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CHEMICAL CLEAVAGE OF PEPTIDE BOND AT TRYPTOPHAN RESIDUE : AN IMPROVED METHOD[1)]

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An improved method for selective chemical cleavage of the tryptophyl bond according to a principle proposed previously is described. By the present method, tryptophyl bonds in several synthetic oligopeptides were cleaved nearly quantitatively.

For selective chemical cleavage of tryptophyl bonds in peptides or proteins, various reactions have been proposed. The major principle for this purpose involves oxidation of the indole nucleus of tryptophan. N-Bromosuccinimide[2)] or its derivatives[3) 4)] were used as a cleaving agent for direct cleavage of the peptide linkage in which tryptophan was concerned. Periodate ions were also used for fragmentation of myoglobin.[5)]

Recently we have established a new method for selective chemical cleavage of the tryptophyl bond. It consists of successive reactions, ozonization of tryptophan-containing peptides and subsequent reaction of the oxidation product with hydrazine.[6)] The second reaction which cleaves the peptide bond relating to the modified tryptophyl residue was studied in detail using benzyloxy-carbonyl-L-alanyl-N'-formyl-L-kynurenyl-L-leucine, as a model. It was found that leucine was released almost quantitatively under mild conditions.[7)] However, upon application of the cleavage reaction to several synthetic tryptophyl amino acids, the over-all cleavage of the tryptophyl bond was less effective. This insufficient cleavage was obviously due to incomplete conversion of the tryptophan residue into N'-formylkynurenine. Then we studied quantitative formation of kynurenine or its N'-formyl derivative from tryptophan by ozonization, and established an improved method for chemical cleavage of the tryptophyl bond.[8)]

When acetyl-L-tryptophan ethyl ester was ozonized in absolute ethylacetate at low temperature around -70°C and the ozonization mixture was reduced with dimethylsulfide,[12)] acetyl-N'-formyl-L-kynurenine ethyl ester[13)] was obtained more effectively than by ozonization of the tryptophan derivative with or without resorcinol in 98-100% formic acid.[14)] But a small amount of red-colored oily by-products was always accompanied. Moreover, ozonization of acetyl-L-tryptophan ethyl ester in absolute methanol in place of ethylacetate gave much better results : acetyl-N'-formyl-L-kynurenine ethyl ester was formed almost quantitatively. Then tryptophan-peptides were oxidized under the same conditions as the first step of the cleavage reaction. An improved method is thus described below. A tryptophan-peptide derivative (10 μmols) was dissolved in absolute methanol (2 ml) and ozone (2-7 μmols/0.5 l oxygen/min.) was bubbled into the methanol solution under cooling at about -70°C (a dry ice-acetone bath). Bubbling was interrupted when a shoulder at 292 nm of the characteristic absorption of tryptophan disappeared almost completely. An aliquot (0.5 ml) withdrawn was immediately mixed with pre-cooled 2% dimethylsulfide in absolute methanol (0.5 ml) and the methanol solution was kept at about -70°C for half an hour. Then the methanol solution was concentrated under reduced pressure to sirup at room temperature, which was dissolved in a glacial acetic acid solution (2 ml)

containing hydrazine (1.6 M) to submit to the cleavage reaction of the kynurenyl bond. The cleavage reaction was carried out at 25-26°C for 24 hours and the cleavage yield was then estimated both by analyzing amino acid released and by measuring absorbance at 353 nm due to the tetrahydropyridazone derivative formed, as described previously.[7] For determination of oxidized tryptophan by the spectral method or amino acid analysis, another portion of the ozonized sample (0.3 ml) was similarly treated (reduction and evaporation of the solvent). The kynurenine derivative thus obtained was dissolved in methanol and one third of the solution was spectrophotometrically examined. Another one third was used for kynurenine determination after evaporation of the methanol and subsequent hydrolysis in constant-boiling hydrochloric acid for 24 hours at 110°C.

The cleavage reaction of the tryptophyl bond in several derivatives of tryptophan-peptides by the improved method was summarized in Table 1. It is seen that the over-all cleavage reaction of the tryptophyl bond could be achieved much effectively when it was determined by means of liberation

Table 1. The chemical cleavage of the tryptophyl bond in several synthetic peptides[a)]

peptide derivative	Oxidation yield determined by		Yield of the over-all cleavage reaction determined by	
	uv-spect.[b)]	Kyn[c)] after acid hydrolysis[d)]	tetrahydropyridazone formation[e)]	amino acid analysis
Z[f)]-Trp-Gly-OH	92%	52%	77%	88%
Z-Trp-Ala-OH	87	72	79	88
Z-Trp-Leu-OH	89	64	83	90
Z-Trp-Phe-OH	91	76	81	88
Z-Gly-Trp-Gly-OH	88	44	77	90
Z-Ala-Trp-Leu-OH	83	66	78	83

a) The data presented were given by duplicate experiments.
b) See text. The yields were calculated on the basis of absorbance at 340 nm (ε, 3200 in methanol)
c) Kyn : kynurenine
d) Uncorrected for decomposition during acid hydrolysis.
e) Determined on the basis of absorbance at 353 nm according to ref. 7.
f) Z : benzyloxycarbonyl

of the amino acid (83-90%). The lower estimation of the cleavage reaction in terms of the tetrahydropyridazone formation may be due to partial degradation of the hetero-ring during the cleavage reaction. Since liberation of leucine from the peptides containing tryptophylleucine was estimated to a similar extent with that in the cleavage reaction of the model tripeptide derivative, benzyloxycarbonyl-alanyl-N'-formylkynurenyl-leucine, it is concluded that the oxidation of the tryptophan residue in the peptide into the kynurenine derivative is nearly complete.

However, spectral determination of N'-formylkynurenine at 325 nm on oxidation of individual tryptophan peptides listed in Table 1 was remarkably low contrary to our expectation. Fig. 1 shows a typical spectral change during ozonization of benzyloxycarbonyl-L-tryptophyl-L-alanine and subsequent

reduction of the ozonization product with dimethyl sulfide. On ozonization of the above tryptophyl-alanine derivative in absolute methanol, an intermediate compound absorbing at both 256 nm and 358 nm (presented as (II) in Fig. 1) was obtained. This compound seems to be a methoxyhydroperoxide which might be formed by reaction of a dipolar intermediate derived from the indole nucleus with methanol on ozonization. Reduction of compound (II) with dimethylsulfide finally gave stable oxidation products absorbing mainly at both 258 nm and 367 nm (presented as (III) in Fig.1) This fact strongly suggests that the major oxidation product of these tryptophan peptides was not N'-formylkynurenine but its deformylated derivative, since N'-formylkynurenine absorbs at both 261 nm and 325 nm, and kynurenine at both 258 nm and 367 nm. The same features were observed on oxidation of acetyl-L-

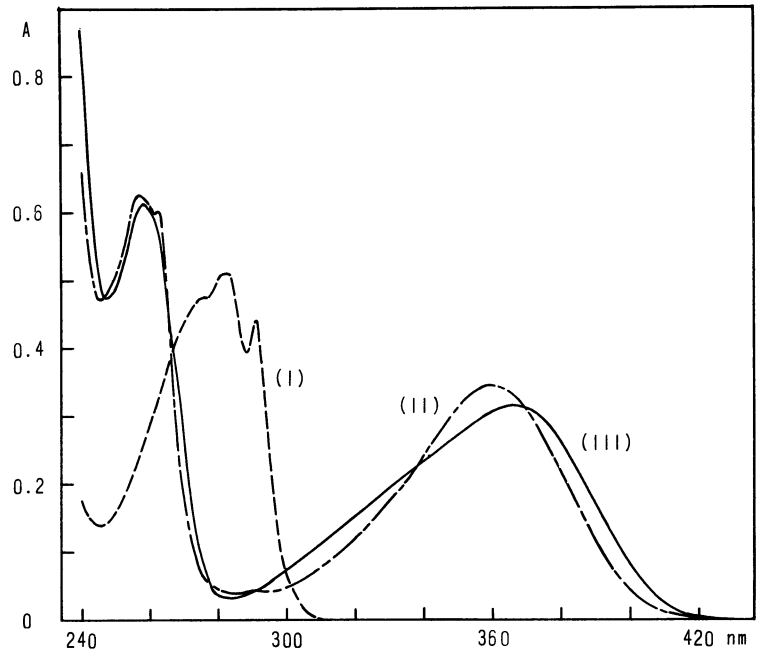

Fig. 1 Spectral change during ozonization of Z-Trp-Ala-OH and subsequent reduction with dimethylsulfide.
(I) : Benzyloxycarbonyl-L-tryptophyl-L-alanine. (II) : ozonization product of (I) in absolute methanol around -70°C. (III) : reduction product of (II) with dimethylsulfide. All spectra (concentration of the peptide derivative, 0.082 mM) were measured in methanol at room temperature within a few minutes after withdrawing individual samples.

tryptophan which was oxidized to give acetyl-L-kynurenine[15] as a major oxidation product (yield, 73%). Thus, the oxidation yield (kynurenine plus its N'-formyl derivative) determined by ultraviolet spectroscopy in Table 1 was calculated on the basis of the absorbance at 340 nm which was an isosbestic point between kynurenine and its N'-formyl derivative. Then it is formulated that in the present cleavage of the tryptophyl bond in acyl derivatives of H-Trp-X-OH (X : amino acid residue) the reaction mainly involves the oxidation into the N-α-acylkynurenine derivative, and not the N-α-acyl-N'-formylkynurenine derivative, at the first step.

The direct formation of kynurenine from tryptophan by oxidation with ozone under the present conditions is an interesting problem which is now being studied what factors lead to the formation of

kynurenine on one hand, and of its N'-formyl derivative on the other.[16]

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13) m.p 119-119.5°C. $[\alpha]_D^{18}$ = +72.7 (c 1, glacial acetic acid). $\lambda_{max.}$ (methanol). 324.5 nm (ε 4.53 x 10^3) and 260.5 nm (ε 12.1 x 10^3)
Anal., found, C, 58.53; H, 5.98; N, 9.04% Calcd. for $C_{15}H_{18}O_5N_2$:
C, 58.81; H, 5.92; N, 9.15%.

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15) m.p 187°C (dec). $[\alpha]_D^{18}$ = +247.0 (c 0.5, glacial acetic acid). $\lambda_{max.}$ (methanol), 367 nm (ε 5.60 x 10^3) and 257.5 nm (ε 7.29 x 10^3).
Anal., found, C, 57.46; H, 5.75; N, 11.07% Calcd. for $C_{12}H_{14}O_4N_2$:
C, 57.59; H, 5.60; N, 11.20%.

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