Biotechnol. Bioprocess Eng. 2000, 5: 61-64

Inhibition of Prolyl 4-Hydroxylase by Oxaproline Tetrapeptides *In Vitro* and Mass Analysis for the Enzymatic Reaction Products

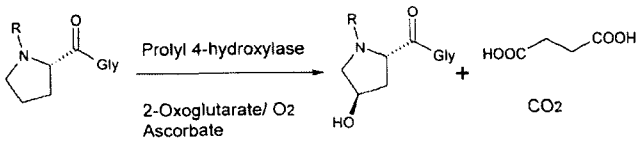
Hong-sik Moon[1]* and Tedhg P. Begley[2]

[1] Cheiljedang Institute of Technology, 522-1, Dokpyong-Ri, Majang-Myon, Ichon, Kyonggi-Do 467-810, Korea
[2] Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, U.S.A.

Abstract A series of 5-oxaproline peptide derivatives was synthesized and evaluated for its ability to inhibit the prolyl 4-hydroxylase *in vitro*. Structure-activity studies show that the 5-oxaproline sequences, prepared by the 1,3-dipolar cycloaddition of the C-methoxycarbonyl-N-mannosyl nitrone in the presence of the ethylene, are more active than the corresponding proline derivatives. Prolyl 4-hydroxylase belongs to a family of Fe^{2+}-dependent dioxygenase, which catalyzes the formation of 4-hydroxyproline in collagens by the hydroxylation of proline residues in -Gly-Xaa-Pro-Gly- of procollagen chains. In this paper we discover the more selective N-Cbz-Gly-Phe-Pro-Gly-OEt (K_m = 520 μM) sequences which are showed stronger binding than others *in vitro*. Therefore, we set out to investigate constrained tetrapeptide that was designed to mimic the proline structure of peptides for the development of prolyl 4-hydroxylase inhibitor. From this result, we found that the most potent inhibitor is N-Dansyl-Gly-Phe-5-oxaPro-Gly-OEt (K_i = 1.6 μM). This has prompted attempts to develop drugs which inhibit collagen synthesis. Prolyl 4-hydroxylase would seem a particularly suitable target for antifibrotic therapy.

Keywords: oxaproline, prolyl 4-hydroxylase, collagen, antifibrotic therapy

Inhibitors of prolyl 4-hydroxylase have received much interests as potential fibrosuppressive agent [1], due to the fact that inhibitors of prolyl 4-hydroxylase strikingly and selectively reduce the biosynthesis of collagens. The activity of this enzyme is essential for the formation of the triple helices of collagens and its subsequent secretion into the extracellular matrix [2]. The stability of the triple-helical domains is known to be dependent on the extent of the conversion carried out by prolyl 4-hydroxylase [3]. The hydroxylation of prolyl residues is very important for the triple-helical formation in procollagen to prevent the undesirable accumulation of newly synthesized collagen in fibrotic diseases.

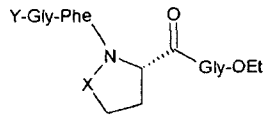


Scheme I.

* Corresponding author
Tel: +82-331-230-6513 Fax: +82-331-234-0210
e-mail: hsm@cwp.co.kr

Prolyl 4-hydroxylase belongs to a family of Fe^{+2}-dependent dioxygenases which couples the oxidative decarboxylation of 2-oxoglutarate to the hydroxylation of the primary substrate. In the case of prolyl 4-hydroxylase the targets of the hydroxylation are prolyl residues in the sequence -Gly-Xaa-Pro-Gly- of procollagen chains.

The active prolyl 4-hydroxylase (human) is an $\alpha_2\beta_2$ tetramer, which has a molecular weight of about 228 kDa (α = 59,000 Da, β = 55,000 Da) and requires Fe^{2+}, 2-oxoglutarate, oxygen, and ascorbate for activity [4]. The large catalytic site may be cooperatively built up of both the α and β subunits, but the α subunit appears to contribute the major part [5]. The free radical intermediate has been implicated in the mechanism of these catalysts and its strategy has been used with some success in using a cyclopropane as a substrate analog to trap the radical form. However, it is unlikely that the enzyme will tolerate the large cyclopropyl substituent and the derivatives in which the three membered ring is incorporated into the proline ring are stable in aqueous buffer. We will therefore explore the use of 5-oxaproline derivatives which are capable of forming prolyl like heteroatom stabilized radicals. Very recently, syncatalytic inhibitors [6] have been described which are analogues of 5-oxaproline or 3,4-dehydroproline peptides [7]. Nevertheless, the mechanism of all these compounds has not been completely described.



X = C (proline), O (oxaproline)
Y = Cbz, Dansyl

Scheme II.

In the present study, we have investigated potency of several 5-oxaproline-containing peptides and their derivatives as inhibitors of prolyl 4-hydroxylase, and elucidated their inactivation mechanism. By virtue of their primary structure, the 5-oxaproline tetrapeptide appears to be a potent and specific syncatalytic inactivator of prolyl 4-hydroxylase. Here we described the synthesis of 5-oxaproline derivatives (Scheme II) and evaluated their activities as an inactivator. N-Dansyl-Gly-Phe-5-oxaPro-Gly-OEt was prepared as previously reported [8]. The 1,3-dipolar cycloaddition [9] of the C-methoxycarbonyl-N-mannosyl-nitrone, formed *in situ* from the partially protected D-mannose-oxime and methylglyoxylate, to ethylene gave preferentially the (3S)-N-glycosyl-isoxazolidine (69%) which was transformed into the 3-isoxazolidine (L-5-oxaproline ester). Isoxazolidinecarbonyl-glycine ethyl ester was synthesized from the 3-isoxazolidine through the hydrolysis and carbodiimide coupling steps. The N-*tert*-butoxycarbonyl-Phe-5-oxaPro-Gly-OEt (76% yield) was obtained from N-*tert*-Boc-phenylalanine and isoxazolidinecarbonyl-glycine ethyl ester by the carbodiimide coupling reaction. Finally, Dansyl-Gly-Phe-5-OxaPro-Gly-OEt (70% yield) was prepared using a protection with di-*tert*-butyl dicarbonate, a cleavage by trifluoroacetic acid, and a carbodiimide coupling strategy with N-Dansyl-(or-benzyloxycarbonyl)glycine.

Determination of enzyme activity was carried out as described previously [7]. Prolyl 4-hydroxylase (60 μg, 0.12 mg/mL, 0.526 μM) was incubated in 500 μl of 50 mM Tris/HCl (pH=7.8), containing 0.05 mM $FeSO_4$, 2 mM ascorbate, 0.1 mM 2-oxoglutarate, 0.1 mM dithiothreitol, 0.1 mg/mL catalase, and 2 mg/mL bovine serum albumin and N-Cbz-Gly-Phe-Pro-Gly-OEt (0.2 mg/mL, 371 μM) at 37°C for 30 min. After 30 min. incubation, 250 μL of the reaction mixture was removed from 500 μL of the incubation solution and it was mixed with 250 μL methylene chloride in order to separate the organic phase. The enzymatic reaction was confirmed by TLC (7% methanol in methylene chloride) and FAB mass spectroscopy [N-Cbz-Gly-Phe-Pro-Gly-OEt : HRMS (M+1) = 539.2509 to N-Cbz-Gly-Phe-4-hydroxyPro-Gly-OEt: HRMS(M+1) = 555.2456, Fig. 1].

To assay the competitive activity, the two mixtures of 0.2 mg/mL of 5-oxaproline (N-Dansyl-Gly-Phe-oxaPro-Gly-OEt) peptide as an inactivator and 0.2 mg/mL of the substrate (N-Dansyl-Gly-Phe-Pro-Gly-OEt) incubated at 37°C for 30 min. The competitive assay was also examined with N-Cbz or Dansyl-Gly-Phe-3,4-dehydroPro-Gly-OEt in comparison with the N-Cbz or Dansyl-Gly-Phe-Pro-Gly-OEt respectively.

Most of the oxaproline-containing peptides used in this study caused irreversible inhibition of prolyl 4-

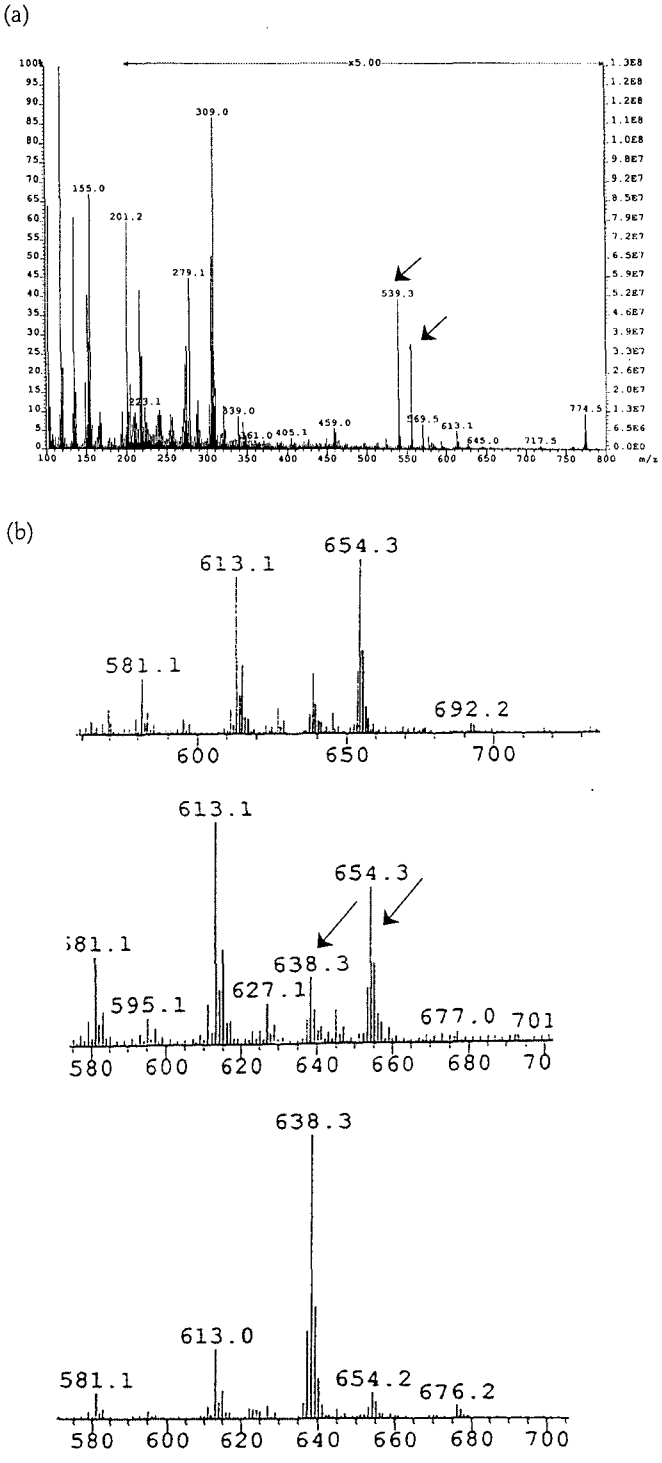


Fig. 1. Mass spectrum of the enzymatic conversion; (a) Mass data of N-Cbz-Gly-Phe-Pro-Gly-OEt[(M+1) = 539.2509] / N-Cbz-Gly-Phe-4-hydroxyPro-Gly-OEt [(M+1) = 552.2456], (b) Mass data of N-Dansyl-Gly-Phe-Pro-Gly-Oet(M+1 = 638.3) / N-Dansyl-Gly-Phe-4-hydroxyPro-Gly-Oet (M+1 = 654.3) depended on the concentration (313, 31, 3 μm) of P 4-H.

Table 1. Comparison of K_m and ID_{50} values of selected oxaproline peptides

No.	Compound	[a]K_m (μM)	[b]ID_{50} (μM)	[c]V_{max} (%)
1	Ac-Pro-5-oxaPro-Gly-N(Et)$_2$	2,100	16,000	24
2	Ac-Pro-5-oxaPro-Gly-OBzl	70	60	4.8
3	Z-Val-5-oxaPro-Gly-OBzl	40	40	14
4	Z-Phe-5-oxaPro-Gly-OBzl	5.2	0.8	1.5
5	Z-Gly-Phe-5-oxaPro-Gly-OBzl	5.0	0.6	2.2
6	Z-Gly-Phe-5-oxaPro-Gly-OEt	4.8	0.2	1.2
7	Z-Gly-Phe-3,4-dehydroPro-Gly-OEt	35	20	3.5

[a] Derived from the enhancement of 2-oxo[1-^{14}C]glutarate decarboxylation (1~4 data were quoted from reference 7)

[b] After 1 h of preincubation.

[c] V_{max} is referred to as "apparent" because no correction for the loss of enzyme activity during the incubation period due to irreversible inhibition was made. V_{max} is expressed as a percent of the V_{max} obtained with $(PPG)_{10}$ as substrate.

Ac = acetyl, Bzl = benzyl, OEt = ethoxy, Z = bezyloxycarbonyl(Cbz)

hydroxylase. Nevertheless, their inactivating efficiency, measured by ID_{50}, varied within wide limits. Likewise, inactivating potency was affected by the amino acid used in the individual peptides. The ID_{50} of Z-Gly-Xaa-oxaPro-Gly-OEt was found to be 0.2 μM (Xaa = Phe) and phenylalanine was found to be most effective in position Xaa (Table 1).

The interaction of the proline sequence of Xaa-Pro-Gly with prolyl 4-hydroxylase is influenced by the Xaa amino acids. It was found that Gly-Xaa-Pro-Gly tetrapeptides were slightly better affinity than tripeptides (Xaa-Pro-Gly) *in vitro*, but there is no significant differences in K_m. On the basis of the inhibitory activity of 5-oxaproline peptides, we decided to investigate the Gly-Xaa-Pro-Gly as a minimal sequence because the presence of glycine as every third amino acid in the repeating sequence of collagen [10] is essential and the K_m decreases markedly with an increase in the number of repeated -Xaa-Pro-Gly- units. The catalytic site of prolyl 4-hydroxylase is thought to comprise a set of separate locations for the binding of peptide substrate and the various cosubstrates.

The Fe^{2+} is thought to be located in a pocket coordinated with the enzyme by three ligands and binding of the 2-oxoglutarate. Binding of molecular oxygen and decarboxylation of 2-oxoglutarate is thought to lead to the formation of a highly reactive iron-oxo complex which subsequently hydroxylase the proline residues in the polypeptide substrate [11]. Thus, the reaction rate of a syncatalytic inactivator must be of the first order [8].

Mass spectrometry is more sensitive technique and is potentially useful for the conversion analysis as long as the converted peak show sufficiently high. Incubation of N-Cbz-Gly-Phe-Pro-Gly-OEt, N-Dansyl-Gly-Phe-Pro-Gly-OEt with prolyl 4-hydroxylase at 37°C for 30 min. gave optically pure *trans*-4-hydroxyprolyl residues (N-Cbz-Gly-Phe-4-hydroxyPro-Gly-OEt, N-Dansyl-Gly-Phe-4-hydroxyPro-Gly-OEt) respectively. But incubation of N-Cbz-Gly-Phe-oxaPro-Gly-OEt, N-Dansyl-Gly-Phe-oxa-Pro-Gly-OEt with prolyl 4-hydroxylase over the same time period showed no hydroxyl prolyl residues at all. In connection with our initial result on the enzyme catalyzed hydroxylation of prolyl residue, we made the expected observation that P 4-H was able to interconvert the *trans*-4-hydroxyprolyl residue of the product. Thus, HRMS studies showed that the conversion of hydroxylation (N-Dansyl-Gly-Phe-Pro-Gly-OEt) is depended on the concentration of prolyl 4-hydroxylase respectively (Fig. 1(b)).

In summary, the inactivation of the enzyme by Ac-Pro-5-oxaPro-Gly-Bzl (No. 2, Table 1) was significant (ID_{50} = 60 μM), the more effective 5-oxaproline derivative needed for these studies. Here, we report that the effective tetrapeptides (No. 5, 6, 7, Table 1) show more significant data (ID_{50} = 0.2 μM, No. 6, Table 1). The inactivation of prolyl 4-hydroxylase by N-Dansyl-Gly-Phe-5-oxaPro-Gly-OEt follows nonpseudo-first-order kinetics due to consumption of the inhibitor. We have estimated the rate constant for the inactivation at several inhibitor concentration (0.5, 1.0, 1.5, 2.0, and 5 μM) by determining the remaining activity after two minutes using the previously described assay procedure [7]. From these data, we determine that k_{inact} = 0.6 min^{-1} and K_i =1.6 μM [8]. The enzyme is protected from inactivation by the substrate $(Pro\text{-}Pro\text{-}Gly)_{10}$. In near future, suicide substrates will provide a useful test of our mechanistic understanding of this enzyme and enable us to identify some of the catalytically important residues at the active site. We recently reported that the oxaproline moiety has been easily oxidized to aspartic acid through the β-scission of the weak N-O bond of oxaproline [8].

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[Received December 29, 1999; accepted February 20, 2000]