Stability of Tryptophan-Containing Peptides in the Presence of an L-Ascorbic Acid–Ferric Ion System

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The stability of free or peptide-bound L-tryptophan (Trp) was studied in the presence of an L-ascorbic acid–ferric ion system. Trp was present in three different forms of peptides: in peptides with amino-terminal Trp, with carboxy-terminal Trp, or with Trp in a middle position. These peptides were synthesized using fluorenylmethoxycarbonyl polyamide solid phase active ester chemistry. Decreases in peptide content as well as the stability of neighboring amino acids were determined. In each case the rate of oxidation of amino-terminal Trp was higher than in the corresponding peptides with carboxy-terminal Trp. Losses of Trp and the peptides themselves depend upon neighboring amino acid residues.

Keywords: *Tryptophan; oxidation system; peptide stability*

INTRODUCTION

L-Tryptophan (Trp) is an important target for protein modification, due to the high reactivity of the indole ring. During food processing and storage many chemical reactions can occur between the different food components (Hurrell, 1980). Specific modifications can be induced by light in the presence of oxygen and vitamins as a photosensitizer (Kanner and Fennema, 1987), oxidized lipids (Krogul and Fennema, 1987), or reducing sugars (Finot et al., 1982). The stability of free or protein-bound Trp depends on the temperature, pH, and presence of oxygen or other oxidizing agents (Friedman and Cuq, 1988). Grossweiner et al. (1976) suggested that Trp photolysis is dependent upon neighboring amino acid residues and other microenvironmental factors. A correlation between stability and position of Trp in peptides was described only for the case of photochemistry of free and peptide-bound Trp (Templer and Thistlethwaite, 1976; Dillon, 1980, 1981; Hibbard et al., 1985; Kanner and Fennema, 1987).

The influence of the concentration of Trp and L-ascorbic acid on Trp degradation has been described (Steinhart et al., 1993). The purpose of this study was to investigate the stability of free Trp and peptide-bound Trp in correlation with neighboring amino acids alanine (Ala), leucine (Leu), and phenylalanine (Phe). Particularly the effects of amino-terminal Trp, carboxy-terminal Trp, and Trp in a middle position were compared. Peptides showing the lowest Trp damage were determined.

MATERIALS AND METHODS

Reagents. Trp was purchased from Degussa AG (Hanau, Germany); Ala-Trp, Trp-Ala, Ala-Trp-Ala, Leu-Trp, Trp-Leu, Leu-Trp-Leu, Phe-Trp, and Trp-Phe were from Bachem Biochemica GmbH (Heidelberg, Germany). Acetonitrile, piperidine, and dimethylformamide were each freshly distilled. Reagents for peptide synthesis were purchased from Pharmacia LKB GmbH (Freiburg, Germany), Fluka AG (Neu-Ulm, Germany), and Riedel-de Haen (Seelze, Germany).

Citric acid buffer was prepared as follows: 9.61 g of citric acid was dissolved in 100 mL of distilled water, and the pH was adjusted with 0.1 N NaOH to 6.0.

Procedures. *Gas Chromatographic Determinations.* The amino acids were determined using a Carlo Erba gas chromatograph (GC) VEGA 6130 (FID detector) and separated on a 50 m DB-5 fused silica column (0.32 mm i.d., 0.25 μm film thickness). Quantitative determinations were made using phloroglucin as an internal standard.

For derivatization, 1 μmol of amino acid was weighed into 4 mL amber vials with Teflon-lined screw caps and 200 μL of dichloromethane was added. The solution was evaporated to dryness with nitrogen. One hundred microliters of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), containing the internal standard phloroglucin, and 100 μL of acetonitrile were added. The vials were sealed and heated at 140 °C for 30 min.

Liquid Chromatographic Determinations. Trp and peptides were determined using a high-performance liquid chromatograph (HPLC) system from Merck/Hitachi (Darmstadt, Germany). The HPLC system was composed of a Model L-6200 solvent delivery system, a Rheodyne injector with a 20 μL sample loop, and a reversed phase Nucleosil 120-3C_{18}, 125 × 4 mm, column (CS Chromatographie Service GmbH, Eschweiler, Germany). A variable wavelength Merck/Hitachi Model L-4000 UV photometric detector set at 219 nm was connected at the column outlet. The chromatographic response was recorded and integrated by using a Merck/Hitachi Model D-2000 integrator. Quantitative determinations were made using external standards.

The HPLC solvents were filtered through a 0.45 μm pore size filter (Schleicher & Schuell, Dassel, Germany), and degassed with helium before use. The mobile phase consisted of bidistilled water (A) and acetonitrile/water azeotrope (B), each containing 0.1% trifluoroacetic acid (v/v). The gradient program used was as follows:

time (min)	solvent A (%)	solvent B (%)
0	100	0
5	100	0
40	50	50
50	100	0
60	100	0

The solvent flow was 1 mL/min at room temperature.

Solid Phase Peptide Synthesis. Peptides were synthesized using a Pharmacia LKB Biolynx 4175 controlled by continuous spectrometric monitoring.

The resin used in the fluorenylmethoxycarbonyl (Fmoc) polyamide solid phase active ester chemistry was a cross-linked and functionalized poly(dimethylacrylamide) gel held within the pores of kieselguhr, in addition to the internal reference-spacer norleucine and a reversible acid-labile peptide–resin linkage agent (Ultrosyn A, LKB Biochrom Ltd., Cambridge, U.K.). Pentafluorophenyl ester derivatives were

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Table 1. Peptide System for the Investigation of Trp Stability As Affected by Neighboring Amino Acids

Trp C-terminal	Trp in the middle	Trp N-terminal
Ala-Trp	Ala-Trp-Ala	Trp-Ala
Ala-Ala-Trp	Ala-Ala-Trp-Ala-Ala	Trp-Ala-Ala
Ala-Ala-Ala-Trp		Trp-Ala-Ala-Ala
Leu-Trp	Leu-Trp-Leu	Trp-Leu
Leu-Ala-Trp	Leu-Ala-Trp-Ala-Leu	Trp-Ala-Leu
Leu-Ala-Ala-Trp		Trp-Ala-Ala-Leu
Phe-Trp		Trp-Phe
Phe-Ala-Trp	Phe-Ala-Trp-Ala-Phe	Trp-Ala-Phe
Phe-Ala-Ala-Trp		Trp-Ala-Ala-Phe

used in every step, including the carboxy-terminal amino acid. The crude peptides were detached from the resin with trifluoroacetic acid for 90 min at room temperature in the presence of ethanediol and anisole. The free peptides were lyophilized and their purities determined by HPLC.

Oxidation of Trp and Peptides by Iron-Catalyzed Autoxidation of L-Ascorbic Acid. Trp or peptide (18.75 μmol) was dissolved in 3 mL of a 0.1 M citric acid buffer (pH 6.0) containing 0.1% trifluoroacetic acid using an ultrasonic bath. To this solution was added 1 mL of 0.1 M citric acid buffer (pH 6.0) containing 0.013 mmol of $FeCl_3{\cdot}6H_2O$ and 0.065 mmol of EDTA. The reaction was started by adding 0.15 mmol of ascorbic acid in 1 mL of citric acid buffer (pH 6.0) and stirred under atmospheric conditions. The reaction was carried out for 5 h at room temperature.

Hydrolysis of Peptides. The procedure according to Yokote et al. (1986) as modified by Kell and Steinhart (1990) was used for hydrolysis of the peptides as follows: 1 μmol of peptide was weighed into 4 mL amber vials with Teflon-lined screw caps. Two hundred microliters of concentrated HCl, 100 μL of trifluoroacetic acid, and 20 μL of butyl mercaptan were added and the vials sealed. The solution was heated at 160 °C for 25 min and evaporated to dryness using nitrogen gas at 80 °C. The resulting residues were used for derivatization for GC determination.

Estimation of Trp Content in Oxidized Peptides. Degradation of Trp in the peptides is in each case less than the degradation of the peptide itself, because of oxidative damage of the neighboring amino acids Ala, Leu, and Phe. A correlation between the unmodified peptide and Trp content was established in a mathematical model. The Trp content is a function of the stability of the amino acids Ala, Leu, and Phe and of the peptide content determined by HPLC (Meyer et al., 1995).

RESULTS

Peptide Synthesis and Peptide System. Sixteen peptides were synthesized with an average yield of 40% (7–87%) in relation to the linkage of Ultrosyn A. Purity of the peptides determined by HPLC varied from 72% up to 98%. For peptides containing C-terminal Trp a low cleavage yield was observed because reaction with the resin-bound cation occurs intramolecularly. The reaction of the indole ring with resin-bound benzylic cations of the linkage results in reattachment of the peptide to the resin. Under these circumstances, added scavengers such as ethanethiol and anisole were unlikely to eliminate the side reaction completely. The peptide system is shown in Table 1. Three different amino acids were used for peptide synthesis: Ala as a short-chain aliphatic; Leu as a long-chain aliphatic amino acid, and Phe as an aromatic amino acid. The position of Trp in the various peptides is either at the C-terminal, N-terminal, or middle position.

Hydrolysis of Peptides and Determination of Neighboring Amino Acids. Butyl mercaptan used as a scavenger did not protect Trp against oxidation during acid hydrolysis, because of the presence of ferric ions

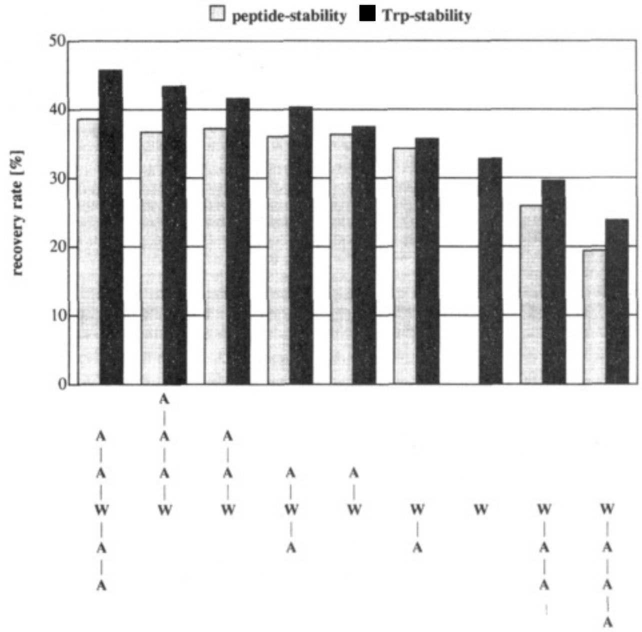

Figure 1. Recoveries of peptides after reaction in the L-ascorbic acid–ferric ion system and estimated Trp content (Trp determined by HPLC, peptides using the mathematical model). Experimental values are means of three determinations. A = alanine, W = tryptophan.

from the L-ascorbic acid–ferric ion system. Similar problems were observed during alkaline hydrolysis. Recoveries after acid hydrolysis of unoxidized dipeptides and tripeptides were 93.5% for Ala, 99.7% for Leu, and 95.9% for Phe.

Oxidation of Aqueous Solutions of Trp and Peptides. Recovery rates of 23 peptides and free Trp after reaction in the L-ascorbic acid–ferric ion system were determined by HPLC. Recoveries were 33.9% for free Trp (determined directly by HPLC) and between 11.5% and 37.8% for peptides (determined using the mathematical model; Meyer et al., 1995). Differences in the stabilities are shown in Figures 1–3. Peptides with C-terminal or N-terminal Phe show the highest losses, with recoveries between 11.5% and 30.3%. The lowest losses were determined for peptides containing only Ala and Trp. The rate of oxidation for peptides with N-terminal Trp was higher than that found in the corresponding peptide with the C-terminal Trp. Figure 4 shows the estimated recovery for N-terminal and C-terminal peptide-bound Trp.

Stability Factors of Neighboring Amino Acids. The recovery rates of the peptides were lower than the hypothetical recovery of Trp after hydrolysis because the peptide loss is due not only to loss of Trp but also to loss of the neighboring amino acids. The destruction of Ala, Leu, and Phe was less than the destruction of Trp itself; however, it could by no means be ignored. As shown above, Trp could not be determined by HPLC after hydrolysis. Neighboring amino acids could be determined by GC after acid hydrolysis and silylation. However, it was possible to estimate the recovery rate of peptide-bound Trp from the recovery rates of peptide and neighboring amino acids. Correlations between the oxidation rates of Trp and the oxidation rates of the neighboring amino acids were investigated. The quotients of oxidation rate of Trp and a given neighboring amino acid were nearly constant for different peptides. These quotients or stability factors were determined for Ala, Leu, and Phe, and it was possible to develop a mathematical model to determine the Trp content from

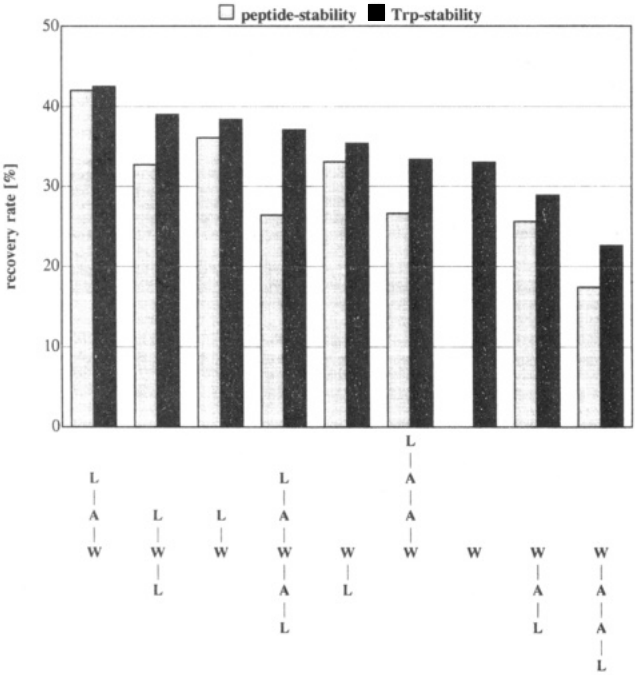

Figure 2. Recoveries of the peptides after reaction in the L-ascorbic acid–ferric ion system and estimated Trp content (Trp determined by HPLC, peptides using the mathematical model). Experimental values are means of three determinations. A = alanine, L = leucine, W = tryptophan.

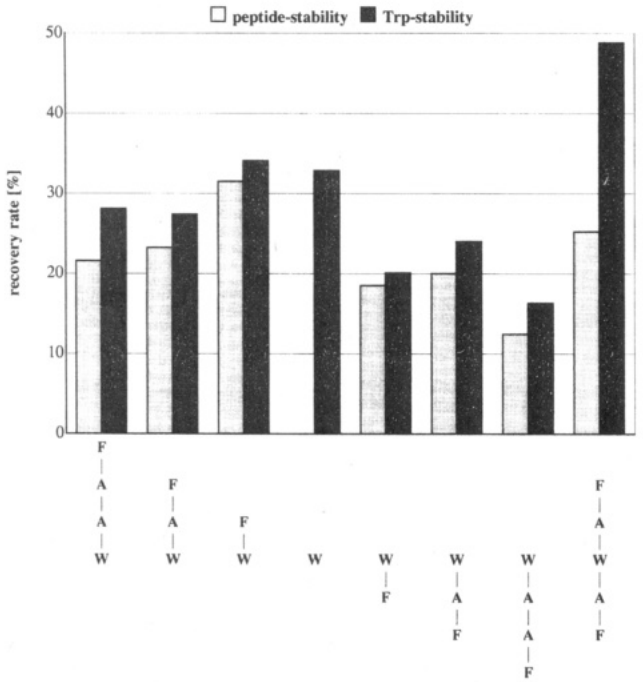

Figure 3. Recoveries of the peptides after reaction in the L-ascorbic acid–ferric ion system and estimated Trp content (Trp determined by HPLC, peptides using the mathematical model). Experimental values are means of three determinations. A = alanine, F = phenylalanine, W = tryptophan.

the peptide recovery and the recovery of neighboring amino acids (Meyer et al., 1995).

DISCUSSION

In the present study, the stability of free and peptide-bound Trp was investigated under the oxidative influence of an L-ascorbic acid–ferric ion system in aqueous solutions at room temperature [for the influence of

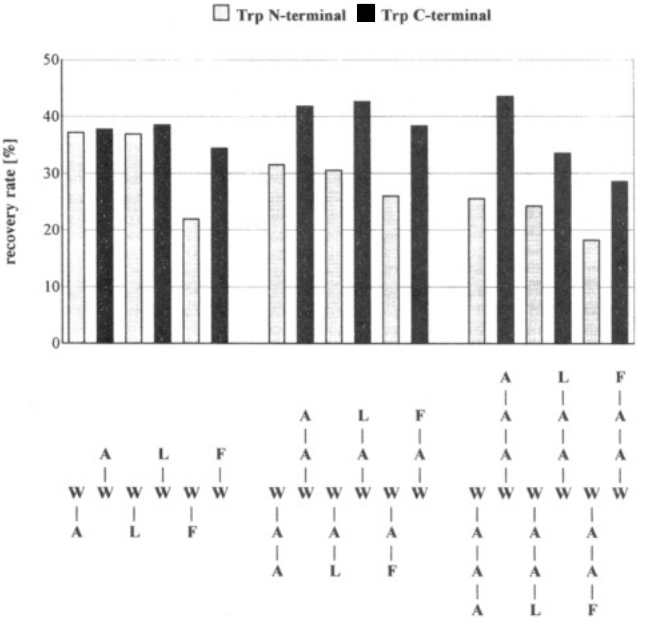

Figure 4. Estimated Trp content (determined using the mathematical model) of corresponding peptides after reaction in the L-ascorbic acid–ferric ion system. Experimental values are means of three determinations. A = alanine, L = leucine, F = phenylalanine, W = tryptophan.

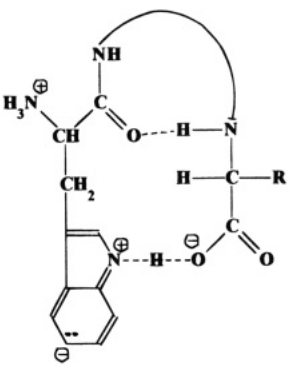

Figure 5. Interresidue hydrogen bonds from the imino proton on the indole ring to the carbonyl oxygen on the C-terminal amino acid.

L-ascorbic acid concentration, pH value, and Trp concentration, see Steinhart et al. (1993)]. The recovery rates of the peptides were determined, and from these data, the recovery rate of peptide-bound Trp was estimated using a mathematical procedure.

Determination of unmodified neighboring amino acids after acid hydrolysis shows a correlation between oxidation of Trp and oxidation of the neighboring amino acids. The stability factors for Ala, Leu, and Phe did not depend on the position of these amino acids in the peptide. For example, there are no differences between the stability factors for C-terminal and N-terminal Ala. The stability of Trp seems to be not directly influenced by a neighboring amino acid; however, the stability of the whole peptide does depend on it. Differences in recovery rates of peptides should correlate with the conformational energy.

Corresponding peptides with N- and C-terminal Trp were the main focus in this stability investigation. In each case, N-terminal Trp is less stable than C-terminal Trp (Figure 4). These differences in stability could be explained by an intramolecular interaction of the N-terminal Trp (Figure 5). Anderson et al. (1983) carried out conformational energy calculations of the zwitterionic forms of Trp and some Trp-containing peptides.

Resulting low-energy conformations were analyzed for the presence of hydrogen bonds, the distances between the N-terminal amino group and the indole ring. The tripeptide Trp-Gly-Gly has interresidue hydrogen bonds, one from the amino proton on the C-terminal Gly to the Trp carboxyl oxygen and another from the imino proton of the indole ring to the carboxyl oxygen on the C-terminal Gly. The two hydrogen bonds hold the molecule in a fairly compact shape. The hydrogen bond of the imino proton on the indole ring increases the electrical charge of the aromatic system; thus, an electrophilic attack is easier. This interaction could explain the difference in stability of corresponding peptides with C-terminal and N-terminal Trp.

Peptides with the lowest possible Trp damage and high recovery rates of the peptide itself were determined to be Ala-Trp-Ala, Ala-Ala-Trp, Ala-Ala-Ala-Trp, and Leu-Ala-Trp. The amino group of Trp participates in a peptide bond, and Ala is a neighboring amino acid with a high stabilizing effect. These peptides may be a good nutritional source for Trp if it should be desired.

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