Synthesis and Enzymic Hydroxylation of Protocollagen Model Peptide Containing a Hydroxyproline Residue

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1. Two protocollagen model peptides, Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ (Z, benzyloxycarbonyl) and AOC-(Pro-Pro-Gly)₆ (AOC, *tert*-pentyloxycarbonyl), were synthesized and hydroxylated with protocollagen proline hydroxylase.
2. The two model peptides were hydroxylated equally. The results suggest that the hydroxyl group of hydroxyproline contained in the *N*-terminal region of the peptide has no effect on the enzymic hydroxylation of the model peptide.

Hydroxyproline in collagen is synthesized biologically by the hydroxylation of proline residues in protocollagen, a large polypeptide precursor of collagen (Prockop & Kivirikko, 1967). The hydroxylating enzyme, protocollagen proline hydroxylase, is an oxygenase (Fujimoto & Tamiya, 1962, 1963) and hydroxylates the proline residues not only in protocollagen but also in synthetic polypeptides (Pro-Gly-Pro)_n (Kivirikko & Prockop, 1967).

Studies of the enzymic hydroxylation of the protocollagen models with well-defined molecular weights (Kikuchi, Fujimoto & Tamiya, 1969*a,b*) suggested that the triple-stranded conformation in larger model peptides was unfavourable for the hydroxylation and that the proline residue in non-terminal -Pro-Pro-Gly- sequences was more easily hydroxylated than those in terminal ones.

The present work was carried out to examine the effect of a hydroxyl group in a neighbouring -Gly-X(=Pro)-Y(=Hyp)- sequence. The work was undertaken because in native collagen hydroxy amino acids such as serine and threonine were found at the Y-position of the above sequence (von der Mark, Wendt, Rexrodt & Kuhn, 1970) and it was thought that the hydroxyl group might 'initiate' the hydroxylation of proline in the neighbouring -Gly-Pro-Pro- sequence.

As shown in Scheme 1, Z*-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ was enzymically hydroxylated and the hydroxylated peptide was degraded with collagenase. The hydroxyproline content in the neutral fragments was compared with that of corresponding fragments from AOC-(Pro-Pro-Gly)₆.

* Abbreviations: AOC, *tert*-pentyloxycarbonyl; t-Bu, *tert*-butyl; DCHA, dicyclohexylamine; OEt, ethyl ester; OBzl, benzyl ester; TosOH, toluene-*p*-sulphonic acid; Z, benzyloxycarbonyl; ONP, *p*-nitrophenyl ester.

MATERIALS AND METHODS

Two collagen model peptides, Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and AOC-(Pro-Pro-Gly)₆, were prepared as shown in Scheme 2. The products were purified by gel filtration on Sephadex LH-20 and by zone electrophoresis on cellulose powder. Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and (Pro-Pro-Gly)₅ were synthesized by fragment condensation on Merrifield's resin as described by Sakakibara, Kishida, Kikuchi, Sakai & Kakiuchi (1968). The details of the synthesis are described below.

Synthesis of the peptide fragments

AOC-Pro-Hyp(t-Bu)-DCHA. Z-Hyp(t-Bu)-OEt was prepared by esterification (Greenstein & Winitz, 1961, p. 925) and benzyloxycarbonylation (Greenstein & Winitz, 1961, p. 887) of hydroxyproline followed by treatment with isobutylene and sulphuric acid (Wünsch & Jentsch, 1964). The benzyloxycarbonyl group was cleaved off by hydrogenolysis. The product, Hyp(t-Bu)-OEt (0.1 mol), and AOC-Pro (0.1 mol) were coupled with dicyclohexylcarbodi-imide (0.1 mol) in chloroform (70 ml) and an oily preparation of AOC-Pro-Hyp(t-Bu)-OEt was obtained. The ethyl ester group in AOC-Pro-Hyp(t-Bu)-OEt was saponified in a mixture of 1M-NaOH (110 ml) and acetone (400 ml). The AOC-Pro-Hyp(t-Bu) thus obtained was crystallized from ether as the dicyclohexylammonium salt (33g, 58% of theory based on Hyp-OEt.HCl). Recrystallization from ethyl acetate gave an analytically pure preparation, m.p. 157-158°C (Found: C, 66.4; H, 10.1; N, 7.2. Calc. for C₃₂H₅₇N₃O₆: C, 66.3; H, 9.9; N, 7.2%).

AOC-Pro-Hyp(t-Bu)-Gly-DCHA. AOC-Pro-Hyp(t-Bu)-DCHA (2.32g, 4mmol) was dissolved in ethyl acetate (10ml) and mixed with 0.5M-HCl (10ml). The dicyclohexylammonium chloride was filtered off and AOC-Pro-Hyp(t-Bu) was extracted with ethyl acetate (30ml) from the filtrate. The oily AOC-Pro-Hyp(t-Bu) (4mmol) was coupled with Gly(OBzl)-TosOH (1.35g, 4mmol) in the presence of triethylamine (0.56ml, 4mmol)

Scheme 1. Expected course of enzymic hydroxylation and collagenase digestion of protocollagen model peptide.

and dicyclohexylcarbodi-imide (0.92 g, 4 mmol) in chloroform (30 ml), and an oily preparation of AOC-Pro-Hyp(t-Bu)-Gly-OBzl (2.43 g) was obtained. The benzyl ester group was cleaved by hydrogenolysis and AOC-Pro-Hyp(t-Bu)-Gly was crystallized from ethyl acetate and light petroleum (b.p. 50–90°C) as the dicyclohexylammonium salt. Recrystallization from the same solvent mixture gave pure AOC-Pro-Hyp(t-Bu)-Gly-DCHA (2.1 g, 81% of theoretical yield), m.p. 150–151°C (Found: C, 64.4; H, 9.4; N, 9.0. Calc. for $\text{C}_{34}\text{H}_{60}\text{N}_4\text{O}_7$: C, 64.1; H, 9.5; N, 8.8%).

AOC-Pro-Pro and AOC-Pro-Pro-Gly. AOC-Pro-Pro and AOC-Pro-Pro-Gly were synthesized as described by Sakakibara & Inukai (1966).

Peptide couplings on Merrifield's resin

(Pro-Pro-Gly)₅. AOC-Gly-resin was prepared by refluxing, for 48 h, a mixture containing chloromethylated co-poly(styrene-divinylbenzene) polymer (chlorine content, 0.42 mmol/g of resin; 10 g) (Merrifield, 1963), AOC-Gly (1.9 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) in ethanol (15 ml) and chloroform (15 ml). The resin was thoroughly washed with ethanol, water and methanol and dried over P_2O_5 under reduced pressure. AOC-Gly-resin (glycine content, 0.06 mmol/g of resin; 10 g) was treated with anhydrous HCl in acetic acid to remove the AOC group and was then coupled with AOC-Pro-Pro (0.78 g, 2.4 mmol) with dicyclohexylcarbodi-imide (0.94 g, 2.4 mmol). The AOC group was removed similarly and four coupling cycles with AOC-Pro-Pro-Gly (each 2.4 mmol) were repeated. All coupling reactions were carried out with dicyclohexylcarbodi-imide in methylene chloride as solvent at room temperature for 4 h. One half of the resulting peptide-resin, AOC-(Pro-Pro-Gly)₅-resin, was kept for further elongation (see below), and the other half was dried over P_2O_5 under reduced pressure. The protecting group and the resin were cleaved from the peptide with anhydrous HF in the presence of anisole (Sakakibara, Shimonishi, Kishida, Okada & Sugihara, 1967). After removal of HF under reduced pressure, the residue was dried over NaOH pellets under reduced pressure and extracted with water (total 50 ml). The remaining HF in the extract was removed by passage

Scheme 2. Synthesis of the model peptides.

through a column (1.2 cm × 7 cm) of Amberlite CG-45 (OH⁻ form) and the neutral eluate was freeze-dried. A crude preparation of (Pro-Pro-Gly)₅ (278 mg) was obtained.

Pro-Hyp-Gly-(Pro-Pro-Gly)₅. AOC-Pro-Hyp(t-Bu)-Gly (1.5 mmol) was coupled in the same way to (Pro-Pro-Gly)₅-resin, which was derived by the removal of the AOC group from the AOC-(Pro-Pro-Gly)₅-resin in the same way. The peptide-resin, AOC-Pro-Hyp(t-Bu)-Gly-(Pro-Pro-Gly)₅-resin, was dried and treated with anhydrous HF in the presence of anisole. A crude preparation of Pro-Hyp-Gly-(Pro-Pro-Gly)₅ (376 mg) was obtained by the same procedure as described above.

Purity and purification of the crude peptide preparations

The purity of the crude preparations of (Pro-Pro-Gly)₅ and Pro-Hyp-Gly-(Pro-Pro-Gly)₅ was checked by electrophoresis on Toyo no. 50 filter paper (Toyo-Roshi Co., Tokyo, Japan) in 1 M-acetic acid, pH 2.4, at 1500 V/50 cm for 2.5 h. The authentic samples were synthesized by the classical solution method as follows. Pro-Pro-Gly was prepared by deprotection of AOC-Pro-Pro-Gly with trifluoroacetic acid, and (Pro-Pro-Gly)₃ by anhydrous HF treatment of AOC-(Pro-Pro-Gly)₃-OBzl, which was synthesized as described previously (Kikuchi *et al.* 1969b). The peptides were detected on the paper as yellow spots by ninhydrin spray followed by the Cl_2 -KI-starch method (Rydon & Smith, 1952). Both preparations contained a trace amount of glycine but no shorter peptides. The crude preparations were purified by gel filtration through a column (2.8 cm × 55 cm) of Sephadex G-25 with 0.2 M-acetic acid, and freeze-dried. Recoveries of (Pro-Pro-Gly)₅ and Pro-Hyp-Gly-(Pro-Pro-Gly)₅ from the column were 83.0 and 83.5% respectively.

Modification of the N-terminal residues of the peptides

Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅. A mixture containing Pro-Hyp-Gly-(Pro-Pro-Gly)₅ (53.8 mg, 34.8 μmol) and Z-Gly-ONP (53.2 mg, 161 μmol) in 0.2 ml of pyridine-water (2:1, v/v) was incubated at 40°C. After 3 days, Z-Gly-ONP (13.2 mg, 40 μmol) was added and the mixture was left at 40°C for 3 days. Water (10 ml) was added and the mixture freeze-dried. The residue was dissolved in methanol-methylene chloride-water (7:2:1, by vol.) and fractionated by gel filtration on a column (1.5 cm × 130 cm) of Sephadex LH-20. The elution curve is shown in Fig. 1(a). Peak A, containing the product and the starting peptide, was pooled and freeze-dried after organic solvents had been evaporated off. Peak B was identified as Z-Gly by t.l.c. and peak C was *p*-nitrophenol. The freeze-dried peak A preparation (Fig. 1a) was subjected

to zone electrophoresis on a slab of cellulose powder at pH 5.0. This separation pattern is shown in Fig. 2(a). The blocks containing peaks I, II and III were extracted thoroughly with water (100 ml per peak). The extracts were freeze-dried to give the residues from peaks I, II and III (41, 5 and 2 mg respectively). The purity of the products was checked by paper electrophoresis at pH 5.0 (Fig. 3a). Peak I (Fig. 2a) contained a single component, which was characterized as the expected product by amino acid analysis (Table 1). The total yield of the product was 68%, based on Pro-Hyp-Gly-(Pro-Pro-Gly)₅. Peak II (Fig. 2a) contained a small amount of the product and another component that was less mobile towards the anode (Fig. 3a). Peak III (Fig. 2a) contained the starting material and another component that was less mobile towards the cathode (Fig. 3a). These components were not identified further.

AOC-(Pro-Pro-Gly)₆. A mixture containing (Pro-Pro-Gly)₅ (46.5 mg, 36.5 μmol) and AOC-Pro-Pro-Gly-ONP (100 mg, 200 μmol) in 0.3 ml of pyridine-water (2:1, v/v) was incubated at 40°C for 3 days. To the mixture was added AOC-Pro-Pro-Gly-ONP (22.5 mg, 45 μmol) and the reaction mixture was kept at 40°C for 4 days. The product was isolated from the mixture in a similar manner to that used for Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅. The results are shown in Figs. 1(b) and 2(b). The purity of the electrophoretically fractionated product was checked by paper electrophoresis (Fig. 3b). Peak I (Fig. 2b) contained a single component, which was shown to be the expected product by amino acid analysis (Table 1). Peaks II and

Fig. 2. Zone electrophoresis of crude preparations of (a) Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and (b) AOC-(Pro-Pro-Gly)₆. Electrophoresis was carried out on slabs (40 cm × 2 cm × 1 cm) of cellulose powder in 0.1 M-pyridine-acetate buffer, pH 5.0 (450 V and 40–50 mA, for 10 h), in a cold-room (4°C). The slabs were cut into blocks (1 cm thick), which were suspended in water (2.5 ml/block). The amino acid content of a 25 μl portion of each supernatant was measured, after alkali hydrolysis (Hirs *et al.* 1956), by ninhydrin colorimetry (Chinard, 1952).

Fig. 1. Gel filtration of the reaction mixtures of (a) Z-Gly-ONP and Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and (b) AOC-Pro-Pro-Gly-ONP and (Pro-Pro-Gly)₅ on Sephadex LH-20. Elution was carried out with methanol-methylene chloride-water (7:2:1, by vol.) for (a) and with methanol-water (9:1, v/v) for (b); 5 ml fractions were collected. The amino acid content of the alkali hydrolysate (Hirs, Moore & Stein, 1956) of a 10 μl portion of each fraction was measured by ninhydrin colorimetry at 570 nm for (a) and at 515 nm (Chinard, 1952) for (b). For the detection of *p*-nitrophenol, a 10 μl portion of each fraction was diluted with 5 ml of 0.1 M-NaOH and the E_{395} was measured. ○, E_{570} for (a), E_{515} for (b); ●, E_{395} .

Fig. 3. Paper electrophoresis of peaks I, II and III from (a) Fig. 2(a) and (b) Fig. 2(b). Electrophoresis was carried out on Toyo no. 50 filter paper in 0.1 M-pyridine-acetate buffer, pH 5.0 (1500 V/40 cm, 15–20 mA/7 cm, for 120 min). The peptides were detected by *tert*-butyl hypochlorite and starch-iodine (Mazur, Ellis & Cammarate, 1962).

Table 1. Amino acid composition of Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and AOC-(Pro-Pro-Gly)₆

Amino acid	Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly) ₅		AOC-(Pro-Pro-Gly) ₆	
	Ratio to	Theoretical	Ratio to	Theoretical
	hydroxyproline	ratio	glycine	ratio
Pro	11.8	11	1.8	2
Gly	6.6	7	1.0	1
Hyp	1.0	1		

III (Fig. 2b) were impure and not examined further. The total yield of the product was 48% based on (Pro-Pro-Gly)₅.

Effect of the hydroxyl group on the enzymic hydroxylation

Protocollagen proline hydroxylase. Protocollagen proline hydroxylase was prepared from 12-day-old chick embryos by ammonium sulphate fractionation followed by calcium phosphate gel fractionation (Kivirikko & Prockop, 1967). In accord with the modification described by Halme, Kivirikko & Simons (1970), 10 mM-tris-HCl buffer, pH 7.8, containing 50 mM-glycine and 50 mM-KCl, was used for dialysis, 20 mM-potassium phosphate buffer, pH 7.8, containing 7 mM-glycine, was used for the first extraction of the protein adsorbed to the calcium phosphate gel and 0.15 M-potassium phosphate buffer, pH 7.8, containing 50 mM-glycine, for the second extraction. The activity of the enzyme was assayed by the hydroxylation of reduced and carboxymethylated collagen from *Ascaris cuticle* (Fujimoto & Prockop, 1968) (100 µg) with 2 mg of enzyme protein in an incubation mixture (4 ml) as described below. The fraction with the highest specific activity was used for the following reaction.

Enzymic hydroxylation of the peptides. A mixture (total vol. 36 ml) containing substrate peptide, Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ (9.75 mg, 5.6 µmol) or AOC-(Pro-Pro-Gly)₆ (9.44 mg, 5.7 µmol), enzyme protein (33 mg), 2 mM-ascorbic acid, 0.5 mM-α-oxoglutarate and 0.04 mM-FeSO₄ in 50 mM-tris-HCl buffer, pH 7.8, was incubated at 37°C for 60 min in contact with the atmosphere and freeze-dried.

Recovery of the hydroxylated peptides. The dried residue was extracted with 2.0 M-acetic acid (10 ml), and passed through two columns (Sephadex G-25, 2.8 cm × 55 cm; Sephadex G-50, 2.8 cm × 59 cm) connected in series and eluted with 0.2 M-acetic acid (Fig. 4). The eluate containing the peptide and free from the enzyme and salts (the peak P of Fig. 4a or 4b) was concentrated at below 30°C under reduced pressure and freeze-dried.

Purification of the peptides. The peptides recovered were chromatographed on a column (1.25 cm × 12 cm) of DEAE-cellulose equilibrated with 10 mM-pyridine-acetate buffer, pH 5.0. The peptides in the eluate were detected by alkali hydrolysis (Hirs *et al.* 1956) on 25 µl portions of fractions followed by a ninhydrin colorimetric determination of proline (Chinard, 1952). The fractions containing acidic peptides were pooled and freeze-dried.

Collagenase digestion. Portions (about 2 mg) of each of the hydroxylated peptides, and of the unhydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ (2.26 mg, 1.3 µmol), were dissolved in 50 mM-tris-HCl buffer, pH 7.8 (0.5 ml), and 20 µl of collagenase solution (0.7 mg/0.14 ml of water) and toluene (1 drop) were added. The mixture was incubated at 37°C for 24 h, then the reaction was stopped by freezing.

Fractionation of the collagenase digest. The thawed collagenase digest was diluted with water (0.5 ml) and added to 0.1 M-pyridine-acetate buffer, pH 5.0 (0.1 ml). After a portion (0.2 ml) of the solution had been taken out and kept for analysis, the remaining solution was applied to a column (1.25 cm × 13 cm) of DEAE-cellulose equilibrated with 10 mM-pyridine-acetate buffer, pH 5.0. The neutral fragments were eluted with the same buffer

Fig. 4. Gel filtration of hydroxylase reaction mixture of (a) Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and (b) AOC-(Pro-Pro-Gly)₆. The extract (10 ml) was passed through consecutive columns of Sephadex G-25 (2.8 cm × 55 cm) and G-50 (2.8 cm × 59 cm). Fractions of volume 10 ml were collected and the amino acid content of the alkali hydrolysates of a 0.5 ml portion of each fraction was measured by ninhydrin colorimetry at 570 nm. Peaks E, P and G were enzyme protein, peptide and glycine in buffer respectively.

solution and the acidic fragments were eluted with 0.1M-HCl. The peptides in the eluate were detected by the same method as described above under 'Purity and purification of the crude peptide preparations'. The fractions containing the fragments were pooled and concentrated to dryness with a rotary evaporator.

Amino acid composition of the fragments. The fractionated fragments (all of those recovered) and unfractionated mixtures were hydrolysed with 6M-HCl (4 ml) in sealed tubes at 110°C for 48 h. Proline and glycine in the hydrolysates were assayed by an automatic amino acid analyser (type JLC-5AH; Japan Electron Optics Laboratory Co., Tokyo, Japan) and hydroxyproline was assayed as described by Kivirikko, Laitinen & Prockop (1967).

RESULTS AND DISCUSSION

The enzymic hydroxylation of the peptides was carried out at concentrations of substrates and enzyme protein (0.15–0.16 $\mu\text{mol/ml}$ and 0.98 mg/ml respectively) similar to those used in the reaction described previously (Kikuchi *et al.* 1969a,b). The hydroxylated peptides were freed from enzyme protein and salts by gel filtration on Sephadex G-25 and G-50. Although the blocking AOC and Z groups were not expected to be cleaved off by the above treatment, the hydroxylated peptides were chromatographed on a DEAE-cellulose column. The blocking groups were stable and no neutral components were recovered from the column. Although, in the preliminary test, the collagenase digestion of the peptides was almost complete within 10h, the hydroxylated peptides were digested for 24h. The separation of the acidic and neutral fragments in the collagenase digest was carried out successfully by DEAE-cellulose column chromatography at pH 5.0. The amino acid compositions of the fractionated fragments and unfractionated mixtures are shown in Table 2.

The non-treated peptide, Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅, was used as the control for collagen-

ase digestion and separation of the fragments. The amino acid composition of the fragments from the unhydroxylated peptide showed that the procedure was successful (Table 2c). Although the glycine content was slightly less than one-third of the expected value, the proline and hydroxyproline contents were as expected in the acidic fragment. In the neutral fragment, the proline/glycine ratio ($5.40/3.19 = 1.69$) agreed with the theoretical value ($10/6 = 1.67$) and the contamination with hydroxyproline was negligible. Moreover, the ratio of proline in the acidic fragment to that in the neutral fragment ($0.600/5.40 = 0.11$) agreed with the theoretical value ($1/10 = 0.10$).

The degree of hydroxylation of the second proline residues of the -Pro-Pro-Gly- sequence, defined as $100(\%) \times 2 \times \text{Hyp}/(\text{Pro} + \text{Hyp})$, was calculated on the basis of the amino acid composition (Table 3). The degree of hydroxylation for acidic and neutral fragments of unhydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ (Table 3c: 91.4 and 0% respectively) agreed with the theoretical values (100 and 0% respectively). The values for the whole molecule of hydroxylated peptides (*W*) were calculated from the values for the acidic fragments (*A*) and those for the neutral fragments (*N*) as follows:

$$W = (A + 5N)/6$$

The calculated values for hydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and AOC-(Pro-Pro-Gly)₆ (21.2 and 4.24% respectively) were in good agreement with the observed values (20.8 and 4.60% respectively).

The above results indicate that the procedure shown in Scheme 1 was carried out as expected.

As shown in Scheme 1, the enzyme action on the peptide was assayed by the degree of hydroxylation of the neutral fragment. The values for the neutral

Table 2. *Amino acid composition of the peptides and collagenase fragments*

The arrows indicate the linkages between acidic and neutral fragments. (a) Hydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅; (b) hydroxylated AOC-(Pro-Pro-Gly)₆; (c) unhydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₄. Experimental details are given in the text.

	Composition (μmol)								
	Fragment								
	Acidic			Neutral			Whole molecule		
	Pro	Gly	Hyp	Pro	Gly	Hyp	Pro	Gly	Hyp
(a) Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly) ₅	0.779	0.219	0.840	7.77	5.11	0.18	1.97	1.26	0.23
(b) AOC-Pro-Pro-Gly-(Pro-Pro-Gly) ₅	1.390	0.082	0.010	7.55	4.50	0.19	1.95	1.10	0.046
(c) Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly) ₅	0.600	0.189	0.504	5.40	3.19	0.01			

Table 3. Degree of hydroxylation of second proline residues of -Pro-Pro-Gly- units in the peptides and their collagenase fragments

Degree of hydroxylation was defined as $100(\%) \times 2 \times \text{Hyp}/(\text{Pro} + \text{Hyp})$ and calculated on the basis of the amino acid composition in Table 2. The arrows indicate the linkages between acidic and neutral fragments. (a) Hydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅; (b) hydroxylated AOC-(Pro-Pro-Gly)₆; (c) unhydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅.

	Degree of hydroxylation (%)		
	Fragment		Whole molecule
	Acidic	Neutral	
(OH) (a) Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly) ₅	103.8	4.68	20.8
↑			
(OH) (OH) (b) AOC-Pro-Pro-Gly-(Pro-Pro-Gly) ₅	1.42	4.80	4.60
↑			
(c) Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly) ₅	91.4	0	
↑			

fragments of hydroxylated Z-Gly-Pro-Hyp-Gly-(Pro-Pro-Gly)₅ and AOC-(Pro-Pro-Gly)₆ (4.68% and 4.80% respectively) indicate that there is no difference between the hydroxylation of the two peptides. The hydroxyproline residue in the N-terminal region of the peptide had no effect on the enzymic hydroxylation of the molecule.

Juva & Prockop (1969) reported that procollagen proline hydroxylase had less affinity for the partially hydroxylated substrate. The above results suggest that their conclusion does not apply to the low-molecular-weight (about 1500) synthetic models of procollagen.

The degree of hydroxylation for the acidic fragment of hydroxylated AOC-(Pro-Pro-Gly)₆ (1.42%) was less than one-third of that for the neutral fragment (4.80%) (Table 3b). The results agree with the previous observation (Kikuchi *et al.* 1969b) that the terminal -Pro-Pro-Gly- sequence was hydroxylated to a smaller extent than were the non-terminal ones.

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