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Analysis of isomeric glutamyl peptides by capillary electrophoresis Application to stability studies

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Abstract

Capillary electrophoresis has been used for the separation of the glutamyl tripeptides Gly–α-Glu–Phe–NH_2 and Phe–α-Glu–Gly–NH_2 including their potential degradation and isomerization products Gly–γ-Glu–Phe–NH_2, α-Glu–Phe–NH_2, γ-Glu–Phe–NH_2 and Phe–NH_2 as well as Phe–γ-Glu–Gly–NH_2, Phe–Glu and Phe, respectively. Between pH 2.2 and pH 10.0 the effective mobilities of the glutamyl peptides have been investigated. Using histidine hydrochloride as internal standard at pH 2.2 linear calibration curves for both assays were obtained for a concentration range from 10 μg ml^{-1} to 3.5 mg ml^{-1}. The assay was applied to analyze the degradation of the tripeptides in solution at pH 7 and pH 3 at 70°C. Hydrolysis and isomerization of the glutamyl peptides were found in the incubation mixtures.

Keywords: Stability studies; Glutamyl peptides; Peptides

1. Introduction

Hydrolysis and isomerization of amino acid residues have been recognized as mechanisms involved in the process of aging of natural peptides and proteins and in inactivation pathways of peptide pharmaceuticals during production and storage [1,2]. Among the amino acids that are sensitive to isomerization when incorporated in peptides, glutamine and glutamic acid have been less studied than asparagine and aspartic acid, for example. To the best of our knowledge only a study by Capasso et al. describes the deamidation and isomerization of the glutamine residue in a dipeptide derivative upon incubation in solution at pH 7.4 and 37°C [3]. The stability of Gln and the dipeptide Ala–Gln upon heat sterilization has been studied by isotachophoresis [4].

Capillary electrophoresis (CE) has become a powerful tool for the analysis of polar compounds such as peptides and proteins and has been successfully applied to the separation of closely related peptides including peptide diastereomers and enantiomers [5–9]. In the present study the analysis of two glutamyl tripeptides with opposite amino acid sequence, Gly–α-Glu–Phe–NH_2 and Phe–α-Glu–Gly–NH_2 including their potential hydrolysis and

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isomerization products was performed by CE. Peptides with opposite sequences were selected as the degradation and isomerization of Asp peptides is known to depend on the primary sequence [2,10]. The CE method was applied to investigate the degradation of the compounds upon incubation in aqueous solution.

2. Experimental

2.1. Chemicals

Phe, Phe–Glu and protected amino acid derivatives were obtained from Bachem (Heidelberg, Germany). All other chemicals were of analytical grade. The di- and tripeptides were prepared in solution using *N*-*tert*.-butyloxycarbonyl- (Boc) or *N*-benzyloxycarbonyl-protected amino acid derivatives and *N*-(3-dimethyl-aminopropyl)-*N*-carbodiimide as coupling reagent [11]. Boc-deprotection was performed using 4 *M* HCl in dioxane while *N*-benzyloxycarbonyl groups and benzylesters were deprotected by hydrogenolysis using a palladium on carbon catalyst [11]. The identity of the peptides was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The purity was determined by nuclear magnetic resonance (NMR) or reversed-phase high-performance liquid chromatography (HPLC) [RP C_{18} column, 0.1% trifluoroacetic acid in a water–acetonitrile gradient] to be at least 95%.

2.2. Instrumentation

All experiments were performed on a Beckman P/ACE 5510 instrument (Beckman Coulter, Waldbronn, Germany) equipped with a diode array detector at 20°C using 50 μm I.D.×375 μm O.D. untreated fused-silica capillaries (BGB Analytik, Schlossböckelheim, Germany) with an effective length of 30 cm, total length 37 cm. UV detection was carried out at 215 nm at the cathodic end of the capillary. Sample solutions prepared in water (100 μg ml^{-1}) were introduced by hydrodynamic injections at the anodic end at a pressure of 0.5 p.s.i. for 3 s (1 p.s.i.=6894.76 Pa). Initial separations of the isomeric Glu peptides were performed in 50 m*M* sodium phosphate buffer adjusted to the final pH by addition of 0.1 *M* H_3PO_4 or 0.1 *M* NaOH. The quantitative analyses were performed in 50 m*M* sodium phosphate buffer, pH 2.2. The applied voltage was 20 kV resulting in a current of 62 μA. Between analyses the capillary was flushed with 0.1 *M* NaOH for 1 min followed by the run buffer for 2 min.

2.3. Incubations

Solutions containing initial concentrations of 4 mg ml^{-1} of Gly–α-Glu–Phe–NH_2 or Phe–α-Glu–Gly–NH_2 were prepared in 50 m*M* phosphate buffer, pH 7 and pH 3, and stored at 70°C. At selected time intervals aliquots were withdrawn and analyzed after the addition of histidine–HCl as internal standard. The identity of the incubation products was confirmed by spiking with the respective pure peptides. Concentrations were calculated by the peak area ratio method using calibration curves obtained with the individual pure compounds.

3. Results and discussion

The structures of the glutamyl tripeptides Gly–α-Glu–Phe–NH_2 and Phe–α-Glu–Gly–NH_2 and their respective isomeric γ-glutamyl peptides Gly–γ-Glu–Phe–NH_2 and Phe–γ-Glu–Gly–NH_2 are depicted in Fig. 1. The migration behavior of these isomeric compounds was studied between pH 2 and pH 10 (data not shown). Below pH 4 the amino groups are fully protonated and the separation is achieved due to differences in the pK_a values of the glutamic acid side chain carboxyl groups. Thus, the α-isomers migrated faster than the respective γ-glutamyl peptides due to the lower acidity of the side chain carboxyl group of the α-isomers. In contrast, the amino groups of the γ-isomers are more basic than the amino groups of the α-peptides resulting in the CE separation above pH 7. Due to their electrophoretic mobility the peptides migrated in front of the electroosmotic flow (EOF) below pH 4 while they migrated as anionic species after the EOF above pH 7. Between pH 5 and 6 the amino groups are still protonated while the carboxyl groups are fully deprotonated resulting in a very low electrophoretic

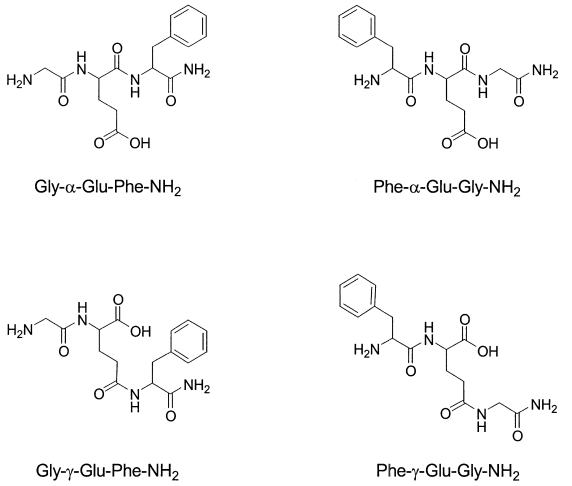

Fig. 1. Structures of the isomeric α- and γ-glutamyl peptides.

mobility. In this case the peptides migrated with the EOF. This is in agreement with the migration behavior of related aspartyl peptides [8,12].

Electropherograms of standard mixtures of Gly–α-Glu–Phe–NH_2 and Phe–α-Glu–Gly–NH_2 including potential hydrolysis and isomerization products using a run buffer at pH 2.2 are shown in Fig. 2. The analyses are complete within 15 min and 10 min, respectively. Only compounds containing a phenyl ring as chromophore were included. Thus, hydrolysis of Gly–α-Glu–Phe–NH_2 may yield α-Glu–Phe–NH_2 and Phe–NH_2 while isomerization results in Gly–γ-Glu–Phe–NH_2 and γ-Glu–Phe–NH_2. Phe–α-Glu–Gly–NH_2 can degrade to Phe–Glu, Phe and the isomeric Phe–γ-Glu–Gly–NH_2. Using histidine–HCl as internal standard good linearity of the detector response was observed for the compounds in a concentration range from 10 μg ml^{-1} to 3.5 mg ml^{-1} (Table 1). The limit of quantitation was 10 μg ml^{-1} except for Phe, Phe–α-Glu–Gly–NH_2 and Phe–γ-Glu–Gly–NH_2 which had a limit of quantitation of 7 μg ml^{-1}. The precision and accuracy of the CE assay was estimated using concentrations of about 50 μg ml^{-1}, 400 μg ml^{-1} and 1.6 mg ml^{-1}. Each concentration was injected three to four times. The relative standard deviation (RSD) of the ratios of the area of the compounds to the area of the internal standard ranged between 1.34 and 6.48%, 0.97 and 2.55%, and 0.45 and 3.16%, respectively, at the three concentrations tested.

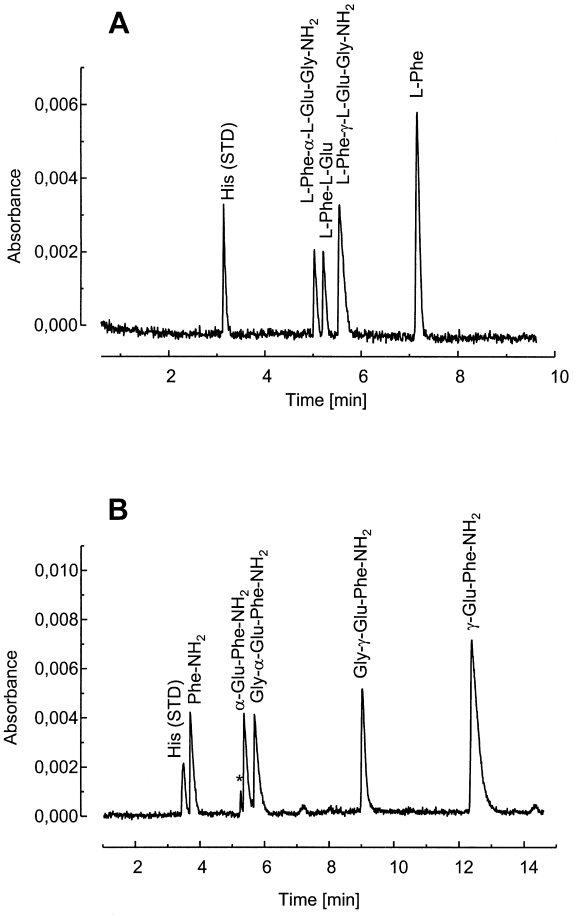

Fig. 2. Electropherograms of standards (A) Gly–α-Glu–Phe–NH_2 and potential degradation products; (B) Phe–α-Glu–Gly–NH_2 and potential degradation products. Conditions: 37 cm (effective length 30 cm) fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 2.2, 20 kV, 62 μA. The asterisk denotes a synthetic impurity.

Table 1
Linearity and slope of the regression curves obtained for CE analysis for the concentration range 10 μg ml^{-1} to 3.5 mg ml^{-1}

Compound	r^2	Slope
Gly–α-Glu–Phe–NH_2	0.9925	0.2159
Gly–γ-Glu–Phe–NH_2	0.9999	0.1763
Phe–α-Glu–Gly–NH_2	0.9998	0.1234
Phe–γ-Glu–Gly–NH_2	0.9996	0.1709
α-Glu–Phe–NH_2	0.9982	0.1785
γ-Glu–Phe–NH_2	0.9995	0.1572
Phe–Glu	0.9985	0.1835
Phe–NH_2	0.9998	0.1364
Phe	0.9956	0.1170

In order to evaluate the applicability of the CE assay to study the degradation of Gly–α-Glu–Phe–NH_2 and Phe–α-Glu–Gly–NH_2, incubations of each tripeptide in 50 m*M* phosphate buffer at pH 7 and pH 3 were performed and analyzed. In incubations of Gly–α-Glu–Phe–NH_2, α-Glu–Phe–NH_2, Phe–NH_2 and Phe as well as the isomeric Gly–γ-Glu–Phe–NH_2 could be identified as degradation products. Incubation of Phe–α-Glu–Gly–NH_2 yielded Phe–Glu, Phe and the isomeric Phe–γ-Glu–Gly–NH_2. A representative electropherogram of an incubation of Gly–α-Glu–Phe–NH_2 at pH 7 is shown in Fig. 3 while the time dependency of the concentration of the products is summarized in Fig. 4. Generally, identical hydrolysis and isomerization products were found at both pH values investigated but the amount of the individual compounds varied. At pH 7 the degradation proceeded faster compared to pH 3 (data not shown). Fig. 5 shows the time course of the degradation products in the incubation of Phe–α-Glu–Gly–NH_2, the tripeptide with inverse amino acid sequence, at pH 3. Hydrolysis to Phe–Glu and Phe as well as isomerization to the corresponding γ-isomer were observed. Incubation at pH 7 yielded the same products (data not shown). Again, the degradation proceeded faster at pH 7 compared to pH 3. Thus, as demonstrated for Asp peptides [2,10] the degradation of Glu peptides also appears to depend on the amino acid sequence of the respective peptides.

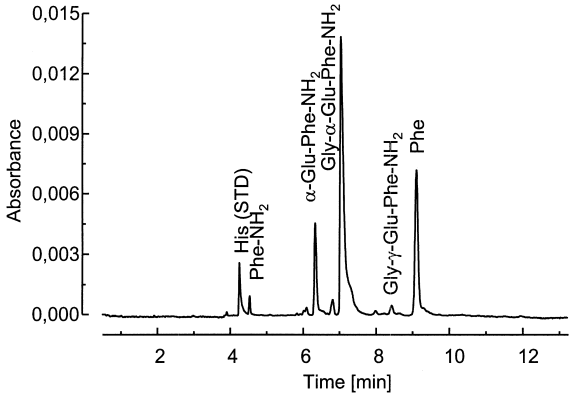

Fig. 3. Electropherogram of an incubation mixture of Gly–α-Glu–Phe–NH_2 following incubation at pH 7 and 70°C for 33 days. Conditions: 37 cm (effective length 30 cm) fused-silica capillary, 50 m*M* sodium phosphate buffer, pH 2.2, 20 kV, 62 μA.

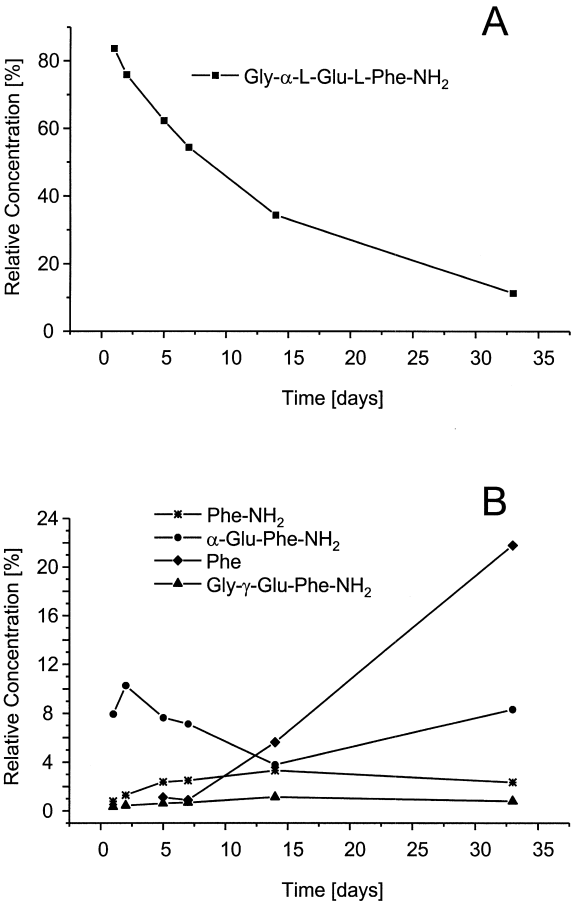

Fig. 4. Time course of the concentration of Gly–α-Glu–Phe–NH_2 (A) and degradation products (B) following incubation at pH 7 and 70°C. The data points represent the mean of two determinations.

In conclusion, CE can be applied to the analysis of closely related glutamyl peptides. The sensitive and reproducible assay allows the detection of the degradation as well as isomerization products of the peptides.

Acknowledgements

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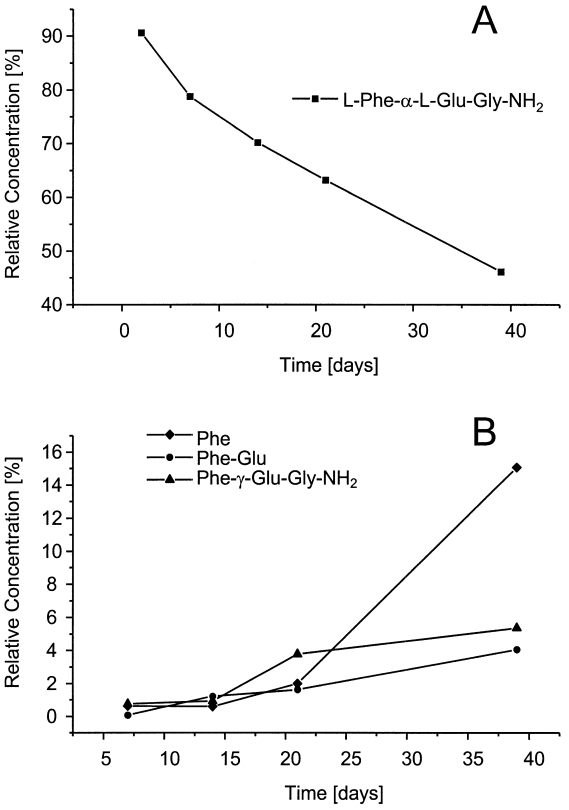

Fig. 5. Time course of the concentration of Phe–α-Glu–Gly–NH_2 (A) and degradation products (B) following incubation at pH 3 and 70°C. The data points represent the mean of two determinations.

References

[1] T. Geiger, S. Clarke, J. Biol. Chem. 262 (1987) 785.
[2] M.C. Manning, K. Patel, R.T. Borchardt, Pharm. Res. 6 (1989) 903.
[3] S. Capasso, L. Mazzarella, F. Sica, A. Zargari, J. Chem. Soc., Chem. Commun. (1991) 1667.
[4] P. Stehle, P. Pfaender, P. Fürst, J. Chromatogr. 294 (1984) 507.
[5] V. Kasicka, Electrophoresis 20 (1999) 3084.
[6] A. Cifuentes, H. Poppe, Electrophoresis 18 (1997) 2362.
[7] H. Wan, L.G. Blomberg, J. Chromatogr. A 792 (1997) 393.
[8] S. Sabah, G.K.E. Scriba, J. Chromatogr. A 822 (1998) 137.
[9] H. Wan, L.G. Blomberg, J. Chromatogr. A 875 (2000) 43.
[10] C. Oliyai, R.T. Borchardt, Pharm. Res. 11 (1994) 751.
[11] M. Bodanszky, A. Bodanszky, The Practice of Peptide Synthesis, 2nd ed., Springer, Berlin, 1994.
[12] D.J. Skanchy, R. Wilson, T. Poh, G.-H. Xie, C.W. Demarest, J.F. Stobaugh, Electrophoresis 18 (1997) 985.