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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 49–56
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Capillary electrophoresis analysis of hydrolysis, isomerization and enantiomerization of aspartyl model tripeptides in acidic and alkaline solution

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Received 9 April 2006; received in revised form 30 May 2006; accepted 4 June 2006
Available online 18 July 2006

Abstract

In order to investigate the degradation of two aspartyl tripeptides, Gly-Asp-PheNH$_2$ and Phe-Asp-GlyNH$_2$ in solution capillary, electrophoresis methods were developed and validated. Separation of most degradation products including those arising from isomerization and enantiomerization of the Asp residues was achieved in a 50 mM sodium phosphate buffer, pH 3.0. Resolution of comigrating compounds could be achieved by addition of cyclodextrins to the background electrolyte. For tripeptide derivatives the assays were linear in the range of 0.015–3.0 mmol/l. Some dipeptides and amino acids exhibited a narrower linear range due to low UV absorbance. The limits of detection were in the range of 0.005–0.1 mmol/l. Incubation of the model peptides was carried out at pH 2 and 10. At pH 2, degradation of the peptides proceeded via C-terminal deamidation and peptide backbone hydrolysis. In contrast, isomerization and enantiomerization were observed in combination with deamidation at pH 10. Generally, degradation of Phe-Asp-GlyNH$_2$ proceeded faster compared to Gly-Asp-PheNH$_2$ due to steric hindrance by the phenyl side chain.

Keywords: Aspartyl peptide; Peptide degradation; Isomerization; Enantiomerization; Capillary electrophoresis

1. Introduction

Peptides and proteins are increasingly used as pharmaceuticals due to the high activity, specificity and relatively low systemic toxicity. The advancement of recombinant DNA technology dramatically increased the number of peptide and protein drugs. During the years 2000–2003, a total of 64 protein-based biopharmaceuticals was approved for human use in North America and Europe [1]. This represents over a quarter of all new drug approvals in this period of time. Hormones and cytokines represent the largest product categories; additional classes are recombinant blood coagulation factors, subunit vaccines and monoclonal antibodies [2].

Peptides and proteins are susceptible to chemical and physical degradation, such as deamidation, oxidation, proteolysis, racemization, aggregation and surface absorption [3]. 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 [3,4]. 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 (Fig. 1). 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 [5,6]. Hydrolysis of the succinimide produces either the native aspartyl residue (α-Asp) or the iso-aspartyl residue (β-Asp). Degradation via succinimide formation was found, for example, in synthetic peptide drugs such as klerval [7] and pramlintide [8]. Moreover, 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, ocular lens which may increase under pathological conditions [9].

While the parameters that influence the formation of the aspartyl succinimide have been the subject of many papers [3,4,10–13], only few studies were published on the enantiomerization of Asp residues in peptides. D-Asp containing peptides

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doi:10.1016/j.jpba.2006.06.012

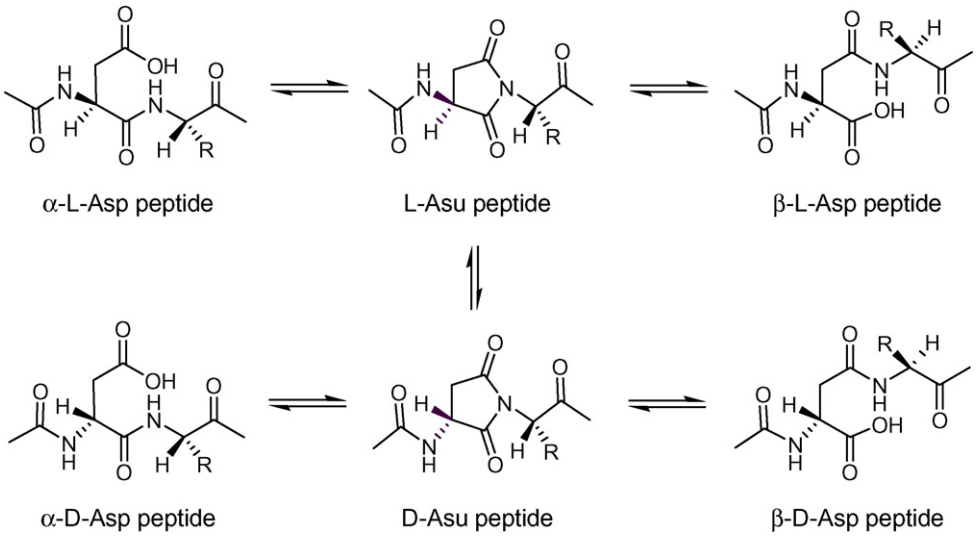

Fig. 1. Scheme of the isomerization and enantiomerization of Asp residues in peptides.

were detected after incubation of α-A-crystallin [14,15]. Oliyai and Borchardt [11] observed D-Asp in model hexapeptides, and Geiger and Clarke [6] demonstrated that about 28% of the degradation products of an adrenocorticotropic hormone-derived aspartyl hexapeptide possessed D-configured Asp after incubation at pH 7.4 at 37 °C.

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 [16]. 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 confirmed by the resistance of β-Asp bonds to Edman degradation [17]. We recently reported 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 [18,19]. As these methods appeared to be especially suitable for the analysis of Asp isomerization and enantiomerization in the tripeptides the present study was conducted in order to investigate the time course of the degradation specifically with respect to the enantiomerization of aspartic acid. For recent reviews on the analysis of peptides by CE including stereoisomer analysis, see references [20,21]. CE has also been recently applied to study the degradation of pseudopeptidic cross linkers in aqueous solutions [22].

2. Experimental

2.1. Chemicals

Commercially available amino acids, dipeptides and protected amino acids were purchased from Bachem AG (Heidelberg, Germany), 4-(aminomethyl)benzoic acid (pAMBA) was from Fisher Scientific (Schwerte, Germany). All other chemicals were of analytical grade. Buffers and solutions were prepared in double distilled, deionized water, filtered (0.45 μm) and degassed. Peptides with amidated carboxyl end groups were synthesized in solution using *N*-*tert*-butyloxycarbonyl- or *N*-benzyloxycarbonyl-protected amino acid derivatives and *N*-(3-dimethyl-aminopropyl)-*N*-carbodiimide as coupling reagent according to reference [23]. Deprotection was performed by hydrogenolysis or by treatment with 6 M hydrochloric acid in dioxane. Peptides with free carboxyl group 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 [24]. Succinimidyl peptides were synthesized by incubation of the corresponding aspartyl peptides in 1.8 M HCl in glacial acetic acid according to reference [25]. Cyclo(Phe-Asp) was prepared by incubation of aspartame in 0.1N sodium hydroxide according to reference [26]. The crude peptides were purified by preparative RP-HPLC and their identity was confirmed by mass spectrometry.

2.2. Capillary electrophoresis

Analyses were performed on a P/ACE™ MDQ Capillary Electrophoresis System (Beckman Coulter, Unterschleißheim, Germany) equipped with a diode array detector set to 215 nm. Data analysis was carried out with 32 Karat™ Software Version 4.0. UV detection was carried out at the cathodic end of the capillary. Untreated fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany) with 50 μm i.d. and 375 μm o.d. had an effective length of 40 cm and a total length of 50.2 cm. Prior to their first use, the capillaries were rinsed for 15 min with 0.1 M sodium hydroxide, 10 min with water, 10 min with 0.1 M phosphoric acid and again 10 min with water. Between analyses the capillary was rinsed for 1 min with 0.1 M sodium hydroxide, 1 min with water and 3 min with the background electrolyte. Sample solutions were injected at the anodic end by hydrodynamic injections at a pressure of 3.45 kPa for 5 s. If not stated otherwise, separations were performed in 50 mM sodium phosphate

buffer, pH 3.0, at an applied voltage of 25 kV and a temperature of 20 °C (CE system 1). Phosphate buffers were prepared by titrating 50 mM phosphoric acid with 0.1 M sodium hydroxide to the respective pH value. Peaks that could not be resolved in this system were analyzed as follows: separations of L-Phe-β-L-Asp-$GlyNH_2$, L-Phe-α-L-Asp-GlyOH and L-Phe-α-D-Asp-GlyOH were achieved in 50 mM phosphate buffer, pH 3.0, containing 16 mg/ml carboxymethyl-β-cyclodextrin and 5% acetonitrile (CE system 2). Separation of Gly-α-L-Asp-L-$PheNH_2$ and Gly-α-D-Asp-L-$PheNH_2$ were achieved in 50 mM phosphate buffer, pH 3.0, containing 3 mg/mL sulfated β-cyclodextrin (CE system 3). Analysis of the diketopiperazine derivatives cyclo(Phe-Asp) and cyclo(Gly-Asp) was performed in 50 mM formic acid, pH 3.0 (pH adjusted with ammonia), containing 10% acetonitrile at a voltage of 30 kV (CE system 4). Separations of the degradation products arising after incubation of Phe-Asu-GlyOH were performed in 50 mM phosphate buffer, pH 2.9, at an applied voltage of 25 kV and a temperature of 15 °C.

2.3. Incubations

The tripeptides Phe-Asp-$GlyNH_2$ and Gly-Asp-$PheNH_2$ were incubated at 80 °C in 50 mM phosphate buffer, pH 2.0, and in 50 mM borate buffer, pH 10.0, in 5 ml glass screw cap vials with Teflon liners. The ionic strength of the buffers was adjusted to 0.2 M with sodium chloride. For every peptide, two different solutions with an initial concentration of 2.0 mg/ml were incubated. The internal standard, pAMBA, had a concentration of 90 μg/ml in the incubation solutions. At selected time intervals, 100 μl aliquots were sampled 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 0.1 M phosphoric acid in case of the incubations at pH 10.

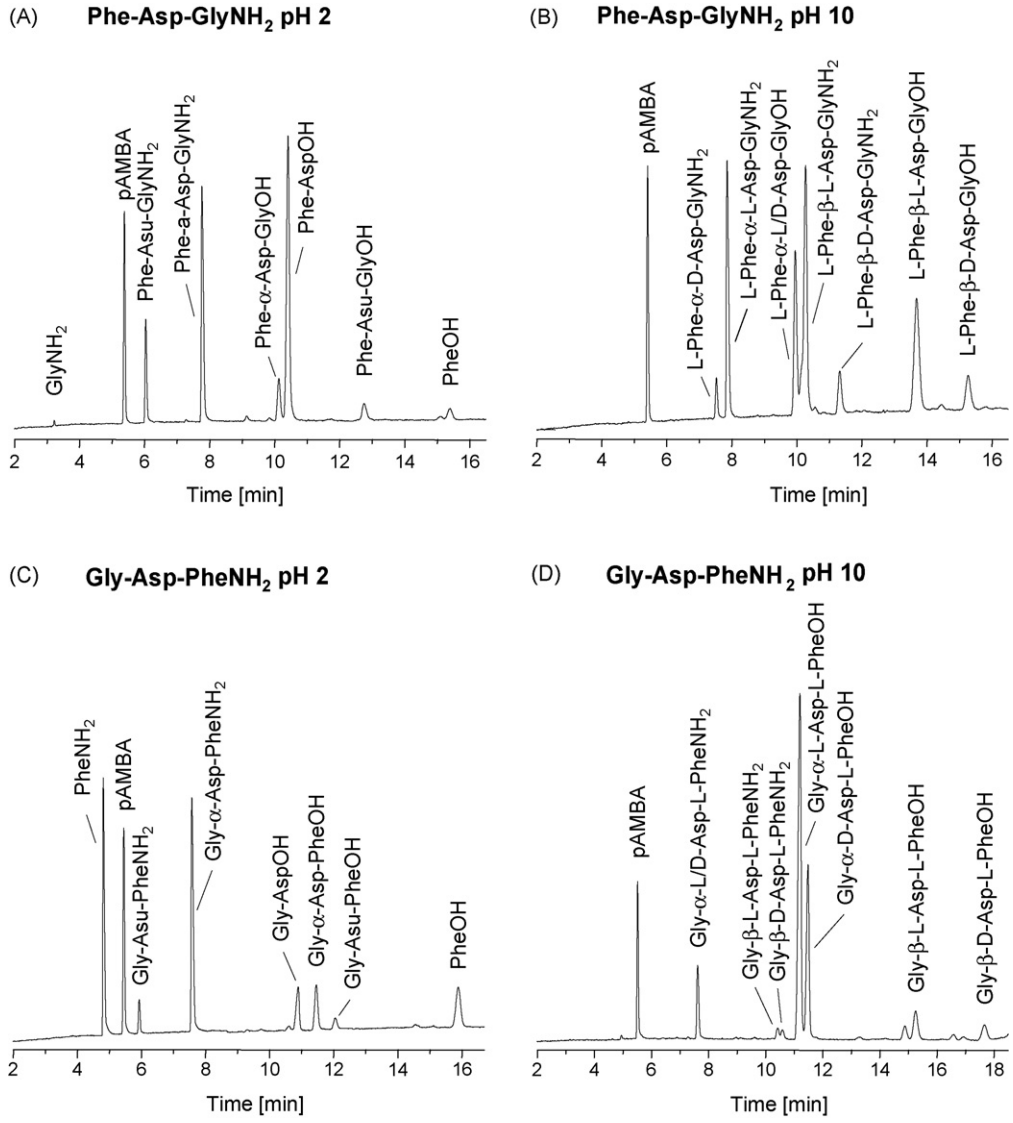

Fig. 2. Representative electropherograms of the CE analysis of incubations of Phe-Asp-$GlyNH_2$ and Gly-Asp$PheNH_2$. (A) Phe-Asp-$GlyNH_2$, pH 2, 12 h; (B) Phe-Asp-$GlyNH_2$, pH 10, 24 h; (C) Gly-Asp$PheNH_2$, pH 2, 36 h; (D) Gly-Asp$PheNH_2$, pH 10, 96 h. *Experimental conditions:* 40/50.2 cm fused-silica capillary, 50 μm i.d., 50 mM sodium phosphate buffer, pH 3.0, 25 kV. For other details see Section 2.

The samples were vortexed and stored at −20 °C until analyzed. Each sample was injected three times.

3. Results and discussion

3.1. Method development and validation

Based on earlier LC-MS and CE-MS analyses of the Asp model tripeptides Gly-Asp-PheNH$_2$ and Phe-Asp-GlyNH$_2$ [18,19] CE was chosen due to the higher peak resolution and lower consumption of chemicals. pAMBA was selected as internal standard based on the migration properties and the fact that the compound was stable under the incubation conditions. The separations were performed at pH 3.0 which allowed the separation of most degradation products studied. Fig. 2 shows representative electropherograms of incubations of the peptides at pH 2 and 10. The identification of the compounds has been performed previously by CE-MS [18]. Only two diastereomeric pairs of compounds, L-Phe-α-L-Asp-GlyOH/L-Phe-α-D-Asp-GlyOH and Gly-α-L-Asp-L-PheNH$_2$/Gly-α-D-Asp-L-PheNH$_2$ could not be separated under these conditions. As pH variation did not lead to significantly improved resolution of these compounds, several cyclodextrins (CDs) were tested. Subsequently, Gly-α-L-Asp-L-PheNH$_2$ and Gly-α-D-Asp-L-PheNH$_2$ were resolved adding 3 mg/ml sulfated β-CD to the run buffer (Fig. 3A) while L-Phe-α-L-Asp-GlyOH and L-Phe-α-D-Asp-GlyOH could be separated in addition to L-Phe-β-L-Asp-GlyNH$_2$ in the presence on 16 mg/ml carboxymethyl-β-CD as buffer additive (Fig. 3B). The diketopiperazines cyclo(Phe-Asp) and cyclo(Gly-Asp) are negatively charged and migrate after the EOF. In order to achieve reasonable analysis times a run buffer consisting of 50 mM formic acid adjusted to pH 3.0 by ammonia and 10% acetonitrile was applied which generates a reasonably high EOF to detect the compounds within 30 min (data not shown).

The methods were subsequently validated in the range of 0.015–3.0 mmol/l with respect to linearity, limit of quantitation (LOQ), limit of detection (LOD) and precision. The data are summarized in Table 1. The lower limit of the calibrated concentration range of 0.015 mmol/l was also the LOQ, except for Gly-Asp and cyclo(Phe-Asp) with an LOQ of 0.06 mmol/l and cyclo(Gly-Asp) with an LOQ of 0.15 mmol/l. Due to the low UV absorbance the LOQ for GlyNH2 was 0.3 mmol/l. The amino acids Gly and Asp as potential hydrolysis products could not be detected at 215 nm due the lack of an UV chromophore and were, therefore, not calibrated. The LODs ranged from 0.005 to 0.1 mmol/l. Precision was estimated by injecting concentrations of 0.06 and 0.75 mmol/l six times on the same day (intraday precision) and three consecutive days (interday precision). Except for the diketopiperazines, the relative standard deviation (R.S.D.) was better than 10% in all cases (Table 1). The larger R.S.Ds. for the diketopiperazines may be due to the fact that the compounds migrated as negatively charged substances after the EOF resulting in relatively broad peaks which causes a larger integration error.

3.2. Tripeptide degradation at pH 2

The tripeptides Phe-Asp-GlyNH$_2$ and Gly-Asp-PheNH$_2$ were incubated in duplicate in 50 mM sodium phosphate buffer, pH 2, with an ionic strength of 0.2 M at 80 °C for 10 days. All incubations contained the internal standard pAMBA to compensate for solvent evaporation during the incubation as well as for migration time fluctuations and injection errors during CE analysis. Representative electropherograms of samples after incubation of Phe-Asp-Gly-NH$_2$ for 12 h and Gly-Asp-PheNH$_2$ for 36 h are shown in Fig. 2A and C, respectively. The peaks were identified by co-injection of reference compounds and/or based on the peak assignment from previous CE-MS studies [18]. The time courses of the incubations are summarized in Fig. 4.

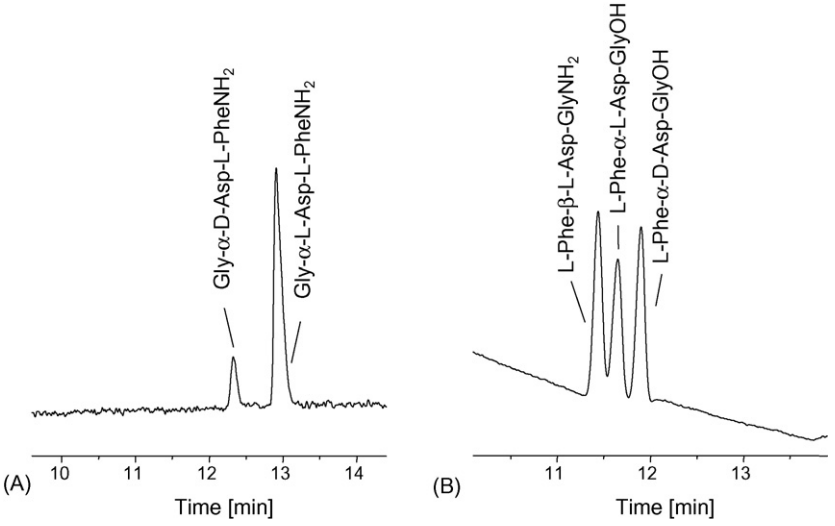

Fig. 3. Representative electropherograms of the CE separation of (A) Gly-α-L-Asp-L-PheNH$_2$ and Gly-α-D-Asp-L-PheNH$_2$ and (B) of L-Phe-β-L-Asp-Gly-NH$_2$, L-Phe-α-L-Asp-GlyOH and L-Phe-α-D-Asp-GlyOH. *Experimental conditions:* 40/50.2 cm fused-silica capillary, 50 μm i.d., 50 mM sodium phosphate buffer, pH 3.0, containing (A) 3 mg/ml sulfated β-CD and (B) 16 mg/ml carboxymethyl-β-CD and 5% acetonitrile. For other details see Section 2.

Table 1
Validation data for Phe-Asp-GlyNH$_2$ and Gly-Asp-PheNH$_2$ and the corresponding degradation products

Compound	System[a]	Range (mmol/l)	Slope	R^2	LOD (mmol/l)	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-GlyNH$_2$	1	0.015–3.0	1.808	1.0000	0.005	1.89	0.99	3.35	0.18
L-Phe-α-D-Asp-GlyNH$_2$	1	0.015–3.0	1.700	0.9999	0.005	4.47	0.96	2.70	1.28
L-Phe-β-L-Asp-GlyNH$_2$	3	0.015–3.0	2.080	0.9949	0.005	6.34	2.46	5.94	3.06
L-Phe-β-D-Asp-GlyNH$_2$	1	0.015–3.0	1.475	0.9996	0.005	9.10	2.00	3.65	0.96
L-Phe-α-L-Asp-GlyOH	3	0.015–3.0	1.548	0.9981	0.005	0.86	2.45	5.73	6.76
L-Phe-α-D-Asp-GlyOH	3	0.015–3.0	1.558	0.9998	0.005	8.87	6.88	6.32	1.96
L-Phe-β-L-Asp-GlyOH	1	0.015–3.0	2.131	0.9999	0.005	1.03	2.05	2.43	0.50
L-Phe-β-D-Asp-GlyOH	1	0.015–3.0	2.129	0.9998	0.005	2.59	1.10	4.22	2.30
Gly-α-L-Asp-L-PheNH$_2$	3	0.015–3.0	2.274	0.9999	0.005	0.48	0.90	2.19	1.47
Gly-α-D-Asp-L-PheNH$_2$	3	0.015–3.0	2.309	1.0000	0.005	3.27	0.11	0.72	0.54
Gly-β-L-Asp-L-PheNH$_2$	1	0.015–3.0	2.299	0.9999	0.005	2.08	0.35	0.28	1.05
Gly-β-D-Asp-L-PheNH$_2$	1	0.015–3.0	2.298	0.9999	0.005	3.15	0.72	0.87	0.37
Gly-α-L-Asp-L-PheOH	1	0.015–3.0	1.885	0.9999	0.005	1.32	0.91	1.09	1.05
Gly-α-D-Asp-L-PheOH	1	0.015–3.0	2.266	0.9998	0.005	2.69	1.55	0.82	0.29
Gly-β-L-Asp-L-PheOH	1	0.015–3.0	2.502	0.9999	0.005	1.14	1.39	1.15	0.92
Gly-β-D-Asp-L-PheOH	1	0.015–3.0	2.607	0.9999	0.005	7.80	2.25	4.60	4.79
Phe-Asu-GlyNH$_2$	1	0.015–3.0	2.329	0.9999	0.005	1.79	0.43	9.59	1.30
Phe-Asu-GlyOH	1	0.015–3.0	2.590	0.9998	0.005	4.75	0.18	1.15	1.31
Gly-Asu-PheNH$_2$	1	0.015–3.0	2.264	0.9999	0.005	2.43	2.88	1.30	1.04
Gly-Asu-PheOH	1	0.015–3.0	2.096	0.9999	0.005	0.76	0.56	0.71	0.34
Phe-AspOH	1	0.015–3.0	2.642	0.9998	0.005	1.52	0.31	3.83	3.86
Gly-AspOH	1	0.06–3.0	0.407	0.9999	0.015	4.72	1.31	5.19	1.37
PheNH$_2$	1	0.015–3.0	1.753	0.9999	0.005	1.39	1.25	1.15	1.53
PheOH	1	0.015–3.0	1.857	0.9999	0.005	3.14	4.36	1.96	0.52
GlyNH$_2$	1	0.3–3.0	0.042	0.9997	0.1	–	4.50	–	3.02
Cyclo(Phe-Asp)	4	0.06–3.0	2.741	0.9986	0.015	2.83	0.31	12.11	3.69
Cyclo(Gly-Asp)	4	0.15–3.0	0.553	0.9992	0.06	–	6.23	–	14.0

[a] CE background electrolytes, 1, 50 mM phosphate buffer, pH 3.0; 2, 50 mM phosphate buffer, pH 3.0, 16 mg/ml carboxymethyl-β-CD, 5% acetonitrile; 3, 50 mM phosphate buffer, pH 3.0, 3 mg/mL sulfated β-CD; 4, in 50 mM formic acid adjusted to pH 3.0 with ammonia,10% acetonitrile.

At pH 2, hydrolysis of the Asp-X peptide bonds constitutes the major degradation pathway. Thus, Phe-Asp-GlyNH$_2$ is primarily hydrolyzed to yield Phe-Asp and GlyNH$_2$ which are further degraded to the individual amino acids Phe, Asp and Gly (Figs. 2A and 4A). Hydrolysis of the C-terminal amide was also observed resulting in Phe-Asp-GlyOH but at a lower rate compared to backbone hydrolysis. Moreover, the aspartyl imide peptides Phe-Asu-PheNH$_2$ and Phe-Asu-GlyOH as well as the slow formation of the diketopiperazine cyclo(Phe-Asp) was observed. Corresponding degradation pathways were found for Gly-Asp-PheNH$_2$ (Figs 2C and 4B) albeit at a lower rate compared to Phe-Asp-GlyNH$_2$. Gly-Asp and PheNH$_2$ were the primary products which subsequently hydrolyzed to yield the respective amino acids. Only low levels of Gly-Asp-PheOH, the Asu peptides Gly-Asu-PheNH$_2$ and Gly-Asu-PheOH as well as of cyclo(Gly-Asp) were observed. Peptide backbone

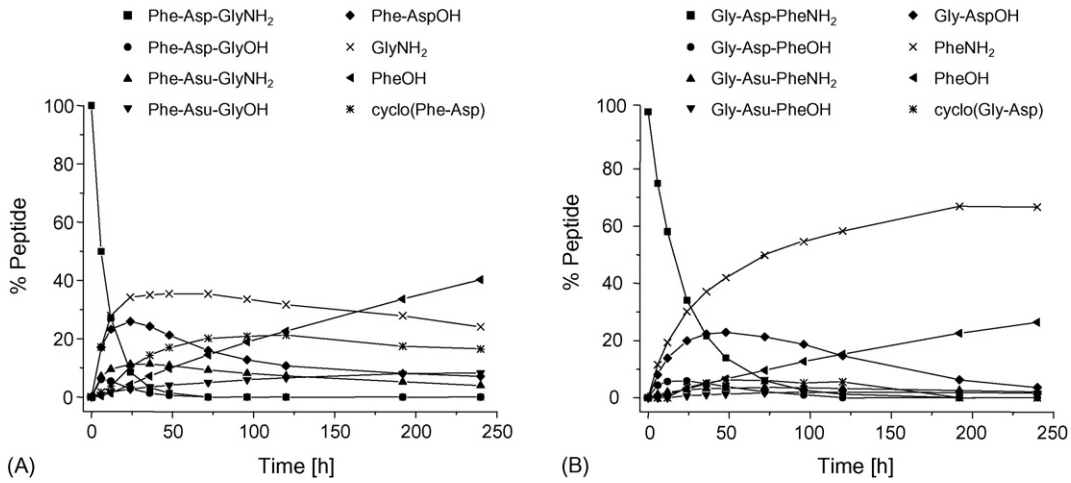

Fig. 4. Time course of incubations of Phe-Asp-GlyNH$_2$ (A) and Gly-Asp-PheNH$_2$ (B) at pH 2 and 80 °C. Each data point represents the mean of two independent incubations.

hydrolysis as the major degradation reaction in acidic solutions was also reported for aspartyl hexapeptides [11] and the pseudo-tetrapeptide klerval [7]. The increased stability of Gly-Asp-PheNH$_2$ compared to Phe-Asp-GlyNH$_2$ can be attributed to the increased steric hindrance of the amino acid side chain of Phe. Decreasing hydrolysis rates of Asp-X peptide bonds with increasing steric hindrance have also been described for hexapeptides [11].

3.3. Tripeptide degradation at pH 10

For an estimation of the degradation of the Asp peptides in alkaline solution, the compounds were incubated in duplicate in 50 mM sodium borate buffer, pH 10, with an ionic strength of 0.2 M at 80 °C for 10 days. All incubations contained the internal standard pAMBA. Representative electropherograms of samples after incubation of Phe-Asp-Gly-NH$_2$ for 24 h and Gly-Asp-PheNH$_2$ for 96 h are shown in Fig. 2B and D, respectively. The peaks were identified by co-injection of reference compounds and/or based on the peak assignment from previous CE-MS studies [18]. The time courses of the degradation products are shown in Fig. 5.

The degradation of the Asp tripeptides at pH 10 involved predominantly hydrolysis of the C-terminal amide as well as the isomerization of Asp to β-Asp. In addition, enantiomerization of Asp was observed. Significant hydrolysis of the peptide backbone could not be detected. Rapid C-terminal deamidation has also been observed for the pseudo-peptide klerval in the alkaline pH range [7]. Under the experimental conditions applied, L-Phe-α-L-Asp-GlyNH$_2$ degraded with a half-life of 14.1 h. The primary major degradation products were L-Phe-α-L-Asp-GlyOH and L-Phe-β-L-Asp-GlyNH$_2$ (Figs. 2B and 5A). During the period of time of 24–36 h where the products reached the highest concentrations, they represented about 36 and 16%, respectively, of the total peptide content in the incubations. Within the amide derivatives, enantiomerization is also observed yielding L-Phe-α-D-Asp-GlyNH$_2$ and L-Phe-β-D-Asp-GlyNH$_2$ although at much lower concentrations. All amides were subsequently deamidated to yield the respective acid derivatives. The initial strong increase of L-Phe-α-L-Asp-GlyOH is followed by degradation due to isomerization and enantiomerization resulting in L-Phe-β-L-Asp-GlyOH, L-Phe-α-D-Asp-GlyOH and L-Phe-β-D-Asp-GlyOH. The percentage of the final deamidated tripeptides after the maximum incubation time of 10 days is summarized in Table 2. Complete equilibrium between the Asp peptides has not been reached even after 10 days. However, the ratio α-Asp peptides to β-Asp peptides was about 1:2.5 which is comparable to the ratio of 1:4 observed for aspartyl hexapeptides containing an Asp-Gly sequence incubated at pH 10 [11] or pH 7.4 [6,27]. A ratio of 1:3.6–3.8 has been reported for α-A-crystallin fragments after incubation at 90 °C for 72 h at pH 7.4 [15]. Modeling studies estimated a ratio of 1:2.2 [28].

About 41.1% of the total peptides contained Asp in the D-configuration resulting in a ratio of L-Asp to D-Asp peptides of about 1.4:1. Cloos and Fledelius [27] reported an L-Asp/D-Asp ratio of 1:1.3 for a collagen-derived hexapeptide after 390 days at 37 °C and pH 7.4. Isomerization and enantiomerization succeeded most likely via the corresponding cyclic succinimides although these compounds could not be detected in the incubation mixtures due to their extremely low stability at pH 10.

Besides a generally slower degradation a different pattern of the resulting products was observed for the tripeptide with the isomeric sequence, Gly-α-L-Asp-L-PheNH$_2$, at pH 10 (Figs. 2D and 5B) compared to L-Phe-α-L-Asp-GlyNH$_2$ due to the steric hindrance of the phenyl side chain. The half-life of Gly-α-L-Asp-L-PheNH$_2$ under the experimental conditions was 26.5 h. Deamidation to Gly-α-L-Asp-L-PheOH was the major degradation pathway followed by Asp enantiomerization and isomerization of the product. None of the peptide amide isomerization and enantiomerization products exceeded 4.7% at any time during the incubation. The primary degradation product Gly-α-L-Asp-L-PheOH proved to be relatively stable. After 10 days at 80 °C, it comprised 60.5% of the peptide content in the incubation mixture. The relative concentrations of the products identified after 10 days are listed in Table 2. In contrast to L-Phe-α-L-Asp-GlyNH$_2$, the concentration of α-Asp peptides

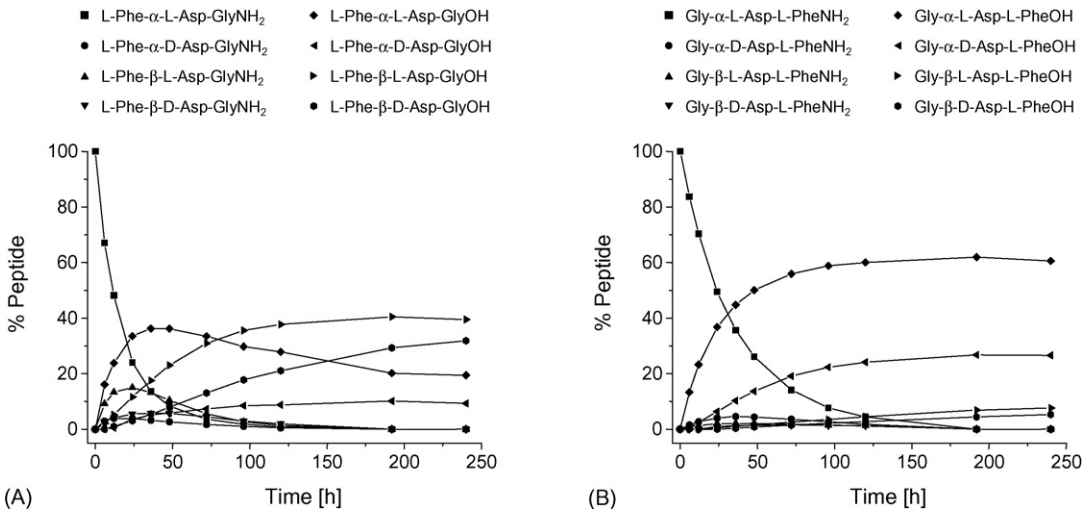

Fig. 5. Time course of incubations of Phe-Asp-GlyNH$_2$ (A) and Gly-Asp-PheNH$_2$ (B) at pH 10 and 80 °C. Each data point represents the mean of two independent incubations.

Table 2
Peptide content of incubations of L-Phe-α-L-Asp-GlyNH$_2$ and Gly-α-L-Asp-L-PheNH$_2$ at pH 10 after 10 days at 80 °C and of L-Phe-L-Asu-GlyOH after 2 h at 30 °C

Incubated peptide	Products	Peptide content (%)	Ratio α-Asp/β-Asp	Ratio L-Asp/D-Asp
L-Phe-α-L-Asp-GlyNH$_2$	L-Phe-α-L-Asp-GlyOH	19.4	1:2.5	1.4:1
	L-Phe-β-L-Asp-GlyOH	39.5		
	L-Phe-α-D-Asp-GlyOH	9.3		
	L-Phe-β-D-Asp-GlyOH	31.8		
Gly-α-L-Asp-L-PheNH$_2$	Gly-α-L-Asp-L-PheOH	60.5	6.8:1	2.1:1
	Gly-β-L-Asp-L-PheOH	7.6		
	Gly-α-D-Asp-L-PheOH	26.6		
	Gly-β-D-Asp-L-PheOH	5.3		
L-Phe-L-Asu-GlyOH	L-Phe-α-L-Asp-GlyOH	18.5	1:3.4	4:1
	L-Phe-β-L-Asp-GlyOH	61.4		
	L-Phe-α-D-Asp-GlyOH	4.2		
	L-Phe-β-D-Asp-GlyOH	15.9		

exceeded the concentration of β-Asp containing peptides with a ratio α-Asp/β-Asp of 6.8:1. Oliyai and Borchardt [11] also found lower concentrations of β-Asp peptide than α-Asp peptide in incubations of Val-Tyr-Pro-Asp-Val-Ala. The reversed α-Asp/β-Asp ratio can therefore also be attributed to steric hindrance by the phenyl side chain of the amino acid adjacent to Asp inhibiting the formation of the succinimide intermediate and subsequently the isomerization reaction. The ratio of peptides with L-Asp versus D-Asp configuration was 2.1:1, i.e. in about the same range as observed for L-Phe-α-L-Asp-GlyNH$_2$ above.

The amount of α-Asp and β-Asp peptide directly formed by hydrolysis of the succinimide as well as the concomitant enantiomerization at pH 10 was studied using L-Phe-L-Asu-GlyOH. Due to the instability of the succinimide at 80 °C, the incubations were performed at 30 °C. At this temperature, the apparent half-life of the degradation was 15.7 min. The time course of the incubation over a period of time of 2 h is shown in Fig. 6. The concentrations of the products did not change significantly when monitored for another 10 h (data not shown). The relative concentration of the resulting products L-Phe-α-L-Asp-GlyOH, L-Phe-β-L-Asp-GlyOH, L-Phe-α-D-Asp-GlyOH and L-Phe-β-D-Asp-GlyOH are summarized in Table 2. As expected, the amount of β-Asp-containing peptides exceeded the amount of α-aspartyl peptides with a ratio of α-Asp/β-Asp of 1:3.4 comparable to the ratio reported for Phe-Asp-GlyNH$_2$ above or for related peptides containing an Asp-Gly sequence [6,11,15,27]. Enantiomerization occurred to a lower extend. Thus, a ratio of L-Asp/D-Asp peptides of 4:1 resulted form the incubation of the Asu peptide compared to ratios of 1.4:1–2.1:1 observed for the peptides at 80 °C. This difference can be attributed to the lower incubation temperature and much shorter incubation time.

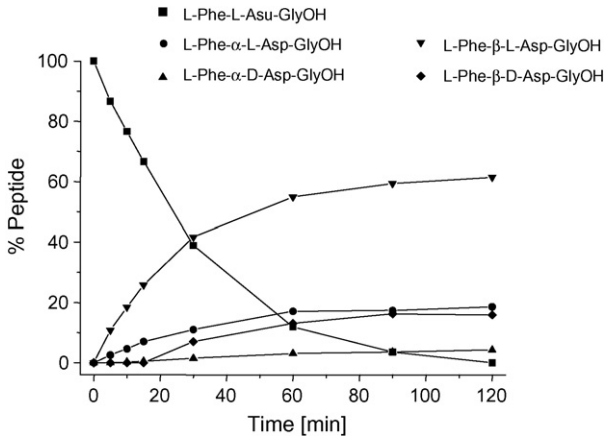

Fig. 6. Time course of the incubation of Phe-Asu-GlyOH at pH 10, 30 °C. Each data point represents the mean of two independent incubations.

3.4. Conclusions

Capillary electrophoresis methods for the analysis of degradation reactions of two tripeptides with isomeric amino acid sequences, Gly-Asp-PheNH$_2$ and Phe-Asp-GlyNH$_2$, were developed and validated. At pH 2 degradation of the peptides proceeded via C-terminal deamidation and peptide backbone hydrolysis. In contrast, isomerization and enantiomerization was observed in combination with deamidation at pH 10. As reported for other aspartyl peptides [11], the ratio of α-Asp to β-Asp peptides depended on steric hindrance by the side chain substituent of the neighboring amino acid. With regard to enantiomerization a ratio of L-Asp/D-Asp of 1.4:1 was observed following incubation for 10 days at 80 °C for Phe-Asp-GlyNH$_2$. The ratio was 2.1:1 for Gly-Asp-PheNH$_2$ under identical experimental conditions. As isomerization and enantiomerization are assumed to proceed via a succinimide intermediate the amino acid sequence of a peptide will apparently not only influence the extent of isomerization but also the enantiomerization of Asp residues. Further studies are currently underway to investigate this dependency.

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