Oxidation of Free Tryptophan and Tryptophan Residues in Peptides and Proteins

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The oxidation of free tryptophan (Trp) and Trp residues in peptides and proteins by hydrogen peroxide as oxidative agent has been used to evaluate Trp losses and the pattern of degradation products formed. Besides free Trp, four Trp-containing peptides and lysozyme were used as substrates in the aqueous model system. The oxidation rate of Trp and the formation of 16 possible degradation compounds were examined using RP-HPLC and UV, fluorescence, and photodiode array detection. The rate of Trp degradation increased from lysozyme to short-chain peptides to unbound Trp. Only ~20% of the total Trp loss could be elucidated by the determined Trp degradation compounds. Oxindolylalanine (Oia), 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (PIC), *N*-formylkynurenine (NFK), dioxindolylalanine (DiOia), kynurenine (Kyn), and 5-hydroxytryptophan (5-OH-Trp) were identified in this order of quantity as degradation compounds, showing the Trp pyrrole moiety to be most susceptible to oxidation. As short peptides such as H-Ala-Trp-Ala-OH were completely hydrolyzed with immobilized Pronase E, Oia and NFK could be identified as main degradation compounds, as could minor amounts of Kyn, DiOia, PIC, and 5-OH-Trp. Acid (6 M HCl), alkaline (4.2 M NaOH), and enzymatic hydrolyses were compared for the determination of Trp degradation compounds in lysozyme. Kyn, Oia, and DiOia could be detected in the hydrogen peroxide treated protein.

Keywords: *L-Tryptophan; high-performance liquid chromatography (HPLC); oxidation; enzymatic hydrolysis*

INTRODUCTION

Proteins in food, feed, or pharmaceuticals are processed or stored under conditions that facilitate oxidation processes. Tryptophan (Trp) losses have been examined using various model systems such as treatment with oxidizing lipids (Yong et al., 1980; Matoba et al., 1984; Nielsen et al., 1985b,d; Krogull and Fennema, 1987), hydrogen peroxide (Nielsen et al., 1985a,c; Weck et al., 1987; Kell and Steinhart, 1990), H_2O_2/peroxidase (Itakura et al., 1994), ozone (Okajima et al., 1990), hypoxanthine/xanthine/Fe(III)/EDTA/O_2 (Itakura et al., 1994), Fe(II)/EDTA/ascorbic acid (Uchida et al., 1990), Fe(III)/ascorbic acid/O_2 (Steinhart et al., 1993), visible light/sodium metabisulfite/O_2 (Lee and Rogers, 1988), visible light/photosensitizer (Nakagawa et al., 1981; Kanner and Fennema, 1987), heat/O_2 (Cuq and Cheftel, 1983; Cuq et al., 1983), light/O_2 (Holt et al., 1977), performic acid (Finlayson, 1969), Cu(II)/ascorbic acid/O_2 (Uchida and Kawakishi, 1988), dimethyl sulfoxide/HCl (Savige and Fontana, 1976), and γ-irradiation (Steinhart, 1991; Wickern et al., 1996).

Manifold oxidation compounds of Trp are reported to be formed during oxidative processes. Physiological metabolites of Trp such as kynurenine (Kyn) and *N*-formylkynurenine (NFK) (Yong et al., 1980; Kanner and Fennema, 1987; Krogull and Fennema, 1987; Uchida et al., 1990) and Kyn, NFK, and 5-hydroxytryptophan (5-OH-Trp) (Wickern, 1996), as well as proteinogenic amino acids, expressly Ala, Gly, Ser, and Asp (Kell and Steinhart, 1990), have been identified. Several nonphysiological compounds, either amino acids such as dioxindolylalanine (DiOia) (diastereomers A and B) (Yong et al., 1980), oxindolylalanine (Oia) (diastereomers A and B), and 7-OH-Trp (Wickern, 1996) or imino acids such as 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (PIC) (diastereomers A and B) (Nakagawa et al., 1981; Uchida et al., 1990) or various compounds such as indole, anthranilic acid (Kell and Steinhart, 1990), and tryptamine (Steinhart, 1991) were detected.

To date, few studies have focused on the identification of compounds formed from Trp residues in peptides or proteins during oxidative treatments. For the identification of Trp degradation compounds in peptides, either the oxidized peptides were isolated and characterized by UV and FAB-MS spectra (Itakura et al., 1994) or reference peptides were synthesized by directed oxidation (Holt et al., 1977) or the oxidized peptides were hydrolyzed enzymatically by Pronase E, releasing the Trp degradation compounds (Kanner and Fennema, 1987; Krogull and Fennema, 1987). During oxidation of Trp-containing peptides, formation of Oia, Kyn, and NFK (Itakura et al., 1994), Kyn and NFK (Kanner and Fennema, 1987; Krogull and Fennema, 1987), and Asp, NFK, and Oia (Holt et al., 1977) could be detected.

In proteins, Kyn has been identified after acid hydrolysis (Finlayson, 1969), as has Oia (Savige and Fontana, 1976). During directed oxidation of Trp in proteins by ozone yielding NFK, the reaction has been monitored spectrometrically by absorption measure-

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Table 1. Nomenclature, Abbreviations, and Peak Numbers of Reference Compounds

peak no.	CAS name	abbrev	common name
1	1,2,3,3a,8,8a-hexahydro-3a-hydroxy**pyrrolo[2,3-*b*]-indole-2-carboxylic acid**	PIC	
2	α,2-diamino-3-hydroxy-γ-oxo**benzenebutanoic acid**	3-OH-Kyn	3-hydroxykynurenine
3, 5	α-amino-2,3-dihydro-3-hydroxy-2-oxo-**1*H*-indole-3-propanoic acid**	DiOia	dioxindolylalanine (diastereomers)
4	2-amino-3-hydroxy**benzoic acid**	3-OH-ABA	3-hydroxyanthranilic acid
6	α,2-diamino-γ-oxo**benzenebutanoic acid**	Kyn	kynurenine
7	5-hydroxy**tryptophan**	5-OH-Trp	
8	α-amino-2-(formylamino)-γ-oxo**benzenebutanoic acid**	NFK	*N*-formylkynurenine
9, 10	α-amino-2,3-dihydro-2-oxo-**1*H*-indole-3-propanoic acid**, 2-hydroxy**tryptophan**	Oia	oxindolylalanine (diastereomers), 2-hydroxy-tryptophan (keto−enol tautomerism)
11	1*H*-indole-3-ethanamine	Tra	tryptamine
12	tryptophan	Trp	
13	4-hydroxy-**2-quinolinecarboxylic acid**	KA	kynurenic acid
14	α-hydroxy-**1*H*-indole-3-propanoic acid**	ILA	indole-3-lactic acid
15	1*H*-indole-3-carboxylic acid	ICA	
16	1*H*-indole-3-acetic acid	IAA	
17	1*H*-indole-3-propanoic acid	IPA	indole-3-propionic acid
18	1*H*-indole	Ind	

ment at 322 nm (Okajima et al., 1990). For the release of labile amino acids of proteins enzymatic hydrolysis has commonly been applied. Incubations with endoproteases and exopeptidases have been used in series for this purpose in either free (Schmitz et al., 1976; Röper et al., 1984) or immobilized (Chin and Wold, 1974, 1984; Matoba et al., 1984; Hauck, 1990) form.

The purpose of the present study was to determine and compare rates of oxidation of free Trp and Trp residues in peptides and proteins in a model system using hydrogen peroxide as the oxidative agent. Relatively strong oxidizing conditions were applied to identify the Trp degradation compounds formed during this process. Therefore, a RP-HPLC separation of 16 Trp-related substances (see Table 1), either known to be Trp metabolites or mentioned as being formed during Trp oxidation, was applied. For peptides and the protein, several hydrolysis procedures were examined to confirm whether they were suitable to release the examined Trp oxidation products without degradation.

REAGENTS

Pronase E (EC 3.4.24.4) (95 000 PUK/g) and aminopeptidase M (EC 3.4.11.2) (3 units/mg) were obtained from Merck (Darmstadt, Germany) and catalase (EC 1.11.1.6) (5000 units/mg) and prolidase (EC 3.4.13.9) (20 units/mg) from Serva (Heidelberg, Germany). Pepsin (EC 3.4.13.9) (30 milliAnson/mg) was from Sigma (Deisenhofen, Germany) and CNBr-activated Sepharose 4B from Pharmacia (Freiburg, Germany).

H-Ala-Trp-OH (AW), H-Trp-Ala (WA), H-Ala-Trp-Ala-OH (AWA), H-Leu-Trp-Leu-OH (LWL), H-Leu-Trp-Met-OH (LWM), and H-Ala-Ala-Trp-Ala-Ala-OH (AAWAA) were purchased from Bachem Biochemica GmbH (Heidelberg, Germany), and lysozyme (EC 3.2.1.17) was purchased from Sigma.

DiOia (diastereomers A and B), Oia, and NFK were synthesized as described in a previous publication (Simat et al., 1994). PIC was synthesized according to the method of Savige (1975), as described; however, only one diastereomer (PIC A) was obtained as authentic sample (Simat et al., 1996).

For purification, methanol (MeOH) was fractionally distilled from sodium hydroxide and sodium borohydride and acetonitrile (MeCN) from potassium carbonate, respectively. Trifluoroacetic acid (TFA) used for chromatography was of HPLC grade (Merck). Deionized water was purified by a Heraeus-Destamat Bi 18E (bidistillator) (Kleinostheim, Germany).

All other chemicals used were of analytical purity.

EXPERIMENTAL PROCEDURES

Immobilization of Enzymes. CNBr-activated Sepharose 4B (1.0 g) was swollen in 30 mL of 1 mM HCl for 15 min (1.0 g yields about 3.5 mL of gel) and filled into a 10 mL column (Bio-Rad Poly-Prep column with end caps, polypropylene; Bio-Rad, München, Germany). The resulting 3.5 mL of swollen gel was washed first with 200 mL of 1 mM HCl and then with 0.1 M borate buffer (pH 8.3) (containing 0.5 M NaCl) until the pH of the eluate was neutral. Enzyme (5−10 mg) was dissolved in 5.0 mL of the borate buffer mentioned above and subsequently added to the Sepharose gel and incubated by gentle rotation on a multiaxle rotating mixer (Hecht, Sondheim, Germany) at 4 °C (refrigerator) for 16−24 h overnight. After deposition of the Sepharose gel, the upper aqueous layer was sucked through the column by vacuum and collected. The remaining active sites of CNBr Sepharose were blocked by addition of 5 mL of 1 M ethanolamine (buffered with 0.1 M borate, pH adjusted to 8.0 with HCl) and using the incubation procedure as described above. The enzyme not covalently bound was finally removed by alternate washings with 5 mL of either 0.1 M borate buffer (pH 8.3, containing 0.5 M NaCl) or 0.1 M acetate buffer (pH 4.5, containing 0.5 M NaCl), each at least five times (aminopeptidase M was entirely washed with the pH 8.3 buffer, since application of the pH 4.5 buffer led to an almost total loss of activity).

The UV_{280nm} absorption of the collected eluates after addition of the enzyme solution to the Sepharose gel was measured by using a Lambda 2 UV/VIS spectrophotometer (Perkin-Elmer, Überlingen, Germany). The immobilized amount of enzyme was determined by subtraction of the eluted amount of enzyme (calculated from UV_{280nm} absorption against blank solutions) from the total applied amount.

Using the procedure described above, 8.1 mg of catalase, 5.3 mg of Pronase E, and 3.3 mg of aminopeptidase M were bound by 1 mL of Sepharose (about 0.3 g of dry weight). The immobilizates exhibited an activity of 2500 units/mL for catalase, 3.3 units/mL for aminopeptidase M, and 6.4 units/mL (aminopeptidase units, substrate Leu *p*-nitroanilide) for Pronase E. The immobilizates were stored at 4 °C in borate buffer, pH 8.3, containing 0.1% sodium azide and 0.5 M NaCl.

Oxidation with Hydrogen Peroxide. *Free Trp, Trp-Containing Peptides, and Lysozyme.* The model solution contained 2 mmol of Trp/L, either unbound Trp or in peptides (AWA, LWL, AAWAA, LWM) or in lysozyme using 0.1 M borate buffer (pH 8.3 and 6.3). For oxidation 40 μL of H_2O_2 (30%) was added to 2 mL of the sample either once at the beginning of the incubation or every hour. For control samples H_2O_2 was replaced by water. The oxidation was performed for 2, 4, and 6 h in 4 mL amber screw-cap vials at 30 and 40 °C. After incubation, the reaction mixture was transferred into a 10 mL column and the reaction was interrupted by 30 min of treatment with 100 μL of immobilized catalase (about 250 units). The absence of H_2O_2 was subsequently checked by potassium iodide/starch test. In the case of lysozyme 2−3 drops of octanol was added prior to catalase treatment to prevent foaming. Since the oxidized lysozyme was partly coagulated, 100 μL of catalase solution (1 mg of catalase/mL

Table 2. HPLC Conditions

equipment	Merck L 6200 pump, Merck L-4000 UV detector, Merck F 1080 programmable fluorescence detector (Darmstadt, Germany); Shimadzu RD-530 fluorescence detector (Duisburg Germany);[a] SCPA Chromstar software (Bremen, Germany); Waters 994 programmable photodiode array detector (Milford, MA)				
mobile phase A	0.1% (v/v) TFA in water				
mobile phase B	methanol				
mobile phase C	acetonitrile				
injection volume	20 μL (Rheodyne valve)				
	HPLC I (Separation of Trp Degradation Compounds)				
column	Nucleosil 120 3-C_{18}	250 × 4 mm	precolumn	20 × 4 mm	
elution profile	min	A (%)	B (%)	C (%)	gradient
	0	95	5	0	linear
	10	86	14	0	linear
	40	46	14	40	linear
	42	26	14	60	linear
	46	95	5	0	step
flow rate	1.0 mL/min				
temp	35 °C				
detection	fluorescence program				
		Ex (nm)	Em (nm)		
	0−20 min	230	342		
	20−46 min	280	335		
	UV, 260 nm				
	HPLC II (Separation of Trp-Containing Peptides)				
column	Spherisorb ODS 2 (C_{18}, 5 μm)	250 × 4 mm	precolumn	20 × 4 mm	
elution	83% A, 17% C, isocratic				
flow rate	1.5 mL/min				
temp	ambient				
detection	fluorescence	Ex 280 nm	Em 356 nm		

[a] Only for HPLC II.

of borate buffer, pH 8.3; about 500 units) was added instead of the immobilizate.

Trp Oxidation Compounds. Trp oxidation compounds were dissolved in 0.1 M borate buffer, pH 8.3, to a final concentration of 10 mg/L (5-OH-Trp, Kyn, PIC A) or 30 mg/L (Oia, DiOia). Two milliliters of the solution was transferred into a 4 mL amber vial, 40 μL of hydrogen peroxide (30%) was added, and the mixture was stirred during incubation for 1 h at 40 °C. After acidification with 200 μL of 2 M acetic acid, the solution was directly used for chromatography (Table 2, HPLC I).

Hydrolysis Procedures. *A. Hydrolysis of Peptides by Immobilized Enzymes.* After catalase treatment and addition of 100 μL of 1-methyltryptophan (1-Me-Trp, 1 mg/L) as internal standard (IS), both oxidized and control samples were hydrolyzed by immobilized Pronase E or aminopeptidase M. Therefore, sample solutions were treated with 100 μL of immobilized enzyme (Pronase E, 0.6 unit; aminopeptidase M, 0.3 unit; both determined as aminopeptidase units) overnight (16 h). Finally, solutions were sucked through the column into a vial and acidified with 200 μL of 2 M acetic acid prior to HPLC analysis (Table 2, HPLC I).

For analysis of the imino acid PIC A, the hydrolysate was additionally incubated for 3 h after addition of 100 μL of prolidase solution (preincubation solution, 0.5 mg of prolidase in 1 mL of borate buffer containing 25 mM $MnCl_2$ and 1 mM gluthathione to develop enzyme activity as recommended by Serva).

1. Stability of Trp and Trp Oxidation Compounds during Pronase E Hydrolysis. Trp and Trp oxidation compounds were dissolved concurrently in bidistilled water to a final concentration of 10 mg/L (Trp, 5-OH-Trp, Kyn, PIC A) or 20 mg/L (Oia, DiOia). After addition of 1-Me-Trp (IS), the solution was submitted to the hydrolysis procedure as described above.

B. Hydrolysis of Lysozyme. 1. Enzymatic Hydrolysis. Enzymatic hydrolysis was performed as described by Schmitz et al. (1976). After oxidation and catalase treatment, the solution was successively incubated at 40 °C with four different enzymes while stirring. After adjustment to pH 2.0 with 1 M HCl, 100 μL of pepsin solution (2 mg/mL 0.01 M HCl) was added and the mixture incubated for 24 h. For further incubations the pH was adjusted to 8.3 with 1 M NaOH. Thereafter, the solution was incubated for 24 h after addition of 100 μL of Pronase E solution (2 mg/mL borate buffer, pH 8.3) and 24 h after addition of 100 μL of aminopeptidase M solution (2 mg/mL borate buffer, pH 8.3). All Trp degradation products except PIC A were determined at this step of hydrolysis by HPLC I (Table 2) and related to the Trp content determined from the alkaline hydrolyzate of the control sample (reference method; BGVV, 1989).

For analysis of PIC A, the hydrolysate was additionally incubated for 24 h after addition of 100 μL of prolidase solution (preincubation solution, 0.5 mg of prolidase/mL).

2. Stability of Trp and Trp Oxidation Compounds during Enzymatic Hydrolysis. Trp and Trp oxidation compounds were dissolved concurrently in bidistilled water to a final concentration of 100 mg/L (Trp, 5-OH-Trp, Kyn, PIC A) or 200 mg/L (Oia, DiOia). The solution was submitted to the hydrolysis procedure as described above and the loss of oxidation compounds related to the Trp content set to 100% at each phase of hydrolysis.

3. HCl Hydrolysis. After catalase treatment, oxidized and control lysozyme samples were transferred into 10 mL Pyrex vials and hydrolyzed under acid conditions (BGVV, 1986). Two milliliters of HCl (37%) was added (final concentration = 6 M HCl), and the resulting solution was subsequently agitated on a vortex mixer. The vials were transferred into a flat flange reaction vessel [155 × 75 mm (d × h), PTFE-coated O-ring] (Schott, Mainz, Germany), which was evacuated (10 mbar) and purged with nitrogen twice. Finally, hydrolysis was performed in the evacuated vessel for 24 h at 110 °C in a drying oven. After aeration of the vessel and cooling, the acid solution was transferred into a 5 mL volumetric flask and diluted to volume with bidistilled water. Two hundred microliters of the resulting solution was filled into a 1 mL vial and brought to dryness using a vacuum centrifuge. The residue was dissolved with 200 μL of 0.1% aqueous TFA and chromatographed as described in Table 2 (HPLC I). The determined amounts of Trp degradation compounds were related to the Trp content determined from the alkaline hydrolysate of the control sample (reference method; BGVV, 1989).

4. Stability of Trp and Trp Oxidation Compounds during HCl Hydrolysis. Trp and Trp oxidation compounds were separately dissolved in bidistilled water to a final concentration

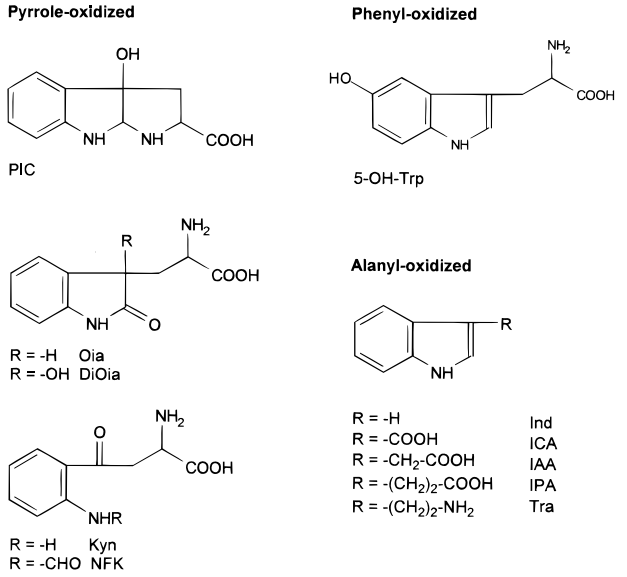

Figure 1. Chemical structures and classification of examined Trp degradation compounds. For abbreviations see Table 1.

of 1 g/L (Trp, 5-OH-Trp, Kyn, PIC A) or 2 g/L (Oia, DiOia). Tyrosine (Tyr) was used as internal standard (1 g/L). Two hundred fifty microliters of a single Trp-derivative containing solution, 250 μL of Tyr solution (IS), and 1.5 mL of 25% HCl were filled into a 4 mL amber vial, mixed thoroughly on a vortex mixer, and submitted to the acid hydrolysis procedure as described above. Losses of compounds during hydrolysis were corrected by IS calculation.

5. Alkaline Hydrolysis. After catalase treatment, oxidized and control lysozyme samples (about 2 mL) were transferred into 5 mL cryovials (polypropylene, Nalgene) and hydrolyzed under alkaline conditions (BGVV, 1989). After addition of 200 μL of 5-methyltryptophan (5-Me-Trp) solution (1 mg/mL) (IS) and 2 mL of 8.4 M NaOH (final concentration = 4.2 M) and subsequent agitation on a vortex mixer, the vials were transferred into the flat flange reaction vessel, which was evacuated (10 mbar) and purged with nitrogen twice. Finally, hydrolysis was performed in the evacuated vessel for 20 h at 110 °C. After aeration and cooling, the alkaline solution was transferred into a 10 mL volumetric flask, acidified by addition of 2.2 mL of 85% phosphoric acid (pH 2), and diluted to volume with bidistilled water. The resulting solutions were chromatographed as described in Table 2 (HPLC I). The Trp losses during hydrolysis were corrected by IS calculation.

6. Stability of Trp and Trp Oxidation Compounds during Alkaline Treatment. Trp and Trp oxidation compounds were separately dissolved in bidistilled water to a final concentration of 1 g/L (Trp, 5-OH-Trp, Kyn, PIC A) or 2 g/L (Oia, DiOia). Two hundred fifty microliters of a single Trp derivative containing solution, 250 μL of 5-Me-Trp solution (IS), 500 μL of bidistilled water, and 1.0 mL of 8.4 M NaOH were filled into a 5 mL cryovial, mixed thoroughly on a vortex mixer, and submitted to the alkaline hydrolysis procedure as described above.

RESULTS AND DISCUSSION

Analytical Features. Sixteen Trp-related compounds (Table 1) that can be classified either in pyrrole-, phenyl- or alanyl-moiety oxidized products (Figure 1) were separated in a single RP-HPLC run (Table 2, HPLC I). Confirmation of substance peaks was done by simultaneous UV and fluorescence detection connecting the monitors in series (Figure 2). Photodiode array (PDA) detection was performed for selected samples for further confirmation of the peak identity. DiOia and Oia gave two peaks (Figure 2a, peaks 3 and 5) or a double peak (Figure 2a, peaks 9 and 10), respectively,

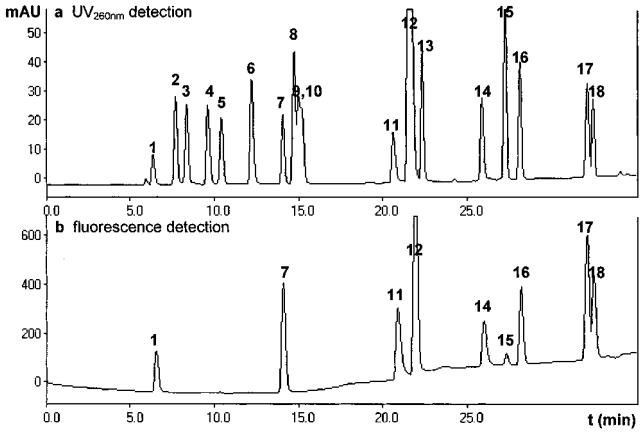

Figure 2. RP-HPLC separation of reference compounds: (a) UV (260 nm); concentrations: Trp (12), 240 mg/L; PIC A (1), 120 mg/L; DiOia A/B (3, 5), Oia A/B (9, 10), 40 mg/L; others, 20 mg/L. (b) Fluorescence program; concentrations: Trp, 3.8 mg/L; PIC A, 1.9 mg/L; others, 0.3 mg/L. See Table 1 for peak identification and Table 2 (HPLC I) for chromatographic conditions.

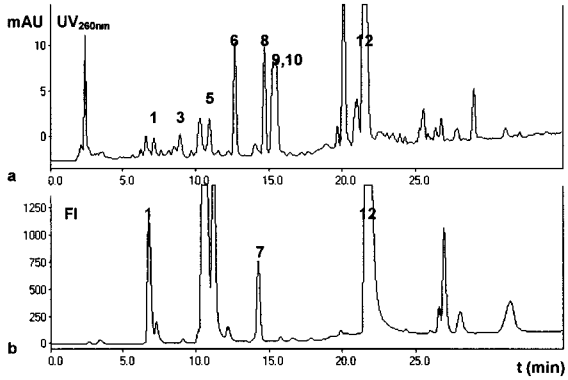

Figure 3. RP-HPLC chromatogram of hydrogen peroxide treated Trp solution: (a) UV (260 nm); (b) fluorescence program. For peak identification see Table 1, and for chromatographic conditions see Table 2 (HPLC I). Hydrogen peroxide treatment consisted of hourly addition of H_2O_2 and incubation at 40 °C for 6 h at pH 8.3.

which refer to their diastereomers (*R*,*S* and *S*,*R*; *R*,*R* and *S*,*S*). The diastereomers of Oia are interconverted by enol tautomerism (Labroo and Cohen, 1990) during chromatography. For that reason, separation was optimized to elute Oia diastereomers as a double peak (Figure 2a, peaks 9 and 10). Under the chosen conditions anthranilic acid (2-aminobenzoic acid, ABA), another Trp oxidation compound, coelutes with NFK (Figure 2a, peak 8). Since ABA generates a strong fluorescence at 325/425 nm (Ex/Em), whereas the fluorescence of NFK is only weak, several samples of 6 h incubated Trp were monitored for the formation of ABA. Its content could be estimated to be <0.1% (moles per 100 mol of Trp). Furthermore, xanthurenic acid (XA) could not be separated from kynurenic acid (KA) (peak 13) by the chosen elution profile. However, neither XA nor KA could be detected in the examined model solutions. The detection of non-UV-active Trp degradation compounds was put aside because this would have made necessary a pre- or postcolumn derivatization to yield detectable products.

Oxidation of Free Tryptophan. The losses of Trp in the model system extend from 3.2 to 68%, dependent on the conditions chosen (Figure 4). The amount of H_2O_2 added, along with the incubation time and tem-

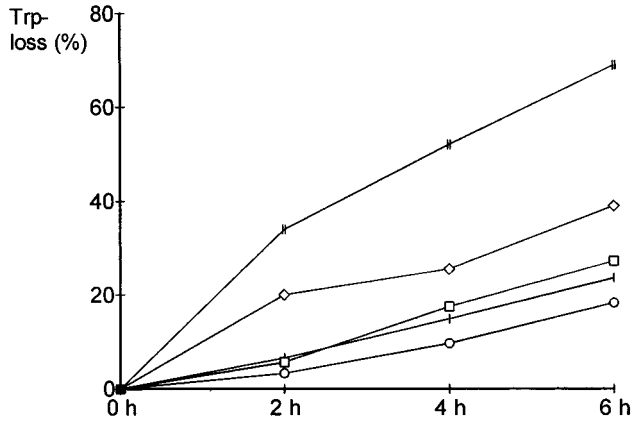

Figure 4. Degradation of tryptophan in model solutions using hydrogen peroxide as oxidative agent at various conditions: ||, 40 °C, pH 8.3, H_2O_2 hourly; ◇, 30 °C, pH 8.3, H_2O_2 hourly; □, 40 °C, pH 8.3, 1 × H_2O_2; |, 40 °C, pH 6.3, 1 × H_2O_2; ○, 30 °C, pH 6.3, 1 × H_2O_2.

Table 3. Oxidation of Trp Degradation Compounds

substance	loss after oxidation[a] (%)	identified degradation products
5-OH-Trp	74 ($n = 2$)[b]	
PIC A	40 ($n = 3$)	Kyn, NFK
Oia	70 ($n = 5$)	DiOia, Kyn
DiOia	70 ($n = 2$)	Kyn
Kyn	42 ($n = 3$)	

[a] One hour incubation at 40 °C, pH 8.3. [b] *n*, number of determinations.

perature, showed the highest influence on the degradation of Trp. The pH value in the examined range and the buffer substance (data not shown) proved to be of minor importance. PIC A, DiOia, Kyn, 5-OH-Trp, NFK, and Oia could be detected in almost all solutions (Figure 3) and their identity confirmed by PDA detector and comparison of the obtained spectra with those derived from a separation of reference substances.

The formation of compounds with intact indolyl and oxidized alanyl moiety such as Ind, IAA, and ICA (Figure 1) could not be proved (practical limit of detection = 0.1 mol/100 mol of Trp), indicating that the indole ring is more susceptible to oxidation than the alanyl moiety. The oxidative attack happened prevalently at the pyrrol ring of Trp. All accessible pyrrole oxidized reference substances (PIC-A, DiOia, Oia, NFK, and Kyn) were found and proved to represent the major group of degradation compounds derived by oxidation. 5-OH-Trp was the only identified substance with an oxidized phenyl moiety (Figure 5).

However, only ~20% of the determined Trp loss could be elucidated by the determined degradation compounds. Some substance peaks (Figure 3) remained unidentified and non-UV-detectable compounds remained disregarded.

Hydrogen peroxide treatment of the identified degradation compounds indicated that they are even more susceptible to oxidation than Trp is itself (Table 3). Whereas under similar conditions 5−15% loss of Trp could be determined after 1 h of incubation time, the oxidation compounds exhibit a 42−74% loss. Identification of degradation compounds was successful for PIC A, Oia, and DiOia (Table 3). Formation of NFK from PIC during oxidation had already been reported by Savige (1975) and Nakagawa et al. (1981); the latter identified 1,2,3,3a,8,8a-hexahydro-3a-hydroperoxypyrroloindole-2-carboxylic acid as intermediate. During oxidation of Kyn no new substance peaks could be detected at UV (260 nm), indicating that the phenyl moiety is cleaved.

When weak oxidative conditions are applied to the oxidation of Trp, PIC A and Oia both increase during the first 6 h of oxidative treatment (Figure 5a) while PIC-A decreases under strong oxidative conditions (Figure 5b) and the amount of Kyn, NFK, and DiOia among the identified degradation compounds is higher. These results indicate that PIC and Oia represent primary Trp oxidation compounds (uncleaved pyrrole), and Kyn and NFK (pyrrole cleaved) represent secondary Trp oxidation compounds, while DiOia occupies a position between them.

Oxidation of Trp Residues in Peptides. *A. Hydrolysis of Peptides.* Investigations were focused on the identified degradation compounds of free Trp with intact alanyl moiety. Various small peptides were oxidized and hydrolyzed enzymatically to determine the released compounds. Pronase E and aminopeptidase M were both immobilized to use the enzymes several times, both in view of the price of aminopeptidase M and to avoid the load of enzymes on the HPLC column. Although the velocity of hydrolysis proved to rise with increasing temperature (about 10%/10 °C), incubations were performed at room temperature to avoid temperature-induced degradation processes and to make use of an easier handling. When Pronase E was used for hydrolysis of AWA, it proved to exhibit an optimum pH at 8.3 and a much higher activity in borate than in phosphate buffer. Both enzymes proved to cleave the peptides after incubation overnight (16 h) for >98% Trp recovery. Monitoring the hydrolysis of AWA with HPLC II (Table 2) permitted small amounts of AW to be detected as intermediate when Pronase E was used for digestion. This reveals that the Pronase E preparation exhibited a minor carboxypeptidase activity, which corresponds to its characterization by Narahashi and Yanagita (1967), whereas during aminopeptidase M digestion WA was the only intermediate. In view of the lower price, most digestions were carried out with immobilized Pronase E.

To prove that immobilized Pronase E is suitable for hydrolysis of peptides containing nonphysiological Trp degradation compounds, oxidized AWA was chromatographed prior to and after Pronase E treatment (Figure 6). If the chromatograms are compared with either UV or fluorescence detection, it can be shown that the AWA peak (Figure 6, peak XII) and most of the peaks related to peptides containing Trp degradation compounds completely disappeared, indicating successful hydrolysis. Using reference substances, the stability of Trp degradation compounds during enzymatic hydrolysis was tested. Whereas PIC-A, DiOia, Kyn, and 5-OH-Trp proved to be stable, NFK and especially Oia were partly destroyed (Table 4). 1-Me-Trp (IS) proved to be less stable than Trp itself. For that reason calculation using the IS was omitted and substance losses were calculated from the determined Trp content of control samples.

B. Trp Degradation in Peptides. Four different peptides were submitted to hydrogen peroxide oxidation. Prior to hydrolysis, peptide samples were examined with HPLC II (Table 2) to determine the loss of the intact peptide. With regard to AWA, LWL, and AAWAA, peptide loss can predominantly be traced back to damage of Trp in the peptide (Table 5). The peptide recovery

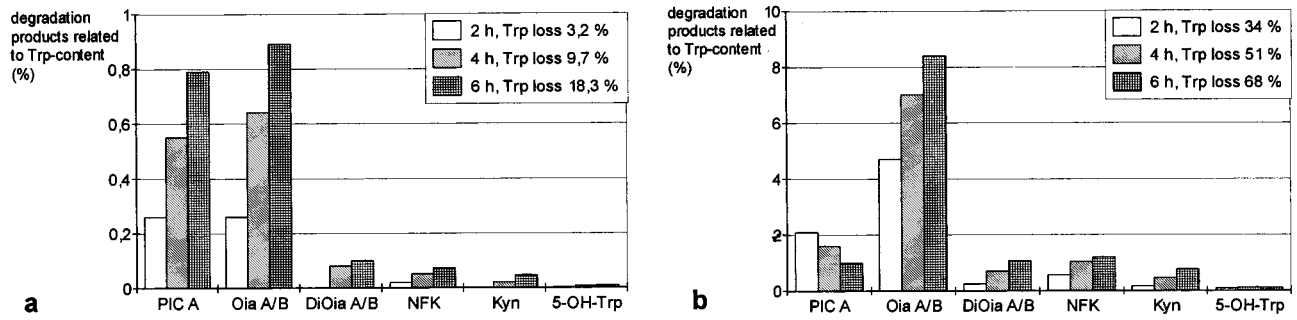

Figure 5. Pattern of tryptophan degradation compounds derived by oxidation of unbound tryptophan by hydrogen peroxide: (a) single addition of H_2O_2 and incubation at 30 °C and pH 6.3; (b) hourly addition of H_2O_2 and incubation at 40 °C and pH 8.3.

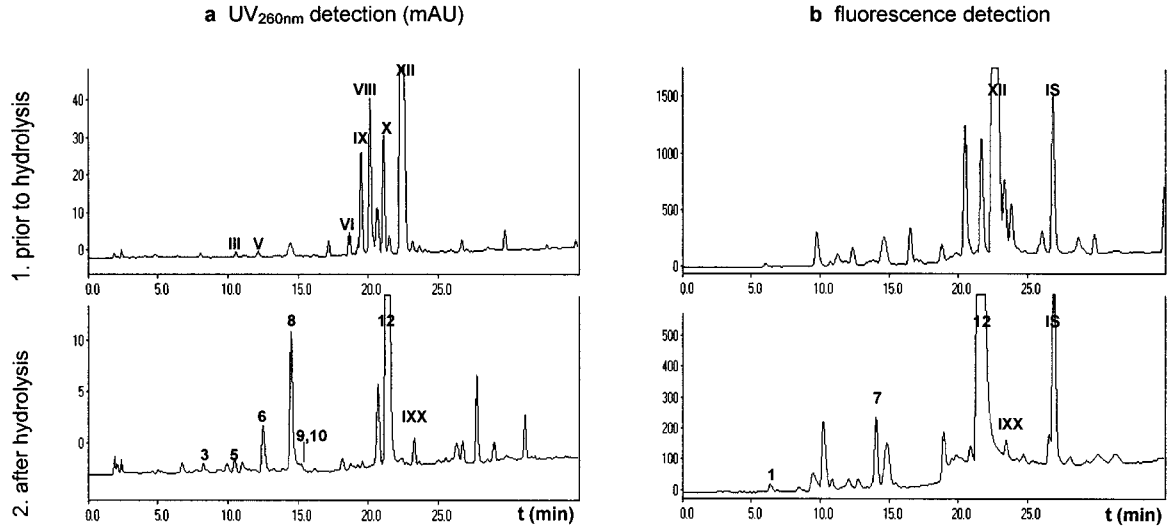

Figure 6. RP-HPLC of AWA after oxidation. Peak identification: for Arabic numbers see Table 1; III, V: H-Ala-DiOia-Ala-OH; VI, H-Ala-Kyn-Ala-OH; VIII, H-Ala-NFK-Ala-OH; IX, X, H-Ala-Oia-Ala-OH; XII, AWA; IXX, AW; IS, 1-Me-Trp. (a) UV_{260nm} detection: (1) prior to hydrolysis; (2) after hydrolysis. (b) Fluorescence detection: (1) prior to hydrolysis; (2) after hydolysis. For chromatographic conditions see Table 2 (HPLC I). Oxidation was brought about by hourly addition of H_2O_2 and incubation at 40 °C for 6 h at pH 8.3.

Table 4. Stability of Tryptophan Oxidation Compounds during Enzymatic Hydrolysis by Immobilized Pronase E[a]

substance	recovery[b] (%)	recovery related to Trp (=100%)
PIC A	88	96
DiOia	92	100
Kyn	93	101
5-OH-Trp	91	99
NFK	69	75
Oia	58	63
Trp	92	100
1-Me-Trp	86	93

[a] Sixteen hour incubation at room temperature with immobilized Pronase E (0.6 unit). [b] Mean of duplicate determinations.

Table 5. Rate of Tryptophan Oxidation[a] in Different Peptide Model Systems

peptide	peptide recovery[b] (%) after oxidative treatment[d]	Trp recovery[c] (%) after oxidative treatment[d]
AWA	48	50
LWL	46	55
AAWAA	30	32
LWM	35	76

[a] Hourly addition of H_2O_2, incubation for 6 h at 40 °C and pH 8.3. [b] After catalase treatment, determined with HPLC II. [c] After catalase treatment and enzymatic hydrolysis, determined with HPLC I. [d] Mean of duplicate determinations.

corresponds to the Trp recovery after hydrolysis. Contrasting LWM showed a remarkable difference between peptide and Trp loss. This is due to the fact that Met is even more susceptible to oxidation than is Trp (Nielsen et al., 1985a).

C. Identification of Degradation Products. The patterns of identified Trp degradation compounds were similar throughout all of the examined peptides. The substance peaks of the oxidized peptides could be identified using PDA detection and the spectra of available non-peptide-bound reference substances (Figure 6a). Unlike unbound Oia, the diastereomers of H-Ala-Oia-Ala-OH are not interconverted by keto−enol tautomerism during chromatography and are separated in two distinct peaks (Figure 6a, peaks IX and X). The tautomerism reaction might be hindered by the neighboring amino acids. Itakura et al. (1994) reported similar chromatographic behavior of *N*-(*tert*-butoxycarbonyl)-Oia. With regard to the loss of Oia during Pronase E incubation (Table 4), the Oia formation was calculated from the oxidized peptide solutions prior to hydrolysis. Oia and NFK were shown to be the main degradation products, followed by Kyn and DiOia (Figure 7). Traces of PIC A and 5-OH-Trp could be detected only by their fluorescence and identified by retention time.

These results are consistent with those of Itakura et al. (1994), who demonstrated the formation of NFK and Oia in several Trp-containing peptides after hypoxanthine/xanthine oxidase/Fe(III)/EDTA and H_2O_2/peroxidase treatment, and Holt et al. (1977), who detected

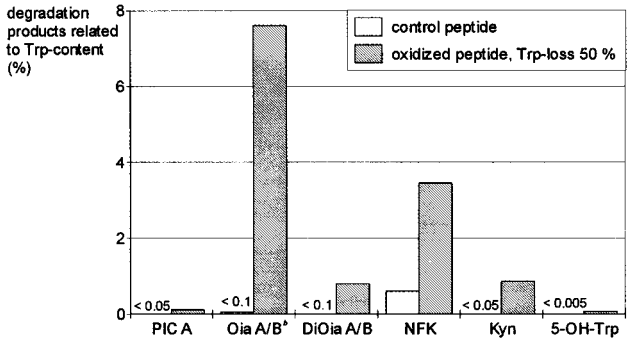

Figure 7. Rate of the formation of Trp degradation compounds in oxidized AWA (mean of triplicate determinations). Oia was determined by RP-HPLC prior to hydrolysis (peak identification by PDA detector). Oxidation was brought about by hourly addition of H_2O_2 and incubation at 40 °C for 6 h at pH 8.3.

Table 6. Stability of Tryptophan Oxidation Compounds during Acid Hydrolysis

substance	recovery[a] (%)	identified degradation products	observations
Trp	81	Oia (4%)[b]	
5-OH-Trp	50		red-brown discoloration
PIC A	<0.1	Oia (88%)[b]	
Oia	87		
DiOia	1.8		yellow-brown discoloration
Kyn	99		

[a] Mean of duplicate determinations. [b] Moles per 100 mol of the initial compound.

NFK, Oia, and Asp after photooxidation of Ala-Gly-Trp-Leu.

The greatest difference between the pattern of degradation compounds of free Trp and Trp residues in peptides is the higher proportion of NFK and Kyn and the lower proportion of PIC A. This may be explained by the involvement of the α-amino N in the formation of PIC, which has already been substituted by the peptide bond to Ala. Uchida et al. (1990) have identified *N*-(*tert*-butoxycarbonyl)-PIC diastereomers after the oxygenation of *N*-(*tert*-butoxycarbonyl)-Trp, indicating the possibility of the formation of PIC in peptides.

Similar to the oxidation of unbound Trp, only 20% of the determined Trp loss could be elucidated by the determined degradation compounds. Some substance peaks (Figure 6a,b) remained unidentified and nonfluorescent or non-UV-detectable compounds were disregarded.

Oxidation of Trp Residues in Proteins. *A. Hydrolysis of Lysozyme.* All degradation compounds that were detected after oxidation of Trp residues in peptides were examined for their stability during standard acid (6 M HCl) and alkaline (4.2 M NaOH) hydrolysis procedures (BGVV, 1986, 1989) as well as an enzymatic procedure described by Schmitz et al. (1976).

Acid (6 M HCl) hydrolysis is commonly used for the determination of amino acids in proteins (BGVV, 1986). Using Tyr as internal standard, Kyn and Oia proved to be stable during acid hydrolysis (Table 6). The Trp loss amounted only to 19%, and Oia was detected as an artifact during hydrolysis. PIC A was established to be almost completely converted to Oia under these conditions, which had been mentioned by Savige (1975) for similar conditions.

Alkaline hydrolysis (4.2 M NaOH) using 5-Me-Trp as IS is recommended for the determination of Trp in proteins since Trp is not stable during acid hydrolysis (BGVV, 1989). Trp and DiOia were shown to be stable, whereas >95% of the initial amount of 5-OH-Trp and Kyn was destroyed and PIC A and Oia were partly converted into DiOia (Table 7).

Table 7. Stability of Tryptophan Oxidation Compounds during Alkaline Hydrolysis

substance	recovery[a] (%)	identified degradation products	observations
Trp	98		
5-OH-Trp	1.5		no formation of fluorescent peaks, red color
PIC A	7.5	DiOia (4%)[c]	formation of a new, strong fluorescent substance, pale yellow
Oia	8.4	DiOia (16%)[c]	
DiOia	121[b]		
Kyn	<0.1		

[a] Mean of duplicate determinations calculated with 5-Me-Trp as IS. [b] DiOia was more stable toward alkaline hydrolysis than IS 5-Me-Trp. [c] Moles per 100 mol of the initial compound.

Table 8. Stability of Tryptophan Oxidation Compounds during Enzymatic Hydrolysis (Series of Four Enzyme Incubations)

	recovery related to Trp (=100%) (%)[a]			
substance	pepsin	Pronase E	AMP	prolidase
5-OH-Trp	104	85	66	<0.1
PIC A	107	62	57	56
Oia	98	<4	<4	<4
DiOia	104	117	115	90
NFK	<1	<1	<1	<1
Kyn[b]	90	73	59	58
Trp	100	100	100	100

[a] Mean of duplicate determinations. [b] Sum of initial amount of NFK and Kyn.

For the release of labile amino acids, frequently a total enzymatic digestion is applied. Since Schmitz et al. (1976) used four enzymes in series, recovery of Trp degradation compounds was determined after each incubation step (Table 8). Surprisingly, 5-OH-Trp was destroyed during prolidase incubation. Oia proved to be unstable during Pronase E incubation. In comparison to the hydrolysis with immobilized Pronase E, this might be traced back to the higher temperature and the extended incubation time. NFK was hydrolyzed yielding Kyn during pepsin incubation at pH 2.0.

B. Degradation of Trp. The oxidation rate of Trp in lysozyme determined after alkaline hydrolysis was much slower than the rate of free Trp or Trp in peptides (Figure 8). This may be due to a steric hindrance of oxidative attack by the protein conformation, since the hydrophobic Trp residues are in the interior of the molecule, and to coagulation of lysozyme that occurred during the oxidative treatment. Additionally, Met, Lys, and His residues are likewise reported to be susceptible to oxidation (Nielsen et al., 1985d) and may compete with Trp residues for the oxidizing radicals.

C. Identification of Degradation Products. Control samples of lysozyme revealed that several of the examined Trp degradation compounds may occur as artifacts of hydrolysis procedures (Table 9). Markedly, ~28% of the initial Trp content of lysozyme converted to Oia during acid hydrolysis, although it was performed in vacuo. This illustrates that the studies on stability of single compounds (Table 6) can only partly be transferred to the protein matrix.

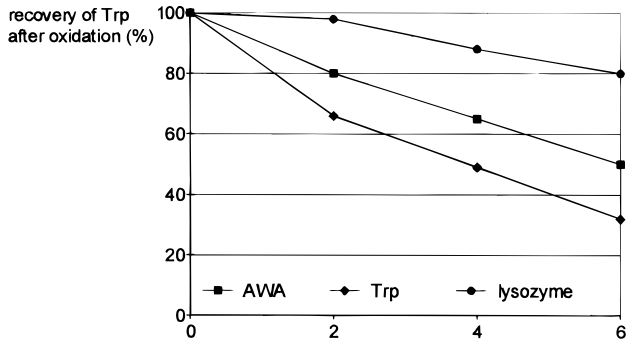

Figure 8. Comparison of the degradation rate of free Trp and Trp residues in AWA and lysozyme. The oxidation procedure included hourly addition of H_2O_2 and incubation at 40 °C for 6 h at pH 8.3. Trp loss in AWA was determined after enzymatic hydrolysis. Trp loss in lysozyme was determined after alkaline hydrolysis.

Table 9. Trp Degradation Compounds in Hydrogen Peroxide Treated Lysozyme[a]

	degradation products related to Trp-content (mol/100 mol of Trp)					
hydrolysis	PIC A	DiOia A/B	Kyn/ NFK	Oia A/B	5-OH-Trp	Trp
enzymatic						
control	0.1	0.2	0.2	–[b]	0.15	100[f]
oxidized[a]	<0.1	0.5	3.2	–	0.09	64
acid						
control	–	–	<0.1	28.2[d]	0.008	41
oxidized[a]	–	–	2.2	35.3[d]	0.05[c]	7.6
alkaline						
control	<0.1[c]	<0.5[e]	–	<0.1[c]	–	100[f]
oxidized[a]	0.4[c]	4.6[e]	–	1.8[c]	–	79

[a] Hourly addition of H_2O_2, incubation for 6 h at 40 °C, pH 8.3. [b] Not stable under conditions of hydrolysis (>95% loss). [c] Under conditions of hydrolysis the compound is partially destroyed (>50% loss). [d] Under conditions of hydrolysis Oia is formed from Trp. [e] Under conditions of hydrolysis DiOia is formed from PIC and Oia. [f] Trp value of control hydolysis was set at 100%.

The sum of initial NFK and Kyn could be shown to be determined after acid hydrolysis since no other compound could be shown to be converted into Kyn under these conditions (Table 6). Oia and DiOia could be detected in oxidized lysozyme; however, their determination was not possible. Oia is formed from Trp during acid hydrolysis but is partly unstable during alkaline hydrolysis, whereas DiOia could be formed from Oia during alkaline hydrolysis (Table 7).

PIC A and 5-OH-Trp could not definitely be detected in oxidized lysozyme since control values were in the same range as sample values. The formation of PIC A is likely to be prevented from the N^{α}-peptide bond of Trp in proteins along with steric effects.

CONCLUSIONS

The formation of 16 possible Trp degradation compounds was examined for the first time in hydrogen peroxide treated solutions of Trp, Trp-containing peptides, and lysozyme.

The results indicate that oxidative attack on Trp occurs first on the pyrrole ring and secondarily on the phenyl moiety. These findings seem to be transferrable to other oxidative processes since the formation of the same Trp-derived oxidation compounds is reported during lipid autoxidation (NFK, Kyn, DiOia) (Yong et al., 1980), photooxidation (Kyn, NFK) (Kanner and Fennema, 1987), and γ-irradiation (Kyn, NFK, Oia, DiOia, 5-OH-Trp) (Wickern et al., 1996).

Immobilized Pronase E and aminopeptidase M facilitate almost complete hydrolysis of either native or oxidized short-chain ***peptides***. Besides Kyn and NFK as frequently mentioned Trp degradation compound, Oia, DiOia, PIC A, and 5-OH-Trp were proved to be formed in Trp-containing peptides under oxidative conditions. Oia was established to be the main degradation compound of Trp in peptides.

None of the applied hydrolysis procedures for ***proteins*** proved to be suitable for all predicted Trp degradation compounds that were identified in oxidized peptides. The sum of NFK and Kyn was shown to be determined after acid hydrolysis, since NFK rapidly undergoes hydrolysis under acid conditions. Since Oia and DiOia are either destroyed or formed during hydrolysis, only detection but no determination was possible in oxidized lysozyme. For PIC A and 5-OH-Trp no reliable detection was achieved considering comparable amounts in samples and controls. The identification of NFK, Oia, and DiOia in oxidized lysozyme is a first step in an advanced description of the oxidative degradation of Trp in proteins.

Perhaps a single- (Garcia and Baxter, 1992; Kim and Haering, 1994) or two-step (Hauck, 1990) enzymatic digestion could improve the results if it were sufficient to release the desired degradation products. Choice of proteases and incubation conditions (pH, temperature, time) have to be empirically established for the individual analytical aim. In contrast, for nutritional evaluations the chosen enzymatic hydrolysis may give some information about the stability of the examined compounds during gastrointestinal passage.

Little is known about the physiological properties of Trp degradation compounds. Studies using rat assays revealed that Trp oxidation products obtained by hydrogen peroxide treatment of free Trp were not utilized for protein synthesis (Weck et al., 1987) and that lipid-oxidized whey protein showed losses of bioavailable Trp (Nielsen et al., 1985b). Oia and Kyn are reported to be inhibitors of tryptophanase (Snell, 1975), and DiOia is an inhibitor of tryptophanase and tryptophan-synthetase (Labroo and Cohen, 1990). Oxidation products of Trp have been associated with the occurrence of cataractous lenses (Elderfield and Truscott, 1996).

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