RESEARCH ARTICLE

Degradation Kinetics of an Aspartyl-Tripeptide-Derived Diketopiperazine under Forced Conditions

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ABSTRACT: The aim of the present study was the determination of the stability of a diketopiperazine (DKP) derived from the aspartyl tripeptide Phe–Asp–GlyOH at pH 10 and 80°C and 25°C as well as at pH 7.4 and 80°C. The analysis was performed using a validated capillary electrophoresis assay. Rapid epimerization of the incubated *cis*-DKP to the *trans*-DKP was observed under all conditions. Linear diastereomeric α-L/D-Asp and β-L/D-Asp peptides were the primary reaction products at pH 10 and 80°C, indicating that a cyclic succinimide is formed in the process. In contrast, the DKPs were by far the major compounds in the incubation solutions at pH 10 and 25°C and at pH 7.4 and 80°C, whereas the linear Asp peptides were found only at low concentrations. A kinetic model was derived to fit the concentration versus time data, which consider the succinimide as central intermediate for DKP formation and for isomerization and enantiomerization of the linear Asp peptides. Besides the back reaction of the DKPs to the succinimides, an additional hydrolysis reaction of the DKP ring was considered to obtain the fit of the experimental data, indicating that additional degradation reactions have to be considered for Asp-derived DKPs. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

Aspartic acid (Asp) and asparagine (Asn) belong to the most unstable amino acids in peptides.[1,2,3,4] Asp and Asn peptides are prone to chemical degradation, for example, enantiomerization, isomerization, and peptide backbone hydrolysis. Enantiomerization and isomerization have been attributed to the formation of a cyclic aminosuccinimidyl (Asu) peptide formed via an intramolecular nucleophilic attack of the side-chain carbonyl group by the α-nitrogen of the peptide backbone amide.[4,5] In the case of Asn, this is associated with the loss of ammonia. Formation of Asu peptides is increased at alkaline pH and suppressed when an amino acid with a sterically hindered side chain is bound to Asn.[3,6,7] The respective reactions of Asp peptides are slower compared with Asn peptides because of the ionization of the Asp β-carboxyl group at alkaline pH.[2,3,5] Because of the increased acidity of the proton of the Asu α-carbon compared with the free amino acids Asp and Asn, the succinimide intermediate is highly prone to enantiomerization, yielding a mixture of L-Asu and D-Asu peptides.[1,8] Li et al.[9] provided evidence that enantiomerization may also occur at the stage of the tetrahedral intermediate preceding the Asu formation from an Asn peptide. An analogous tetrahedral intermediate can also be envisioned in the case of Asu formation from an Asp peptide. Subsequently, the succinimide can hydrolyze to form the "original" Asp (α-Asp) peptide as well as an isoaspartyl (iso-Asp or β-Asp) peptide.[1,10] The β-Asp peptide is favored over the α-Asp peptide at a ratio between 5:1 and 3:1.[6,7] Isomerization and enantiomerization of Asp residues are natural processes of protein aging[11,12,13,14] and have also been implicated in diseases such as Alzheimer's disease.[15,16]

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Another degradation pathway for peptides and proteins, which is also observed as a side reaction in peptide synthesis, is the formation of diketopiperazines (DKPs) resulting from the attack of the N-terminal amino group on the carbonyl group of the second amino acid in the peptide backbone.[17,18] Typically, DKP formation is associated with the loss of the first two amino acids of a protein and, in some cases, with the inversion of the sequence of the first two N-terminal amino acids.[17,19,20] Several factors have been shown to affect the rate of formation of DKPs including pH, temperature, solvent, buffer type, and amino acid sequence.[18,21–25] Cyclization to DKPs is increased at alkaline pH as the amino group has to be present in the nonprotonated form.[17,26] DKPs can subsequently hydrolyze to yield dipeptides with opposite amino acid sequences. For example, Steinberg and Bada[27] explained the extensive inversion of Ile–Gly into Gly–Ile and vice versa over a pH range from 2 to 9 by the hydrolysis of previously formed DKP favoring Ile–Gly over Gly–Ile. DKP hydrolysis may result in the preferred formation of one dipeptide sequence over the other because of increased cleavage of the sterically less hindered amide bond in the DKP as also shown for the hydrolysis of cyclo(Ala–Gly).[24] Furthermore, DKPs exhibit epimerization, that is, enantiomerization of one of the amino acids involved. The initially resulting DKP displays the sterically crowded *cis*-configuration because of the fact that natural amino acids are L-configured. The *cis*-DKP can subsequently isomerize to the energetically favored *trans*-DKP involving the enantiomerization at one of the chiral centers. In some cases, this epimerization appeared to occur faster in comparison with the corresponding dipeptides.[24]

Diketopiperazine formation from peptides containing Asp or Asn as second amino acid is associated with another phenomenon, the occurrence of an Asp-derived DKP peptide with the reminder of the amino acid chain attached to side-chain β-carboxyl group of Asp.[28–32] Jörnvall[31] attributed the formation of the Asp-derived DKP peptides to the involvement of the α-carboxyl group of Asp liberated during β-aspartyl shifts, whereas other groups suggested the intramolecular attack of the N-terminal amino group of the initially generated Asu intermediated on the aminosuccinimidyl carbonyl group as mechanism of DKP formation.[30,32] Studying the degradation of the model tripeptide Phe–Asn–GlyOH at 25°C in alkaline buffers (pH 8.5–10.5) DeHart and Anderson[30] observed that a DKP with Gly attached to the side-chain carboxylic acid of Asp [DKP-GlyOH, cyclo(Phe–Asp)–GlyOH] was the major degradation product, whereas the linear breakdown products Phe–α-Asp–GlyOH and Phe–β-Asp–GlyOH were only found at low concentrations. In ammonia-based buffers, DKP formation exceeded isomerization to linear α-Asp and β-Asp peptides below pH 10, whereas Phe–β-Asp–GlyOH was the major degradation product at pH 10 and above.[30] In contrast, investigating the isomerization and enantiomerization of L-Phe–α-L-Asp–GlyOH in borate buffer, pH 10, at 80°C, Conrad et al.[28] detected only small amounts of *cis*- and *trans*-DKPGlyOH (below 2% of the total peptides), whereas the linear peptides L-Phe–α-L-Asp–GlyOH and L-Phe–α-D-Asp–GlyOH as well as L-Phe–β-L-Asp–GlyOH and L-Phe–β-D-Asp–GlyOH were the major degradation products found in the incubation solutions. Incubations of the succinimide peptide L-Phe–L-Asu–GlyOH at pH 10 and at temperatures between 37°C and 80°C indicated that DKP formation is favored at lower temperatures, whereas at higher temperatures, isomerization to α-Asp and β-Asp peptides dominated.[28] The differences in the reaction products (DKPs versus linear α-Asp and β-Asp peptides) between the two studies may also be caused, at least in part, by different stabilities of the DKPs at 80°C and 25°C. Thus, the aim of the present study was the investigation of the degradation of the cyclic aspartyl tripeptide *cis*-DKPGlyOH as the initially formed DKP from L-Phe–L-Asu–GlyOH at pH 10 and at 80°C and 25°C. Incubations of the DKP at pH 7.4 and 80°C were also included because *cis*/*trans*-DKPGlyOH had been detected as major degradation products of L-Phe–α-L-Asp–GlyOH under these conditions.[29] Analysis of the peptides was performed by a validated capillary electrophoresis (CE) assay.

MATERIALS AND METHODS

Chemicals

9-Fluorenylmethoxycarbonyl (FMOC)–L-PheOH, FMOC–L-Asp(OtBu)-OH, FMOC–D-Asp(OtBu)-OH, FMOC–L-AspOtBu, FMOC–D-AspOtBu, FMOC–GlyOH, and 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, L-Phe–β-D-Asp–GlyOH, and *cis*-DKPGlyOH were synthesized by standard solid-phase synthesis using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate as a coupling reagent and 1-hydroxybenzotriazole as an additive. *cis*-DKP was synthesized according to the literature.[33] L-Phe–D-Asu–GlyOH was prepared from L-Phe–α-D-Asp–GlyOH by the treatment with 1 mol/L HCl in acetic acid.[32] The crude products were purified by preparative reversed-phase high-performance liquid chromatography (HPLC) using an acetonitrile–0.1% aqueous trifluoroacetic acid (TFA) gradient. After combining fractions and lyophilization, the identity of the peptides was confirmed by mass spectrometry (MS). The purity of all peptides was at least 98% as determined by HPLC and CE.