from Merck (Darmstadt, Germany), carboxymethyl-β-cyclodextrin sodium-salt was from Wacker-Chemie GmbH (Burghausen, Germany) and 4-(aminomethyl)benzoic acid (PAMBA) from Fisher Scientific (Schwerte, Germany). All other chemicals were of analytical grade. Buffers and solutions were prepared in double distilled, deionized water, filtered (0.2 μm) and degassed by sonication.

2.2. Instrumentation

Capillary electrophoresis with UV detection was performed on a Beckman P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Unterschleißheim, Germany) equipped with a diode array detector. 50 μm ID fused-silica capillaries (BGB Analytik Vertrieb, Schlossboeckelheim, Germany) with an effective length of 40.0 cm and a total length of 50.2 cm were used. The capillary was thermostated at 20 °C, UV detection was carried out at 215 nm at the cathodic end of the capillary. Separations were performed in 50 mM sodium phosphate buffer adjusted to pH 3.0 by addition of solid sodium hydroxide to 50 mM phosphoric acid. The applied voltage was 25 kV. Sample solutions were introduced at the anodic end by hydrodynamic injections at 0.5 psi for 5 s. Between the analyses the capillary was washed for 2 min with 100 mM sodium hydroxide, 2 min with water and 3 min with the background electrolyte. Separation of the diastereomeric pair L-Phe-α-L-Asp-GlyOH/L-Phe-α-D-Asp-GlyOH was performed in 50 mM phosphate buffer, pH 3.0, containing 16 mg/ml carboxymethyl-β-cyclodextrin and 5% acetonitrile. To avoid the evaporation of acetonitrile the buffer was covered with a thin layer of liquid paraffin.

Capillary electrophoresis-mass spectrometry (CE-MS) experiments were performed using an ATI Unicam Crystal CE system (Prince Technologies, Emmen, The Netherlands) connected to a quadrupole-time-of-flight mass spectrometer (QqTOF MS, Bruker Daltonik, Bremen, Germany) via a sheath liquid interface from Agilent Technology (Waldbronn, Germany). Separation was performed in 50 μm ID fused-silica capillaries with a length of 62 cm using a 100 mM formic acid buffer adjusted to the pH of 2.9 by the addition of aqueous ammonia. The applied voltage was 28 kV. A pressure of 100 mbar for 6 s was used for sample injection. ESI was performed in positive mode, MS inlet set to −4500 V (sprayer on ground). The sheath liquid isopropanol/water (1:1, v/v) containing 0.2% (v/v) formic acid was supplied at a flow rate of 4 μl/min. Nebulizer gas pressure was set to 0.2 psi. MS spectra were acquired in the mass range m/z 50–700 by summarizing 5000 spectra resulting in a time resolution of 1 s. Data-dependent MS/MS experiments were performed for unequivocal identification in combination with accurate mass measurements. Calibration was performed for each CE-MS run using the sodium formate cluster. Data processing was achieved with the DataAnalysis 3.4 software (Bruker Daltonik). The peptide MS fragments were labeled according to standard rules [28,29].

2.3. Peptide degradation

The degradation of the peptides was performed at 80 °C in 50 mM phosphate buffer, pH 2.0 or pH 7.4, adjusted to an ionic strength of 0.2 M by the addition of sodium chloride. The pH was adjusted at 25 °C, the actual pH may be estimated using dpK_A/dT of $-0.0028\,K^{-1}$. The peptides had an initial concentration of 2 mg/ml (5.93 mmol/l) containing 0.09 mg/ml (0.59 mmol/l) PAMBA as internal standard to compensate for solvent evaporation during the incubation process. At selected time intervals, 70 μl aliquots were withdrawn and added to 140 μl ice-cold 100 mM phosphoric acid (pH 7.4) or 140 μl ice-cold water (pH 2.0). The samples were thoroughly mixed, frozen and stored at −20 °C until analyzed.

The experimental data were fitted using the SAAM II v1.2.1 software (SAAM Institute, Seattle, WA, USA).

3. Results and discussion

The CE analysis was based on the experimental procedures of the CE separation of degradation products of Phe-Asp-GlyNH_2 including the isomerization and enantiomerization products which used a phosphate buffer, pH 3.0 [7,24]. All compounds could be separated except for the pair of diastereomers of L-Phe-α-L-Asp-GlyOH and L-Phe-α-D-Asp-GlyOH, which were separated using a phosphate buffer, pH 3.0, containing 16 mg/ml carboxymethyl-β-cyclodextrin and 5% acetonitrile (data not shown). PAMBA was selected as internal standard to compensate for solvent evaporation during the incubations as well as for small injection changes and variability of the electroosmotic flow in CE analysis. The compound was stable under the incubation conditions at elevated temperature [7]. As PAMBA did not reach the detector within 45 min due to strong complexation by the cyclodextrin, the quantification of L-Phe-α-L-Asp-GlyOH and L-Phe-α-D-Asp-GlyOH was based on the sum of analytes using the phosphate buffer without cyclodextrin followed by the determination of the ratio of the compounds obtained in the assay using carboxymethyl-β-cyclodextrin in the background electrolyte.

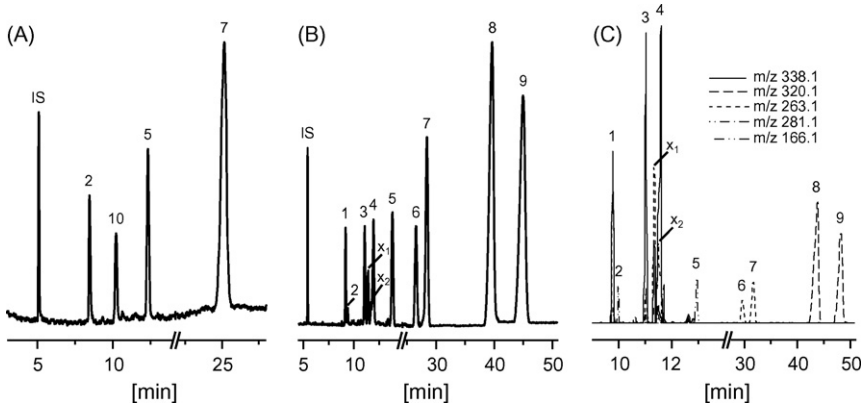

Fig. 1. Electropherograms of incubations of L-Phe-α-L-Asp-GlyOH in phosphate buffer at 80 °C. (A) pH 2.0 after 72 h and (B) pH 7.4 after 168 h. *Experimental conditions*: 40/50.2 cm fused-silica capillary, 50 μm ID; 20 °C; 25 kV; UV detection at 215 nm; background electrolyte: 50 mM sodium phosphate buffer, pH 3.0. (C) Extracted ion electropherogram of an incubation at 80 °C and pH 7.4 after 192 h. *Experimental conditions*: 62 cm fused-silica capillary, 50 μm ID; 20 °C; 28 kV; background electrolyte: 100 nM ammonium formate buffer, pH 2.9, containing 10% acetonitrile. For MS conditions see Section 2. L-Phe-α-L/D-Asp-GlyOH (1), L-Phe-L-AspOH (2), L-Phe-β-L-Asp-GlyOH (3), L-Phe-β-D-Asp-GlyOH (4), L-PheOH (5), *trans*-DKP (6), *cis*-DKP (7), *trans*-DKP-GlyOH (8), *cis*-DKP-GlyOH (9), L-Phe-L-Asu-GlyOH (10), and the internal standard PAMBA (IS).

Fig. 1 shows representative electropherograms of incubations of L-Phe-α-L-Asp-GlyOH at 80 °C and pH 2 for 72 h (Fig. 1A) and pH 7.4 for 168 h (Fig. 1B) analyzed in phosphate buffer, pH 3.0, as background electrolyte. Peak identification was performed by co-injection of reference compounds, in case those were available, and by CE-electrospray ionization-mass spectrometry (CE-ESI-MS) as discussed in detail below. The degradation of L-Phe-α-L-Asp-GlyOH is presented in Section 3.2.

3.1. Peptide identification by CE-MS

For CE-ESI-MS experiments the phosphate buffer, pH 3.0, was replaced by 100 mM ammonium formate buffer, pH 2.9, containing 10% acetonitrile [24]. CE-MS yielded comparable analyte separations as shown by the extracted ion electropherogram in Fig. 1C for an incubation at pH 7.4. All compounds were detected as $[M+H]^+$-ions, summarized in Table 1. MS spectra of linear Asp peptides as well as the Asu peptide are summarized in Fig. 2. Generally, $[M+Na]^+$- and $[M+K]^+$-ions could also be detected. Moreover, α-Asp and β-Asp peptides could be distinguished by their b_2/y_2 fragment ratio. Lehmann et al. distinguished by ESI-MS between α-Asp and β-Asp peptides for a series of 15 peptides based on the ratio of the complementary b- and y-fragment ions generated by cleavage of α/β-Asp-XXX bond and of the XXX-α/β-Asp bond [30]. Generally, a smaller b/y ratio was observed for the β-Asp peptides compared to α-Asp peptides. An analogous MS behavior was observed previously for L-Phe-α/β-D/L-Asp-GlyNH$_2$ peptides [24]. The diastereomers L-Phe-α-L-Asp-GlyOH and L-Phe-α-D-Asp-GlyOH could not be separated by CE so that only a "cumulative" spectrum is shown in Fig. 2A indicating an intense b_2 ion and a low abundant y_2 ion. This clearly identifies the α-Asp linkage. In contrast, the β-Asp peptide diastereomers shown in Fig. 2B and C exhibit more intense y_2 ions than b_2 ions. The Asu peptide could also be unequivocally identified based on the mass spectrum (Fig. 2D) [24]. Furthermore, two peaks with a $[M+H]^+$-ion at m/z 281 were observed. One compound could clearly be assigned L-Phe-L-Asp (Fig. 2E) based on comparison of the migration time of reference material and the mass spectrum. The second compound was present at very low concentrations. Thus, it was only detected in CE-MS but was below the limit of detection in CE with UV detection. The mass spectrum shown in Fig. 2F is in accordance with the sequence L-Asp-L-PheOH.

Table 1
$[M+H]^+$-ions detected by CE-ESI-MS experiments.

Peptide/amino acid	Peak no.	$[M+H]^+$
L-Phe-α-L-Asp-GlyOH	1	338
L-Phe-β-L-Asp-GlyOH	3	338
L-Phe-α-D-Asp-GlyOH	1	338
L-Phe-β-D-Asp-GlyOH	4	338
L-Phe-L-Asu-GlyOH	10	320
cis-DKP-GlyOH	9	320
trans-DKP-GlyOH	8	320
L-Phe-L-AspOH	2	281
α-Asp-L-PheOH	–	281
cis-DKP	7	263
trans-DKP	6	263
PheOH	5	166

One peak could be detected in incubations at pH 2.0 while four peaks were found in incubations at pH 7.4 which migrated after the EOF (Fig. 1, EOF not shown in the figure) indicating that they are at least partially negatively charged at the pH of the background electrolyte. Formation of diketopiperazine derivatives has been reported for Phe-Asp-GlyNH$_2$ at pH 2.0 and 80 °C [7,25] and for Phe-Asp-GlyOH at pH 10.0 and 25 °C [26]. The pairs of compounds in Fig. 1, i.e. compounds 6 and 7 and compounds 8 and 9, respectively, showed identical mass spectra. Only the spectra obtained from peaks 7 and 9 are displayed in Fig. 3A and B, respectively. The proposed fragmentation scheme is shown in Fig. 3C. The mass spectrum of compounds 6 and 7 displayed a $[M+H]^+$-ion at m/z 263 (Fig. 3A) which corresponds to the diketopiperazine cyclo(Asp-Phe). Loss of water results in the ion at m/z 245, while the fragment at m/z 203 can be explained by the loss of the aspartic acid side chain, i.e. loss of ($[CH_2–COOH^\bullet]^+ + H^+$). The commercially available cyclo(L-Asp-L-Phe) comigrated with peak 7 and gave identical mass spectra. Thus, peak 7 was assigned cyclo(L-Asp-L-Phe) (*cis*-DKP) while peak 6 was tentatively assigned the epimer cyclo(D-Asp-L-Phe) (*trans*-DKP). Peaks 8 and 9 showed $[M+H]^+$-ions at m/z 320 (shown for compound 9 in Fig. 3B). This is in accordance with the mass of the diketopiperazine derivative cyclo(Asp-Phe)-GlyOH. The fragment at m/z 245 is due to the loss of glycine, m/z 203 results from further loss of $[CH_2CO^\bullet]^+$. As no reference compounds for any of these derivatives were available the assignment was based on the migration behavior of the cyclo(Asp-Phe) isomers assuming that the *cis*-form migrates slower than the *trans*-isomer. Thus, peak 8 was assigned cyclo(L-Asp-L-Phe)-GlyOH (*cis*-DKP-GlyOH) while peak 9 was assumed to be cyclo(D-Asp-L-Phe)-GlyOH (*trans*-DKP-GlyOH). The fact that *cis*-DKP-GlyOH was observed in higher concentrations compared to *trans*-DKP-GlyOH

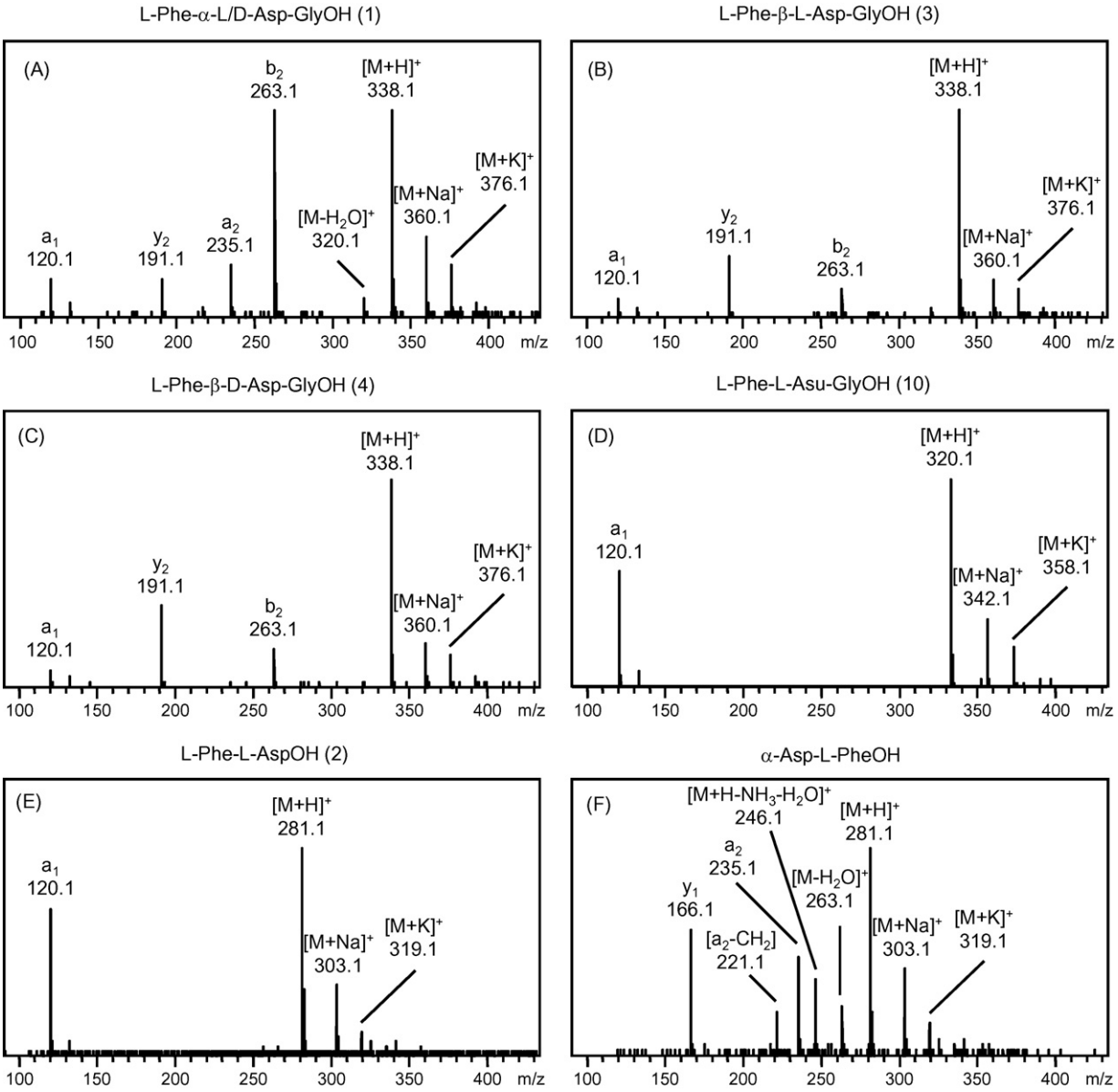

Fig. 2. (A–F) Mass spectra of compounds detected by CE-ESI-MS. For experimental conditions see Section 2.

at early time points of the incubations of L-Phe-α-L-Asp-GlyOH at pH 7.4 as described below supports this assumption. The isomeric compounds *cis*/*trans*-DKP-GlyOH were also reported as degradation products of L-Phe-α-L-Asp-GlyOH in the pH range 7.5–10.5 [26].

The electropherogram of the incubation of L-Phe-α-L-Asp-GlyOH at pH 7.4 for 168 h showed two additional unknown peaks with migration times of about 11.3 min (peak X_1) and 11.6 min (peak X_2) (Fig. 1B). Dominant peaks in the respective mass spectra include *m*/*z* 338 and *m*/*z* 263 (compare Fig. 1C). Both peaks X_1 and X_2 gave identical mass spectra so that they appear to be stereoisomers containing L-Asp and D-Asp, respectively. The mass and tandem mass spectra of peak X_1 are shown in Fig. 4A and B. The compound displayed a $[M+H]^+$-ion at *m*/*z* 338. The ions at *m*/*z* 360 and *m*/*z* 376 correspond to the sodium and potassium adducts, respectively. The masses of the fragments at *m*/*z* 263 and *m*/*z* 235 correspond to the b_2 and a_2 ions of the Phe-Asp-GlyOH peptides (see Fig. 2A–C) resulting from loss of glycine (b_2 fragment) and further loss of CO, respectively. Thus, Gly appears to be the C-terminal amino acid. The absence of a fragment at *m*/*z* 191 suggests that Phe is not attached to the amino group of Asp, i.e. Phe is not the N-terminal amino acid. The MS/MS spectrum of the $[M+H]^+$-ion yielded the highly abundant a_2 ion at *m*/*z* 235 as well as lower abundant ions at *m*/*z* 263 (b_2), *m*/*z* 223, *m*/*z* 175 and *m*/*z* 120. While the ion at *m*/*z* 120 could be attributed to Phe the other ions are inconclusive. Two peptide sequences may be assumed for the unknown products, a branched peptide with Phe attached to the α-carboxyl group of Asp and Gly attached to the β-carboxyl group, i.e. Asp(GlyOH)-PheOH, or a linear peptide with the sequence Asp-Phe-GlyOH (Fig. 4C). The branched peptide can be explained by hydrolysis of DKP-GlyOH while there is currently no explanation for a pathway leading to the linear peptide. Further studies are currently underway in order to unequivocally identify the unknown degradation products.

3.2. Peptide degradation

The CE assay was investigated in the range of 0.015–3.0 mmol/l with respect to range, linearity, limit of quantitation (LOQ), limit of detection (LOD) and precision. The assay was linear in the investi-

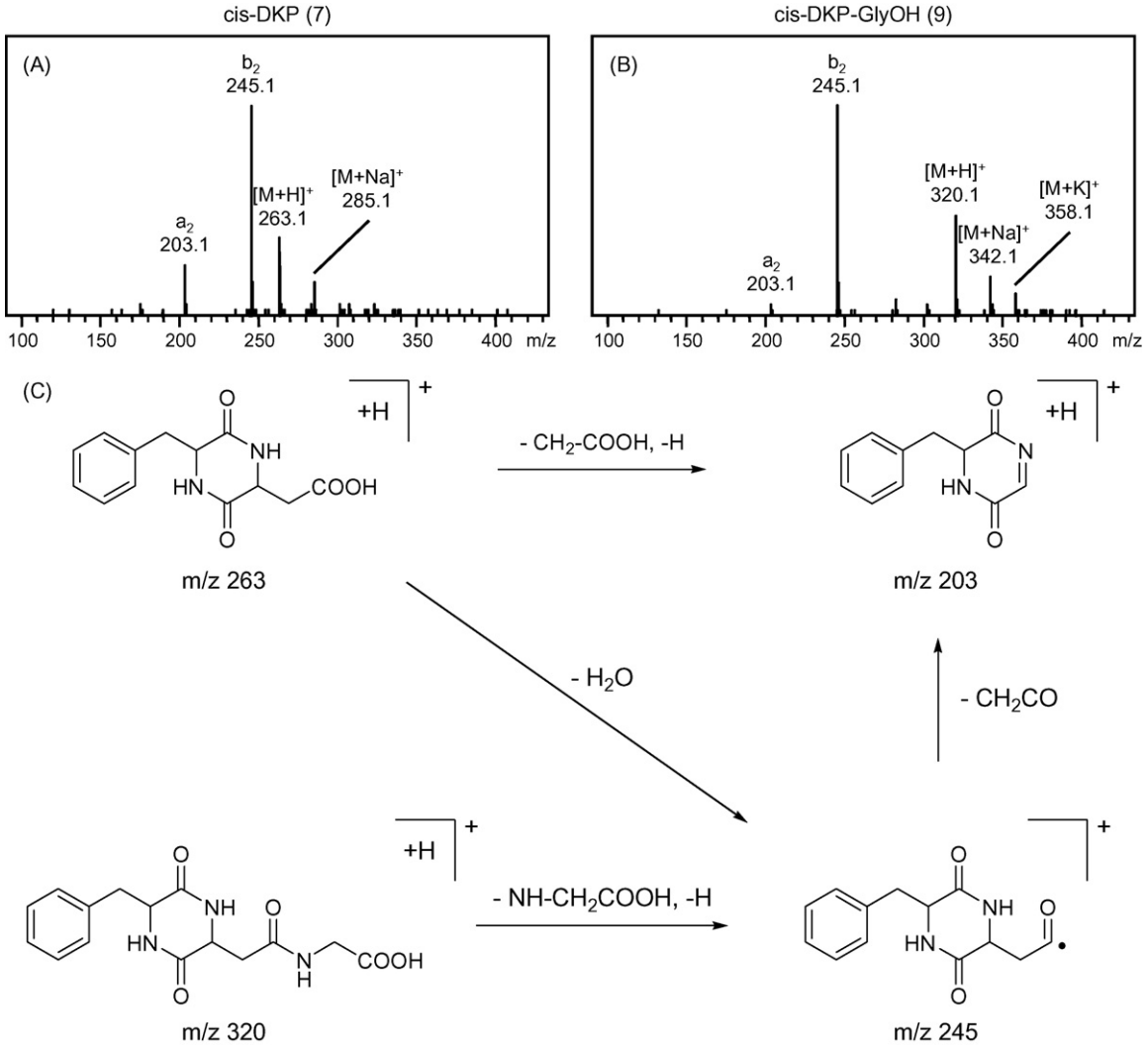

Fig. 3. Mass spectra of diketopiperazine derivatives (A and B) and proposed fragmentation pattern (C).

gated range with correlation coefficients r^2 of at least 0.9979. The lower limit of the calibrated concentration range of 0.015 mmol/l was also the LOQ, the LOD was 0.005 mmol/l for all analytes. Precision was estimated at concentrations of 0.06 and 0.75 mmol/l by six repetitive injections on the same day (intraday precision) and three repetitive injections on 3 consecutive days (interday precision). The relative standard deviation (RSD) was better than 5% in all cases (Table 2).

Representative electropherograms of samples of incubations of L-Phe-α-L-Asp-GlyOH at 80 °C and pH 2.0 and 7.4, respectively, are shown in Fig. 1A and B. At pH 2.0 and 80 °C, degradation is fast so that the starting peptide could not be detected after 48 h. Only few degradation products were found which included the cyclic succinimide L-Phe-L-Asu-GlyOH, L-Phe-L-AspOH and PheOH resulting from backbone hydrolysis as well as *cis*-DKP. This is in accordance with incubations of the related peptide L-Phe-Asp-GlyNH_2 where succinimide formation and hydrolysis reactions dominated at pH 2.0 [7,25].

At pH 7.4, a more complex picture is obtained. The starting peptide could still be detected after 168 h. Besides the hydrolysis

Table 2
Precision data of the capillary electrophoresis assay. The data represent the RSD values of six repetitive injections on 1 day (intraday) and three repetitive injections on 3 consecutive days (interday).

Compound	Intraday precision (R.S.D.) (%)		Interday precision (R.S.D.) (%)	
	0.06 mmol/l	0.75 mmol/l	0.06 mmol/l	0.75 mmol/l
L-Phe-α-L-Asp-GlyOH	3.7	3.9	2.0	2.5
L-Phe-β-L-Asp-GlyOH	1.2	1.1	3.0	1.3
L-Phe-α-D-Asp-GlyOH	1.8	2.4	2.2	1.7
L-Phe-β-D-Asp-GlyOH	3.0	2.3	2.6	2.1
L-Phe-α-L-Asu-GlyOH	2.4	1.8	1.6	3.2
L-Phe-L-AspOH	1.1	2.5	2.1	4.0
cis-DKP	3.5	1.3	3.9	4.6
PheOH	2.3	1.7	2.2	2.5

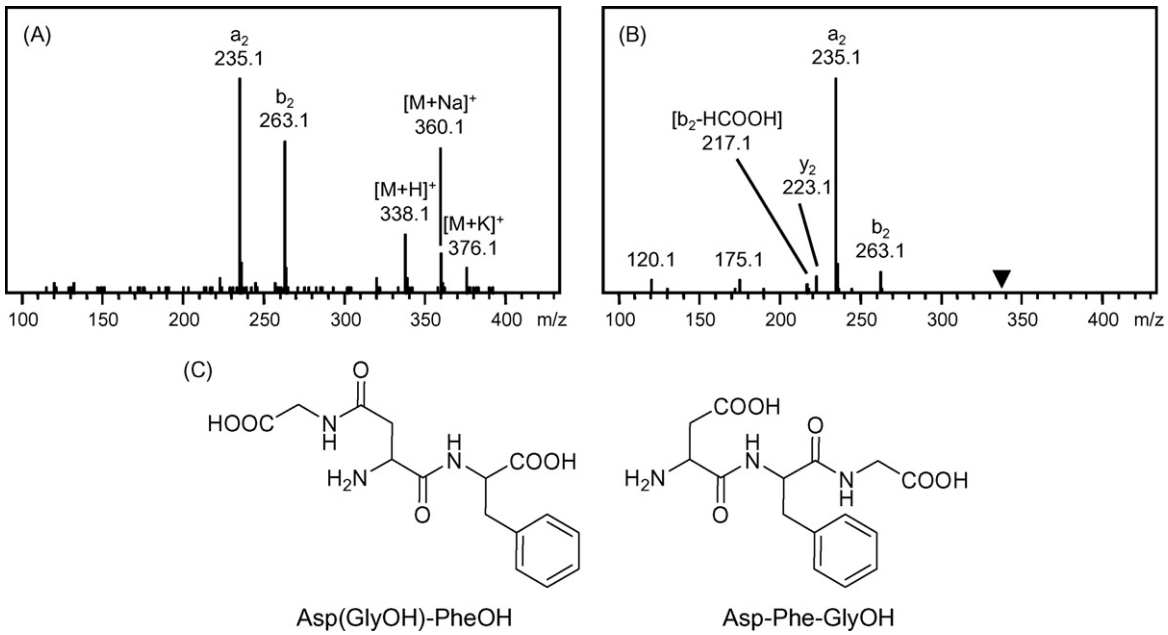

Fig. 4. Mass spectrum (A) and MS/MS spectrum (B) of unknown compound X_1 as well as proposed structures (C). For MS conditions see Section 2.

products Phe-AspOH and PheOH, isomerization and enantiomerization products L-Phe-β-L-Asp-GlyOH, L-Phe-α-D-Asp-GlyOH and L-Phe-β-D-Asp-GlyOH could be detected. The succinimide which is considered to be the intermediate in Asp isomerization and enantiomerization was not found presumably due to its fast hydrolysis to the α-Asp and β-Asp peptides. The diketopiperazines dominated the degradation of the peptide at pH 7.4, especially *cis*-DKP-GlyOH and *trans*-DKP-GlyOH. Formation of the diketopiperazines as major degradation products of L-Phe-α-L-Asp-GlyOH has been reported by DeHart and Anderson in the alkaline pH range at 25 °C [26]. The DKP-GlyOH isomers are assumed to arise from the succinimide intermediates by intramolecular attack by the terminal amino group.

The time courses of the degradation of L-Phe-α-L-Asp-GlyOH and the appearance of the degradation products at pH 2.0 and pH 7.4 at 80 °C are summarized in Fig. 5. The data are also presented with a log scale (Fig. 5B and D) because this provides a better picture of the low concentration degradation products especially

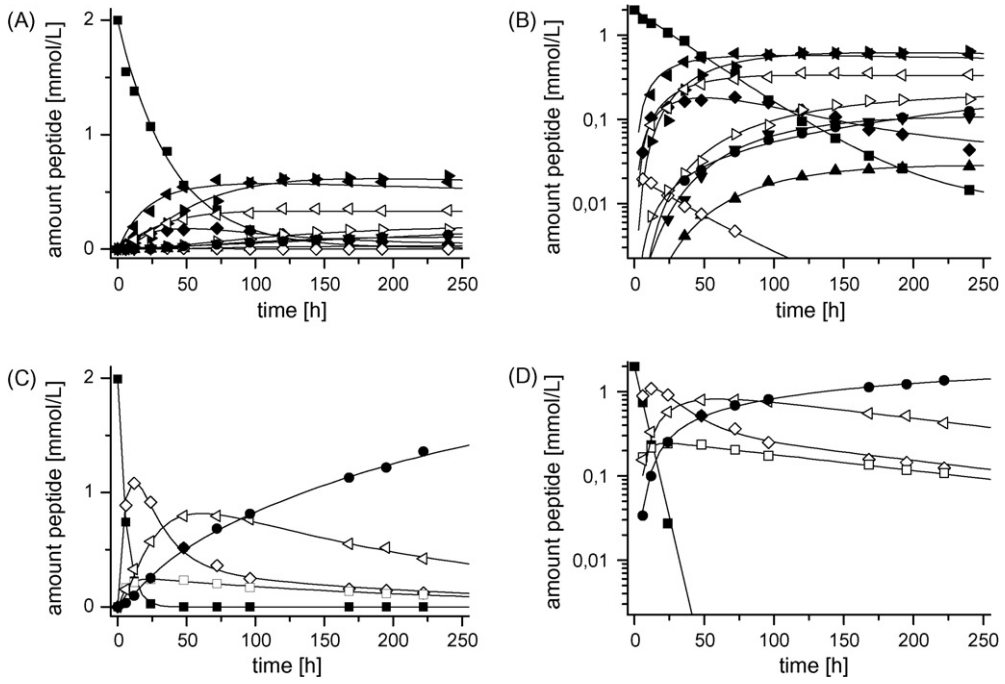

Fig. 5. Time course of the concentration of L-Phe-α-L-Asp-GlyOH and degradation products at pH 7.4, 80 °C (A and B) and pH 2.0, 80 °C (C and D). (B) and (D) present the data with a log scale for better visibility of low concentration products. (■) L-Phe-α-L-Asp-GlyOH; (▲) L-Phe-α-D-Asp-GlyOH; (♦) L-Phe-β-L-Asp-GlyOH; (▼) L-Phe-β-D-Asp-GlyOH; (◄) *cis*-DKP-GlyOH; (►) *trans*-DKP-GlyOH; (●) PheOH; (□) L-Phe-L-Asu-GlyOH; (◊) L-Phe-L-AspOH; (◁) *cis*-DKP; (▷) *trans*-DKP. The data are the mean of three experiments, the S.D. values are smaller than the symbols.

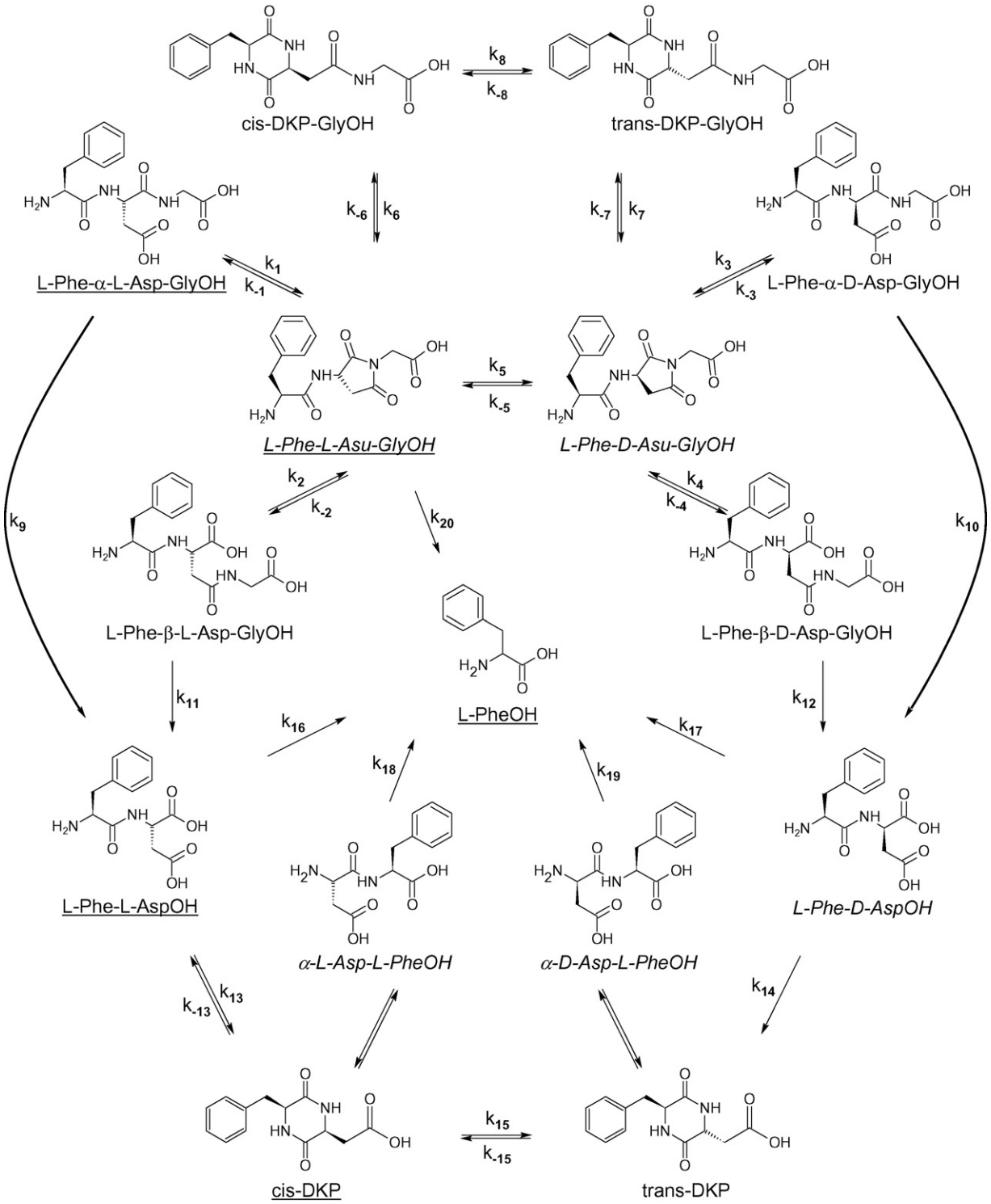

Fig. 6. Cumulative degradation scheme of L-Phe-α-L-Asp-GlyOH. Underlined compounds were detected in incubations at pH 2.0, compounds listed in "normal" print were found in incubations at pH 7.4. Italic compounds were not detected but can be concluded from publications on the degradation of Asp peptides [4,5,7,26,31].

in incubations at pH 7.4. Except for *cis*-DKP no reference compounds were available for calibration of the diketopiperazines. As the stereochemistry should not significantly affect the UV adsorption at 215 nm, *trans*-DKP was quantified using the calibration data of *cis*-DKP. The DKP-GlyOH isomers contain an additional amide bond compared to *cis*-DKP. Comparing the slope of the calibration curves of the linear di- and tripeptides which differ also by one amide group it was noted that the average slope of the tripeptides exceeded the slope of the calibration curve of the dipeptide by about 0.44. Thus, a slope of 3.78 was assumed for the DKP-GlyOH isomers and used for the quantitation of these products. The unknown products X_1 and X_2 were not included in the analysis.

Based on the degradation products observed in the current incubations as well as literature data including related Asp tripeptides [7,25,26] and hexapeptides [4,5,31] the overall degradation scheme summarized in Fig. 6 was constructed. Only the products containing the Phe moiety are considered. Not all compounds could be

detected in the present incubations. For example, only the underlined compounds were detected in incubations at pH 2.0, while the analytes labeled in "normal" print were found in incubations at pH 7.4. Peptides labeled in italic could not be found in incubations but their presumable occurrence was deduced from the literature [4,7,26,31] or the fact that the respective L-Asp containing peptides could be observed. It can be speculated that these compounds appear only at very low concentrations below the LOD of the present analytical method. One example would be Asp-PheOH which could only be seen in CE-MS when analyzing the mass trace of the compound but was below the LOD in CE with UV detection.

The experimental data shown in Fig. 5 were fitted to the corresponding model shown in Fig. 6 in which all compounds are treated as kinetic compartments. Reaction steps, whose kinetic constants were 0 after the fitting at either pH value, are not shown in the scheme, for example, the hydrolysis of glycine from the DKP-GlyOH derivates to yield an isomeric DKP.

The model assumes a central role of the succinimide intermediate in the isomerization and enantiomerization process as well as the cyclization to DKP-GlyOH. Geiger and Clarke concluded from the degradation of an Asp hexapeptide that most of the enantiomerization of Asp occurred by epimerization at the succinimide level but that other mechanisms could be involved as well [4]. Li et al. provided evidence that enantiomerization in the case of Asn peptides can also occur in the tetrahedral intermediate preceding the succinimide [9]. A tetrahedral intermediate can also be formed in the case of Asp peptides so that enantiomerization at this stage could be postulated as well. However, the current model does not distinguish between the individual contributions of these two possible mechanisms so that only an "overall" enantiomerization can be derived independent of the stage (tetrahedral intermediate of succinimide) where enantiomerization occurs. Furthermore, the Asu peptides are the precursors of the DKP-GlyOH isomers [26]. In order to fit the data several assumptions had to be made to simplify the model so that the rate constants of the individual reactions could be estimated even in the case when analytes were below the LOD and could therefore not be detected. Thus, the rate constants for the formation of the Asu peptide from the L-Asp peptides and D-Asp peptides, respectively, were assumed to be identical in accordance with Geiger and Clarke [4], e.g. $k_1 = k_2$ and $k_3 = k_4$. The same applied to the enantiomerization constants of the Asu peptides, i.e. $k_5 = k_{-5}$. Moreover, the rate constants for the hydrolysis of the pairs of linear peptides differing only in the configuration of Asp were set equal, i.e. $k_9 = k_{10}$, $k_{11} = k_{12}$, $k_{16} = k_{17}$ and $k_{18} = k_{19}$. Constants k_{18} and k_{19} represent the "overall" hydrolysis of the DKPs resulting in PheOH via the Asp-PheOH peptides. These dipeptides were detected in minute amounts by CE-MS but were always below the LOD of the assay using UV detection. Thus, it can be assumed that the degradation of α-Asp-PheOH is much faster than the hydrolysis of DKP so that the ring opening reaction is the rate limiting step. Using these conditions, the rate constants summarized in Table 3 were obtained by fitting of the experimental data of the concentration versus time plots shown in Fig. 5. The program also allowed to determine rate constants of reaction pathways where intermediates could not be detected. However, the fitting bears the uncertainty that the DKP-GlyOH derivatives could not be exactly quantified due to the lack of reference compounds. Furthermore, the unknown impurities X_1 and X_2 were not included.

Heating L-Phe-α-L-Asp-GlyOH in aqueous solution at 80 °C in phosphate buffer, pH 7.4, yielded the isomeric β-Asp peptides as well as the L-Phe-D-Asp-GlyOH epimers (Fig. 5A and B). The cyclic DKP derivatives were the major degradation products. According to the kinetic analysis, the succinimide L-Phe-L-Asu-GlyOH which could not be detected is rapidly converted to *cis*-DKP-GlyOH. The rate constant exceeded the constants representing the competing hydrolysis reactions, i.e. k_{-1} and k_{-2}, or the epimerization reaction, i.e. k_5 and k_{-5}, by a factor of 3–10. Equilibrium was not reached after the maximal incubation time of 240 h because the DKP-GlyOH epimers function as a "deep compartment" due to their slow hydrolysis. The formation of the DKPs exceeded their hydrolysis by a factor of about 100–350. The sum of DKP derivatives amounted to about 85% after 240 h. DeHart and Anderson also observed much higher concentrations of DKP-GlyOH compared to linear α-Asp and β-Asp peptides in incubations of L-Phe-α-L-Asp-GlyOH at pH 7.5 and 25 °C [26]. DKPs were not observed in incubations of L-Phe-α-L-Asp-$GlyNH_2$ at pH 10.0 and 80 °C [7]. It may be speculated that the rate of hydrolysis of the succinimide intermediates to yield the linear peptides L-Phe-α-L/D-Asp-GlyOH and L-Phe-β-L/D-Asp-GlyOH exceeds the rate of cyclization to *cis*/*trans*-DKP-GlyOH at pH 10 compared to pH 7.4. In fact, DeHart and Anderson showed for Phe-Asp-GlyOH that at a temperature of 25 °C the formation of DKP-GlyOH was the major degradation product at pH 7.5 while the concentration of the linear Asp peptides exceeded the concentration of the DKPs at pH 10.0 [26].

Table 3
Pseudo-first-order rate constants of the degradation reactions of L-Phe-α-L-Asp-GlyOH at 80 °C according to the scheme shown in Fig. 6. The data are the mean of three experiments ± S.D.

	Rate constant ($\times 10^3$ h^{-1})	
	pH 7.4	pH 2
k_1 and k_2	21.1 ± 0.7	22.1 ± 2.6
k_3 and k_4	15.2 ± 1.0	–
k_5 and k_{-5}	174.7 ± 28.2	–
k_6	5.13 ± 1.38	–
k_7	12.7 ± 1.0	–
k_8	26.8 ± 3.1	–
k_9 and k_{10}	3.94 ± 0.65	142 ± 6
k_{11} and k_{12}	5.60 ± 0.25	–
k_{13}	525 ± 35	29.9 ± 2.1
k_{14}	524 ± 108	–
k_{15}	5.52 ± 0.71	–
k_{16} and k_{17}	30.8 ± 4.6	10.3 ± 1.2
k_{18} and k_{19}	0.98 ± 0.08	2.65 ± 0.68
k_{20}	–	4.36 ± 1.05
k_{-1}	167 ± 10	–
k_{-2}	688 ± 54	–
k_{-3}	108 ± 8	–
k_{-4}	381 ± 29	–
k_{-6}	1891 ± 164	–
k_{-7}	1170 ± 137	–
k_{-8}	20.5 ± 3.2	–
k_{-13}	–	11.3 ± 1.9
k_{-15}	10.3 ± 2.1	–

Generally, only low concentrations of products resulting from backbone hydrolysis such as L-Phe-L-AspOH and PheOH were observed over the period of time investigated. After the incubation period of 240 h about 31% of the compounds comprised compounds that arose from hydrolysis, i.e. Phe-AspOH, PheOH as well as *cis*- and *trans*-DKP. According to the kinetic analysis backbone hydrolysis occurred only in linear peptides. It can be estimated from the rate constants that it will take about 1 year to hydrolyze 99% of the starting peptide to PheOH under the incubation conditions applied.

After incubation for 240 h the ratio of linear α-Asp/β-Asp tripeptides in about 1:4.2. This is consistent with the ratio of 1:4 reported for aspartyl hexapeptides containing the Asp-Gly sequence [4], a collagen fragment [32] or 1:3.6–3.8 found for α-A-crystallin fragments [33]. The D/L ratio of the linear tripeptides was 1:1.2. Cloos and Fidelius found a L-Asp/D-Asp ratio of 1:1.3 for a collagen-derived hexapeptide after 390 days at 37 °C and pH 7.4 [32]. The overall L/D ratio of Asp including the diketopiperazines was 1:0.97. This is caused by the about 5–30 times slower enantiomerization

rate of the diketopiperazines (k_8, k_{-8} and k_{15}, k_{-15}) compared to the enantiomerization rate of the succinimide (k_5, k_{-5}).

At pH 2.0 (Fig. 5C and D), the predominant reaction is the hydrolysis of the peptide backbone resulting in L-Phe-L-AspOH. Thus, based on the rate constants about 87% of the starting peptide degraded via hydrolytic cleavage while the remaining 13% were converted via the succinimide. Interestingly, formation of the succinimide occurred with a rate constant comparable to the constant observed at pH 7.4. The Asu peptide did not isomerize, epimerize or form *cis*-DKP-GlyOH at pH 2.0 but was slowly hydrolyzed to yield PheOH. L-Phe-L-AspOH was either hydrolyzed to yield PheOH or, preferably, cyclized to give *cis*-DKP. Depending on the site of the hydrolytic cleavage of the cyclic amide bonds of *cis*-DKP either L-Phe-L-Asp was formed or the apparently unstable intermediate L-Asp-L-PheOH which was rapidly converted to PheOH. It can be estimated by extrapolating the kinetic data that about 99% of the starting peptide is hydrolyzed to PheOH after about 1000 h.

4. Conclusions

The degradation of the tripeptide L-Phe-α-L-Asp-GlyOH at pH 2.0 and 7.4 at elevated temperatures was investigated by CE. CE-MS was utilized to identify degradation products including four diketopiperazine derivatives. Two further products were detected containing Asp, Phe and Gly but the structure could not be assigned from MS and MS/MS spectra. The compound could be a branched peptide arising from the hydrolysis of DKP-GlyOH but further studies will be required in order to unequivocally identify the compounds.

In accordance with literature data of Asp tripeptides [7] and Asp hexapeptides [5,31] hydrolysis of the peptide backbone is the dominating degradation pathway under strongly acidic conditions. At physiological pH, isomerization to β-Asp peptides as well as concomitant enantiomerization to D-Asp containing peptides was observed but the dominating degradation products were diketopiperazine derivatives. Formation of the DKP-GlyOH epimers was assumed by intramolecular attack of the amino group of Phe-Asu-GlyOH on a succinimide carbonyl. The fact that this cyclization displayed very fast reaction rates compared to the hydrolysis reaction yielding α-Asp or β-Asp peptides explained the dominant formation of the DKP derivatives. Moreover, hydrolysis of the diketopiperazines proved to be relatively slow at pH 7.4.

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