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Development of a Mathematical Model for Estimation of the Tryptophan Content in Peptides

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A mathematical model for the estimation of the tryptophan (Trp) content in oxidized peptides was developed. Analytical parameters were the peptide recovery (determined by HPLC) and the recovery of the neighboring amino acids (determined by GC). The correlation of the peptide recovery with the degradation of the neighboring amino acids was derived by using a model and was applied to calculate the Trp recovery rate. The ratio of the degradation of Trp and the degradation of the neighboring amino acids in the peptides was estimated and defined as a factor named the stability factor. Equations describe the Trp reduction in the model peptides in dependence on the peptide recovery and the respective stability factors.

Keywords: *Tryptophan; peptide; oxidation; mathematical model*

INTRODUCTION

The most widely studied method for the analysis of protein-bound L-Tryptophan (Trp) in food appears to be alkaline hydrolysis (Hugli and Moore, 1972; Lucas and Sotelo, 1980). The acidic hydrolysis procedure is only applicable with total elimination of oxygen and use of reducing substances (Yokote et al., 1986). In the presence of traces of heavy metals the hydrolysis is impossible without making complicated provision against oxidative degradation.

Chrastil (1986) and Verma et al. (1988) suggested the determination of Trp selectively without hydrolysis of the proteins or small peptides. Chemical modification and spectral–photometrical determination of Trp without a chromatographic separation of the reaction mixture gave no satisfactory results because of the great variety of Trp derivatives.

The purpose of this study was to develop a method to determine the Trp content especially for the examination of the stability of Trp-containing peptides used in model systems.

MATERIALS AND METHODS

Reagents. L-Alanine (Ala), L-leucine (Leu), L-phenylalanine (Phe), and Trp were 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 was freshly distilled. All other chemicals used were of analytical purity and obtained from E. Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Fluka AG (Neu-Ulm, Germany), and Riedel-de-Haen (Seelze, Germany).

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

For derivatization of the amino acids, 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.

HPLC Determinations. HPLC determinations of Trp and peptides were carried out with 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},

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Table 1. Recovery of Trp in Percent Determined by HPLC after Basic Hydrolysis of Some Alanylpeptides (Lucas and Sotelo, 1980)[a]

	5 N NaOH, 5% $SnCl_2$ (m/v) $x \pm \sigma_{n-1}$	4 N LiOH $x \pm \sigma_{n-1}$
Trp	62.5 ± 10.62	65.0 ± 9.34
Ala-Trp	81.0 ± 3.06	80.4 ± 6.82
Trp-Ala	66.2 ± 8.32	62.7 ± 6.01
Ala-Trp-Ala	84.1 ± 8.68	87.1 ± 6.04

[a] Average of three determinations, solution containing 1 μmol of $FeCl_3 \cdot 6H_2O$.

125 × 4 mm, column (CS Chromatographic 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 used for determination of Trp and peptides 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. The gradient program used was as follows:

time (min)	solvent A (%)	solvent B (%)
0	100	0
5	100	0
30	50	50
40	100	0
50	100	0

The solvent flow was 1 mL/min.

Peptide Oxidation. Oxidation of peptides through the iron-catalyzed reaction of hydrogen peroxide was carried out for 2 h at 100 °C under atmospheric conditions in vials containing 5 mL of solution of 0.01 mmol of peptide, 0.01 mmol of $FeCl_2 \cdot 4H_2O$, and 0.5 mmol of H_2O_2 in bidistilled water. The reaction was initiated by the addition of the hydrogen peroxide.

Basic Hydrolysis. The procedure according to Lucas and Sotelo (1980) was used for the basic hydrolysis of the peptides as follows: 1 μmol of peptide was weighed into 4 mL amber vials with Teflon-lined screw caps. Two milliliters of 5 N NaOH containing 5% $SnCl_2$ (m/v) or 2 mL of 4 N LiOH was added, and the vials were sealed. The solution was heated at 145 °C for 4 h, and immediately 2 mL of phosphoric acid (25%, m/v) was added. The resulting residues were used for HPLC determination.

Acid Hydrolysis. The procedure according to Yokote et al. (1986) as modified by Kell and Steinhart (1990) was used for the acid 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.

RESULTS AND DISCUSSION

Hydrolysis of Peptides and Recovery Rates of Amino Acids. The methods for basic hydrolysis for determination of Trp with HPLC according to Lucas and Sotelo (1980) gave unsatisfactory results. The ferric ions used in the model reaction catalyze the oxidative degradation of Trp during the hydrolysis. Therefore, traces of ferric ions decrease the recovery of Trp; the recovery rates of some Ala-containing peptides after hydrolysis were between 62% and 87% (Table 1). The variation coefficients of threefold estimation were between 10% and 30%.

Table 2. Recovery Rates of Ala, Leu, and Phe in Percent Determined by GC after Acid Hydrolysis of Some Trp-Containing Peptides (Kell and Steinhart, 1990)[a]

peptide	$x \pm \sigma_{n-1}$	peptide	$x \pm \sigma_{n-1}$
Ala-Trp	92.7 ± 1.52	Leu-Trp-Leu	96.1 ± 3.74
Trp-Ala	94.3 ± 1.65	Phe-Trp	95.7 ± 2.48
Leu-Trp	101.8 ± 1.29	Trp-Phe	96.0 ± 1.47
Trp-Leu	101.1 ± 2.06		

[a] Average of three determinations.

Acid hydrolysis of the peptides according to the procedure of Kell and Steinhart (1990) and determination of amino acids by GC after silylation of the functional groups gave satisfactory recoveries for Ala, Leu, and Phe. The recovery rates were between 93% and 102% (Table 2). Trp could not be detected because of oxidative degradation during the hydrolysis in the presence of traces of ferric ions.

Estimation of the L-Tryptophan Content. Degradation of the peptides catalyzed by ferric ions causes high Trp losses and also some losses of the neighboring amino acids. Recovery of the peptides is therefore always lower than the content of unchanged Trp in these peptides. For the estimation of stability of Trp in model systems, the degradation of neighboring amino acids had to be considered because direct determination of the Trp content was not possible.

The relationship of the Trp content, the peptide recovery, and the degradation of neighboring amino acids will be demonstrated for the peptide Phe-Ala-Trp as an example. Degradation of amino acids under model conditions is a statistical event occurring with a definite probability. Using a model calculation, the influences will be examined. The degradation of neighboringamino acids is defined and the reaction is divided into three steps.

starting point	100% Phe-Ala-Trp	
defined degradation of the amino acids	80% Trp are destroyed (*Trp*) 20% Ala are destroyed (*Ala*) 10% Phe are destroyed (*Phe*)	
final situation	14.4% Phe-Ala-Trp 1.6% *Phe*-Ala-Trp 3.6% Phe-*Ala*-Trp 0.4% *Phe-Ala*-Trp 57.6% Phe-Ala-*Trp* 6.4% *Phe*-Ala-*Trp* 14.4% Phe-*Ala-Trp* 1.6% *Phe-Ala-Trp*	
single steps	100% Phe-Ala-Trp	
	80% of Trp are destroyed	
20.0 Phe-Ala-Trp	80.0 Phe-Ala-*Trp*	
	20% of Ala are destroyed	
16.0 Phe-Ala-Trp 4.0 Phe-*Ala*-Trp	64.0 Phe-Ala-*Trp*	16.0 Phe-*Ala-Trp*
	10% of Phe are destroyed	
14.4 Phe-Ala-Trp 3.6 Phe-*Ala*-Trp 1.6 *Phe*-Ala-Trp 0.4 *Phe-Ala*-Trp	57.6 Phe-Ala-*Trp* 6.4 *Phe*-Ala-*Trp*	14.4 Phe-*Ala-Trp* 1.6 *Phe-Ala-Trp*

This calculation with defined losses of the amino acids demonstrates that the defined Trp recovery (20%) is greater than the calculated recovery of the unchanged peptide (14.4%). The dependence of peptide and Trp recovery upon the degradation of the neighboring amino

Table 3. Recoveries of Peptides and Neighboring Amino Acids after Oxidation with H_2O_2 Catalyzed by Fe^{3+}[a]

	A[b]	B_n[c]	X[d]	X_H[e]
Ala-Trp	36.4 ± 0.80	91.9 ± 1.45	39.6 ± 1.29	27.6 ± 7.28
Leu-Trp	31.8 ± 1.25	88.6 ± 1.65	35.9 ± 1.74	28.6 ± 5.91
Phe-Trp	28.1 ± 0.30	85.5 ± 2.38	32.8 ± 1.23	21.3 ± 3.61
Trp-Ala	27.8 ± 1.06	90.6 ± 2.78	30.7 ± 2.13	22.2 ± 7.78
Trp-Leu	24.1 ± 1.19	87.2 ± 1.80	27.7 ± 1.12	20.0 ± 3.78
Trp-Phe	19.4 ± 1.05	83.6 ± 4.40	23.2 ± 1.21	13.5 ± 3.59

[a] Calculated content of Trp in comparison to the average recovery after basic hydrolysis (averages of three determinations). [b] A, recovery of the peptide determined by HPLC (%). [c] B_n, recovery of the neighboring amino acid after acid hydrolysis and determination by GC (%). [d] X, calculated content of Trp (%). [e] X_H, recovery of Trp after basic hydrolysis and determination by HPLC (%).

Table 4. Stability Factors (F_n)[a] of the Neighboring Amino Acids in the Peptides Ala-Trp, Trp-Ala, Leu-Trp, Trp-Leu, Leu-Trp-Leu, Phe-Trp, and Trp-Phe

	N-terminal	C-terminal	middle position	$F_{\text{amino acid}}$[b]
alanine	7.7 ± 1.58	7.8 ± 2.45		7.8 ± 1.85
leucine	5.0 ± 1.34	5.6 ± 1.57	6.0 ± 1.36	5.5 ± 1.31
phenylalanine	4.8 ± 1.02	4.9 ± 1.50		4.8 ± 1.15

[a] $F_n = (100 - X)/(100 - B_n)$, where F_n is the stability factor of the neighboring amino acid, X is the calculated content of Trp in %, and B_n is the recovery of the neighboring amino acid after acid hydrolysis and determination by GC (%). Average of three determinations. [b] $F_{\text{aminoacid}}$, average of the stability factors for the N- and C-terminal amino acid.

acids could be represented as a function

$$X = A(100/B_1)(100/B_2)$$

where X is the calculated content of Trp in %, A is the recovery of the peptide in % determined by HPLC, B_1 is the recovery of Ala in % after acid hydrolysis and determination by GC, and B_2 is the recovery of Phe in % after acid hydrolysis and determination by GC.

The general equation for the estimation of the Trp content as a function of peptide recovery and the content of the neighboring amino acids is

$$X = A \times 100^n/(B_1 \times \ldots \times B_n) \quad (1)$$

The calculated Trp recoveries of six oxidized dipeptides are shown in Table 3. The recoveries of the neighboring amino acids are estimated after acid hydrolysis and determination by GC. The Trp recovery after basic hydrolysis and determination by HPLC is listed in Table 3 for comparison. In all cases the Trp recovery determined by HPLC after basic hydrolysis is lower than the recovery of the peptide. This contradicts the expectation that the Trp content should be higher than the peptide recovery and demonstrates the Trp degradation occurring during the basic hydrolysis in the presence of ferric ions.

Determination of Stability Factors for Amino Acids. A correlation between Trp degradation and the degradation of neighboring amino acids was demonstrated. The quotient of these parameters defined as a stability factor seems to be constant and independent of the peptide used (Table 4). Ala exhibits the lowest molecular weight and the shortest C-chain and shows the greatest stability factor. Phe has the lowest stability factor of the three amino acids used, possibly due to the aromatic residue, and Leu takes a middle position. All of these amino acids in peptides have greater stability factors than Trp itself.

Functions To Calculate the Trp Content from Dependence on the Recovery of the Peptide and the Stability Factor. The Trp recovery is a function of the peptide recovery (A) and the recovery of the neighboring amino acids (B_n). It is not necessary to estimate the recovery of the neighboring amino acids for every peptide because the recovery can be replaced by the stability factor (F_n). Therefore, the Trp content is a function of the peptide recovery and the stability factors.

$$X = f(A,B_n) = A \times 100^n/(B_1 \times \ldots \times B_n) \quad (2)$$

$$B_n = f(X,F_n) = (100 - (100 - X)/F_n) \quad (3)$$

$$X = f(A,F_n) = A \times 100^n/(100 - (100 - X)/F_1)/\ldots/(100 - (100 - X)/F_n) \quad (4)$$

X is the calculated content of Trp in %, A is the recovery of the peptide in % determined by HPLC, B_n is the recovery of the neighboring amino acid in % after acid hydrolysis and determination by GC, and F_n is the stability factor of the neighboring amino acid.

These functions can only be calculated exactly for Trp in dipeptides because the exponent of the unknown X is due to the number of amino acid residues (3 for tripeptides, 4 for tetrapeptides, etc.) (Table 5). The function can be solved if the terms with exponents of X higher than 2 are discarded (Table 5). The deviation caused by this simplification may be disregarded if the peptide recovery does not exceed 50% (Figure 1).

Table 5. Function of the Stability of Neighboring Amino Acids, Peptide Recovery Rate, and Trp Content (Terms Left for Calculation Are in Italics)[a]

dipeptide with one neighboring amino acid (5)

$$X^2 + X \times 10^2 \times (F_1 - 1) - A \times 10^2 \times F_1 = 0$$

tripeptide with two neighboring amino acids (6)

$$X^3 + X^2 \times 10^2 \times (F_1 + F_2 - 2) + X \times 10^4 \times (F_1F_2 - F_1 - F_2 + 1) - A \times 10^4 \times F_1F_2 = 0$$

tetrapeptide with three neighboring amino acids (7)

$$X^4 + \mathit{X^3 \times 10^2 \times (F_1 + F_2 + F_3 - 3)} + X^2 \times 10^4 \times (F_1F_2 + F_1F_3 + F_2F_3 - 2F_1 - 2F_2 - 2F_3 + 3) + X \times 10^6 \times (F_1F_2F_3 - F_1F_2 - F_1F_3 - F_2F_3 + F_1 + F_2 + F_3 - 1) - A \times 10^6 \times F_1F_2F_3 = 0$$

pentapeptide with four neighboring amino acids (8)

$$X^5 + \mathit{X^4 \times 10^2 \times (F_1 + F_2 + F_3 + F_4 - 4) + X^3 \times 10^4 \times (F_1F_2 + F_1F_3 + F_1F_4 + F_2F_3 + F_2F_4 + F_3F_4 - 3F_1 - 3F_2 - 3F_3 - 3F_4 + 6)} + X^2 \times 10^6 \times (F_1F_2F_3 + F_1F_2F_4 + F_1F_3F_4 + F_2F_3F_4 - 2F_1F_2 - 2F_1F_3 - 2F_1F_4 - 2F_2F_3 - 2F_2F_4 - 2F_3F_4 + 3F_1 + 3F_2 + 3F_3 + 3F_4 - 4) + X \times 10^8 \times (F_1F_2F_3F_4 - F_1F_2F_3 - F_1F_2F_4 - F_1F_3F_4 - F_2F_3F_4 + 2F_1F_2 + 2F_1F_3 + 2F_1F_4 + 2F_2F_3 + 2F_2F_4 + 2F_3F_4 - F_1 - F_2 - F_3 - F_4 + 1) - A \times 10^8 \times F_1F_2F_3F_4 = 0$$

[a] X = Trp content in %, F = stability factor of the neighboring amino acid (Ala = 7.8, Leu = 5.5, Phe = 4.8), A = peptide recovery rate in % determined by HPLC.

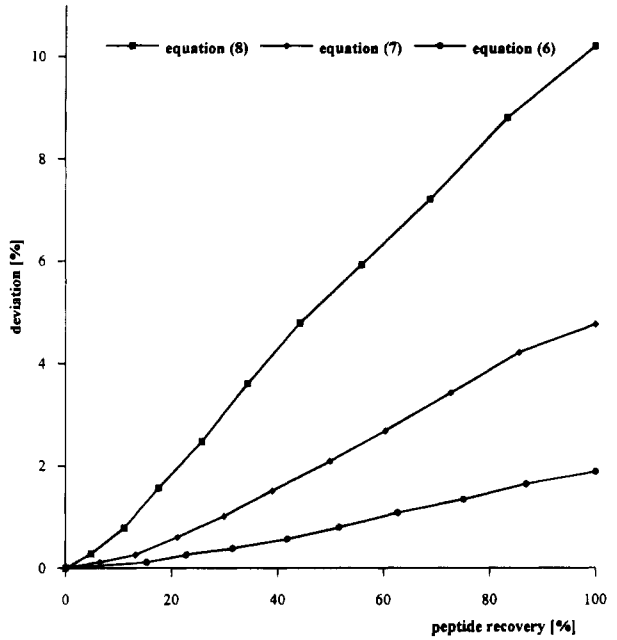

Figure 1. Deviation of simplification of the eqs 6–8 (neglecting all terms with exponents of X greater than 2).

Summary. The instability of Trp even during the basic hydrolysis is the reason for proceeding to an indirect, mathematical method of determination. The calculation of the Trp content of oxidative treated peptides is possible on the basis of a few analytical parameters under model conditions.

The estimation of the degradation rates of the neighboring amino acids and of the peptide recovery allows a calculation of the Trp content on the premise that the degradation is a statistical event with a certain probability.

The newly defined arithmetical parameter of a neighboring amino acid, named the stability factor (F_n), enables calculation of the Trp content in peptides after treatment under model conditions. Therefore, a presupposition is that the stability of Trp is influenced not only by the direct neighboring amino acid but also by the primary structure of the peptide, which determines the stability of all amino acids.

The mathematical consideration of the oxidation of Trp-containing peptides makes it possible to examine stabilizing effects of individual peptides. A transfer of the numerical results to other oxidation systems is not possible. The method of mathematical estimation of the Trp content should be transferable to other oxidation systems also.

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