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A NEW PROLYL HYDROXYLASE ACTING ON POLY-L-PROLINE, FROM SUSPENSION CULTURED CELLS OF *VINCA ROSEA*

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Summary

A new prolyl hydroxylase having a novel substrate specificity was isolated from the suspension-cultured cells of *Vinca rosea*. This enzyme was solubilized with 0.05 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.3 M NaCl and 0.5 mM β-mercaptoethanol from the membrane fractions of the cells, and was partially purified by $(NH_4)_2SO_4$ fractionation and DEAE-Sephadex A-50 column chromatography. The enzyme preparation was found to require O_2, Fe^{2+}, ascorbate, α-ketoglutarate and poly-L-proline to attain maximum activity. The plant enzyme does not hydroxylate free proline and di-, tri- and tetra-L-proline, but hydroxylates octa-L-proline and poly-L-proline ($M_r >$ 2000). Model peptides of unhydroxylated collagen, $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ are poor substrates for the plant enzyme. This means that the plant enzyme has a novel substrate specificity in regard to peptidyl substrate, and this differs from vertebrate prolyl hydroxylase, proline,2-oxoglutarate dioxygenase (prolyl-glycyl-peptide, 2-oxoglutarate: oxygen oxidoreductase, EC 1.14.11.2).

Introduction

The plant cell walls contain a hydroxyproline-rich glycoprotein (extensin) as a structural protein [1,2]. Vertebrates, however, have collagen and elastin for this imino acid-rich protein. Also in the case of both plants [3—5] and vertebrates (for recent reviews, see Refs. 6—8), the hydroxylation of proline

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2(*N*-morpholino)-ethanesulfonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid).

occurred either during or after the process of peptide-bond formation. The hydroxyl group formation of the imino acid has been shown to be derived from the intake of molecular oxygen in plant cell suspensions [9] and chick embryos [10,11].

The vertebrate prolyl hydroxylase, proline,2-oxoglutarate dioxygenase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) has been isolated as a homogeneous protein by affinity chromatography from three sources [12—14] and the mechanism of the enzyme reaction has been investigated in detail [15,16]. The enzyme stoichiometrically converts α-ketoglutarate to succinate during the hydroxylation of a peptidyl substrate, and requires Fe^{2+} and ascorbate as cofactors. This enzyme does not practically hydroxylate free proline, and minimum sequence requirement for the enzyme activity is a -X-Pro-Gly- triplet [17]. Poly-L-proline and oligo-L-proline are effective competitive inhibitors of this enzyme [18,19].

In plants, however, only carrot prolyl hydroxylase has been reported by Sadava and Chrispeels [20]. The carrot enzyme seems to resemble vertebrate prolyl hydroxylase and requires O_2, Fe^{2+}, ascorbate, an α-keto acid (α-ketoglutarate, oxaloacetate or pyruvate) and a peptidyl substrate for activity. In this case, protocollagen, produced in chick embryos by the removal of Fe^{2+}, could serve equally as the plant substrate prepared from carrot disks in a similar manner as that for the carrot prolyl hydroxylase. On the basis of the cross-reactivity of the two substrates, these authors concluded that the enzyme could not recognize a defined peptidyl primary structure. But considering the oligomeric hydroxyprolyl sequences in the cell wall glycoprotein [22], it seems reasonable to assume that the plant prolyl hydroxylase has a unique substrate specificity. In fact, in this paper, we show that a prolyl hydroxylase from *V. rosea* requires oligomeric prolyl sequence as a substrate. This paper is the first report dealing with prolyl hydroxylase capable of hydroxylating poly-L-proline and oligo-L-proline.

Materials and Methods

Materials. V. rosea cells were the generous gift of Dr. M. Misawa, Tokyo Research Laboratory of Kyowa Hakko Co., Ltd. Japan. Poly-L-proline (M_r 2000, 6000 and 12000) and poly-L-hydroxyproline (M_r 30000) were purchased from Sigma Chemicals Co., U.S.A. Di-, tri- and tetra-L-proline were products of Bachem Fine Chemicals, U.S.A. $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4\ H_2O$ and $(Pro\text{-}Pro\text{-}Gly)_{10} \cdot 9\ H_2O$ were obtained from the Protein Research Foundation, Japan. Glycyl-octa-L-proline and *t*-butyloxycarbonyl-octa-L-proline were generous gifts from Dr. S. Sato, of the Central Research Laboratory of Mitsubishi Chemical Industries Ltd., Japan. α-[1-^{14}C]Ketoglutarate was obtained from New England Nuclear Corp., U.S.A.

Culture of plant calluses. Calluses of *V. rosea* were cultured in suspension in the medium of Murashige and Skoog [21] containing 3% sucrose and 0.5 mg/l 2,4-dichlorophenoxyacetic acid, at 27° C in the dark, using a rotary shaker at 180 rev./min. The cells were grown in 100 ml of the medium in a 300 ml Erlenmyer flask and subcultured by 10-fold dilution every 7 days.

Enzyme purification. This procedure of enzyme purification was carried out

at 0—4° C. The fifth-day cells following inoculation were harvested by centrifugation (170 × *g* for 15 min). The cells were washed once in a homogenized buffer containing 50 mM Tris-HCl (pH 7.4)/0.2 M sucrose/20 mM KCl/0.5 mM β-mercaptoethanol. About 70 g of the washed cells were homogenized in 140 ml of the homogenized buffer in a Warling blender with 50 g of glass beads (diameter; 0.2 mm) at a maximum speed for 30 s. After standing for 1.5 min to cool, the homogenization was further repeated twice. In the same manner, a total of 240—250 g of cell homogenate was obtained. This homogenate was centrifuged (1500 × *g* for 15 min), and the sedimented glass beads, cell debris and nuclei were discarded. The supernatant was further centrifuged (105 000 × *g* for 60 min) and the resultant pellet was suspended in 20 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.3 M NaCl and 0.5 mM β-mercaptoethanol. The suspension was sonicated using a sonicator (Branson model W-200P) having a micro-tip with an output control of 6 for 1.5 min. The sonicated suspension was centrifuged (105 000 × *g* for 60 min), and solid $(NH_4)_2SO_4$ was slowly added, with stirring, into the supernatant fraction to a final 65% saturation. After being stirred for an additional 30 min to allow for a complete salting-out, the mixture was centrifuged (22 000 × *g* for 30 min). The salted-out proteins were floated on the surface of the solution of 65% saturated $(NH_4)_2SO_4$ in the centrifuge tube. The clear solution under the protein layer was carefully discarded with a Pasteur pipet. The proteins salted out by 65% saturated $(NH_4)_2SO_4$ were dissolved in a 50 mM Tris-HCl buffer (pH 7.8) containing 50 mM NaCl and 0.5 mM β-mercaptoethanol, and dialyzed against the same buffer containing 10% glycerol. The sample was applied on a column (1.2 × 10 cm) of DEAE-Sephadex A-50 previously equilibrated with the dialyzed buffer. The enzyme was eluted stepwise with the dialyzed buffer containing 0.15 M NaCl. The active fractions were pooled and then dialyzed against 10 mM Tris-HCl (pH 7.4) containing 25% glycerol, 20 mM KCl and 0.2 mM β-mercaptoethanol.

Enzyme assays. Prolyl hydroxylase was assayed by the two following methods: first, the formation of hydroxyproline by this enzyme was determined colorimetrically. Secondly, the activity was assayed by measuring the rate of $^{14}CO_2$ evolution from α-[1-^{14}C]ketoglutarate.

In most experiments, the second method was used under standard conditions, in a final volume of 300 μl containing 1—10 μg of the purified enzyme preparation, 200 μg poly-L-proline (M_r 6000), 150 μg bovine serum albumin, 40 mM Hepes buffer (pH 6.8), 0.15 mM $FeSO_4$, 2 mM ascorbate, 0.1 mM dithiothreitol and 0.1 mM α-[1-^{14}C]ketoglutarate ($7.26 \cdot 10^6$ dpm/μmol). The rate of $^{14}CO_2$ evolution was measured by a slight modification of the procedure of Rhoads and Udenfriend [23]. The reaction was carried out in a 10 ml conical-type test tube for centrifugation. This test tube was equipped with a silicon rubber tube connected inversely to a glass-made scintillation vial. A glass fibre disc, (Whatman GF/C, 25 mm diameter) containing 200 μl NCS-solubilizer (Amersham) was attached to the bottom of the vial. The reaction was started by the addition of 90 μl of a mixture containing α-[1-^{14}C]ketoglutarate, $FeSO_4$, ascorbate, dithiothreitol and Hepes buffer (pH 6.8), to 210 μl of the enzyme preparation, poly-L-proline and bovine serum albumin previously mixed at 0° C. Following incubation for 30 min at 30° C with constant shaking,

800 μl of 4% perchloroacetic acid solution was injected into the reaction mixture through the silicon rubber tube. The acidified mixture was made to stand at room temperature for 60 min to allow for the complete absorption of liberated $^{14}CO_2$.

To make a determination of the enzyme activity of the crude preparation, the first method was employed; that is, hydroxyproline formed by the enzyme was measured under the standard conditions described above, except for the use of 0.5 mM non-radioactive α-ketoglutarate. After incubating for 60 min at 30°C, 300 μl ice-cold 10% trichloroacetic acid was added, and the mixture was allowed to stand for 60 min at 0°C. Poly-L-proline and hydroxylated poly-L-proline were soluble in 5% trichloroacetic acid. The mixture was centrifuged (1500 × *g* for 15 min), and then the supernatant was collected. The precipitate underwent 5% trichloroacetic acid (0.2 ml) extraction once more, and after this, the combined supernatant was loaded on a column (1.5 × 9.0 cm) of Sephadex G-25, previously equilibrated with 0.1 N acetic acid. The void volume fraction was collected and lyophilized. After hydrolizing the fraction in 6 M HCl, the hydroxyproline content was determined by the method of Bondjers and Björkerud [24].

Unless otherwise stated, all assays were carried out under air.

In this paper, 1 unit of enzyme activity is defined as that amount of the enzyme required to synthesize 1 nmol of peptidyl hydroxyproline/h at 30°C, using a saturating concentration of poly-L-proline (M_r 6000) under the standard conditions.

Protein determination. The protein concentration was determined by the method of Lowry et al. modified by Higuchi and Yoshida [25] using bovine serum albumin as the standard.

Results

Subcellular distribution of prolyl hydroxylase

No actual measurement of the activity of *V. rosea* prolyl hydroxylase could be made for the crude cell extracts. But, as shown in Table I, we were able to detect the activity of a prolyl hydroxylase, which hydroxylates poly-L-proline,

TABLE I

SUBCELLULAR DISTRIBUTION OF PROLYL HYDROXYLASE

Activity of prolyl hydroxylase in subcellular fractions except for the soluble fraction was measured following extraction with 0.1% Triton X-100 and fractionation by $(NH_4)_2SO_4$ as described in Materials and Methods. The activity was assayed by determination of peptidyl hydroxyproline formed in an hour. The enzyme of the soluble fraction was directly precipitated by 0.65 saturated $(NH_4)_2SO_4$, following this, determination of activity was made.

Cellular fractions	Total activity (units per 240 g cells)	%
Cell debris, nuclear (1500 × *g* precipitate)	0	0
Mitochondrial (12 000 × *g* precipitate)	357	47.9
Microsomal (105 000 × *g* precipitate)	303	40.7
Soluble (105 000 × *g* supernatant)	85	11.4

in the extracts with 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.3 M NaCl and 0.5 mM β-mercaptoethanol from the mitochondrial, microsomal and supernatant fractions. After precipitation by $(NH_4)_2SO_4$, the activities of these fractions could be determined quantitatively, on the basis of linearity, in regard to both the time course of the enzyme reaction and the dependency of the activity on protein concentration in the extracts.

Purification of prolyl hydroxylase

Following the $(NH_4)_2SO_4$ fractionation of 0.1% Triton X-100 extract from the mitochondrial and microsomal fractions, prolyl hydroxylase was chromatographed on DEAE-Sephadex A-50 (Fig. 1). Elution was performed stepwise, and the enzyme was eluted with 0.15 M NaCl. By pooling the fraction 14—20, 96.7% of the applied activity could be recovered in the form of a 10.7-fold purified enzyme. Assuming that almost all the activity in the initial homogenate was recovered in the $(NH_4)_2SO_4$ fraction except for the remaining activity in 105 000 × *g* supernatant fraction, a purification exceeding that of the initial homogenate by about 600-fold was achieved up to the step of DEAE-Sephadex A-50. It was found that this preparation contained neither hydroxyproline-containing proteins nor nonspecific α-ketoglutarate decarboxylating enzyme, judging from the hydroxyproline determination of this preparation and from the requirements of this enzyme as described below. This preparation was unstable at 4° C, and the activity decreased to about one-half for 3 days at 4° C. Freeze-thawing also resulted in a decrease in activity. The activity was retained in 25% glycerol at —80° C for a month. The preparation dialyzed against 25% glycerol was stored frozen at —80° C in aliquots until use.

Requirement for prolyl hydroxylase

The requirements for prolyl hydroxylase were examined by two methods: the hydroxyproline determination (Table II) and decarboxylation of α-ketoglutarate (Table III). The hydroxylation of poly-L-proline required Fe^{2+},

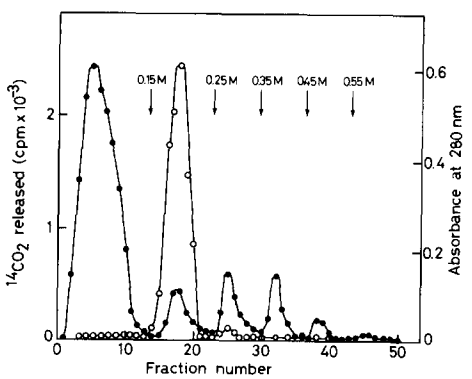

Fig. 1. DEAE-Sephadex A-50 column chromatography of prolyl hydroxylase. The $(NH_4)_2SO_4$ fraction was applied to a DEAE-Sephadex A-50 column (1.2 × 15 cm), previously equilibrated with 50 mM Tris-HCl buffer (pH 7.8), containing 50 mM NaCl, 0.5 mM β-mercaptoethanol and 10% glycerol. Elution was carried out stepwise with the same buffer containing NaCl, as indicated by the arrow in the figure. Fractions of 3.0 ml were collected. Open circles (○———○) and closed circles (●———●) represent the activity of prolyl hydroxylase and absorbance at 280 nm, respectively.

TABLE II

REQUIREMENT FOR HYDROXYLATION OF POLY-L-PROLINE BY PROLYL HYDROXYLASE

The prolyl hydroxylase was assayed by determining the hydroxyproline formed in an hour, using 3 units of enzyme under standard conditions. Poly-L-proline (M_r 6000) was used as substrate. All values are corrected by no incubation control.

Condition	Hydroxyproline formed (nmol)	Relative activity (%)
Complete (duplicates)	3.07, 2,98	100, 97.1
Fe^{2+}	0	0
Ascorbate	0.39	12.7
α-Ketoglutarate	0.32	10.4
Dithiothreitol	1.57	51.1
Poly-L-proline *	0	0
α-Ketoglutarate + pyruvate **	0.25	8.1
α-Ketoglutarate + oxaloacetate **	0.49	16.0

* After the reaction was stopped by the addition of trichloroacetic acid, poly-L-proline was added.
** Pyruvate and oxaloacetate were added to the reaction mixture having a final concentration of 0.5 mM.

ascorbate and α-ketoglutarate. Dithiothreitol activated the reaction. Pyruvate and oxaloacetate could not replace the α-ketoglutarate. We confirmed that α-ketoglutarate was decarboxylated during the hydroxylation of poly-L-proline by using α-[1-^{14}C]-ketoglutarate. In the absence of Fe^{2+}, $^{14}CO_2$ evolution was reduced to a level lower than that of the case in which either enzyme or substrate was omitted. This indicates that Fe^{2+} accelerates the spontaneous decarboxylation of α-ketoglutarate. Enzymic decarboxylation was calculated by subtracting the spontaneous decarboxylation value when either enzyme or substrate was omitted from the observed value. The decarboxylation of α-ketoglutarate required Fe^{2+}, ascorbate and poly-L-proline. In particular, it should be emphasized that enzymic decarboxylation depends completely on the presence of poly-L-proline. In addition, the rate (2.89 nmol/h) of $^{14}CO_2$ evolution from α-ketoglutarate was stoichiometrically in agreement with the rate

TABLE III

REQUIREMENT OF DECARBOXYLATION OF α-KETOGLUTARATE BY PROLYL HYDROXYLASE

The prolyl hydroxylase was assayed by determination of $^{14}CO_2$ released from α-[1-^{14}C]ketoglutarate using 3 units of enzyme under standard conditions, except in the case of the final concentration of α-ketoglutarate of 0.2 mM. The reaction proceeded for 30 min. Poly-L-proline (M_r 6000) was used as the substrate. Relative activities are calculated after the value in no enzyme control is subtracted from the observed values.

Condition	$^{14}CO_2$ released (cpm)	Relative activity (%)
Complete	5820	100
Fe^{2+}	227	—
Ascorbate	1134	1.6
Dithothreitol	3754	43.5
Bovine serum albumin	1935	18.5
Poly-L-proline	999	0
Enzyme	1056	0

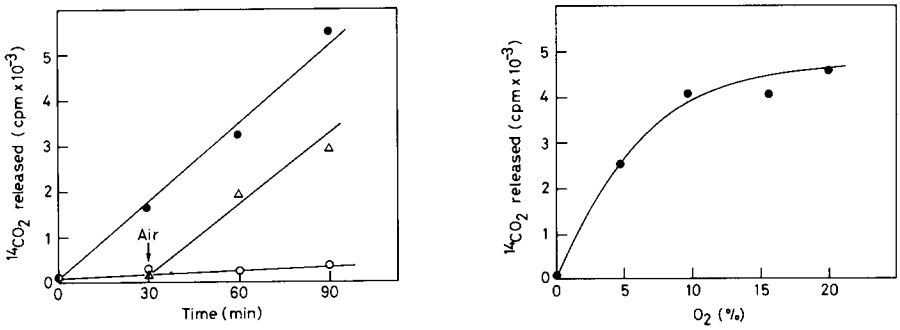

Fig. 2. O_2 requirement for prolyl hydroxylase activity. All the reaction tubes were evacuated at 0°C and filled with N_2. The same operation was then repeated twice to bring about complete replacement with N_2. When the reaction was performed in air, a stream of air was blown into the reaction mixture. Closed circles (●——●) and open circles (○——○) represent the activity of prolyl hydroxylase in air and with N_2. Open triangles (△——△) represent the activity when air was blown into the reaction mixture at 30 min following the start of incubation. All points are corrected by no substrate control under each condition.

Fig. 3. Effect of variation in O_2 concentration on prolyl hydroxylase activity. The reaction tubes were replaced with mixed gasses, having a defined O_2 concentration in N_2, in a similar manner as described in legend to Fig. 2. All points are corrected by no substrate control under each condition.

(3.0 nmol/h) of hydroxylation of poly-L-proline in this enzymic reaction. Since the absolute requirements of molecular oxygen (Figs. 2 and 3) are consistent with the reaction mechanism of vertebrate prolyl hydroxylase, *V. rosea* prolyl hydroxylase may also catalyze the same reaction as that of vertebrate prolyl hydroxylase but not that of the peptidyl substrate.

Properties of prolyl hydroxylase

Effect of pH. The effect of pH on the enzymic activity was examined, using 40 mM Hepes buffer (pH 6.8—7.6), 40 mM Mes buffer (pH 5.5—6.7) and 40 mM Pipes buffer (pH 6.15—7.35). The optimum pH was 6.8 in Pipes buffer. Both potassium phosphate buffer and sodium acetate buffer almost completely inhibited the activity of the enzyme. Tris-acetate buffer (pH 7.0—7.6) reduced to about one-half of the activity of each pH of Hepes buffer.

Michaelis constant. The Michaelis constants for poly-L-proline and α-keto-

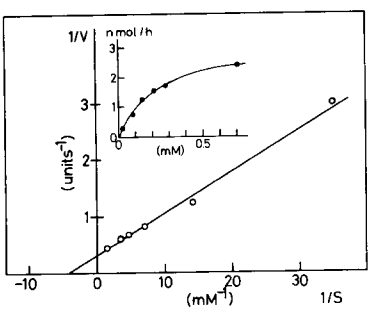

Fig. 4. Lineweaver-Burk and Michaelis-Menten plots illustrating the effect of poly-L-proline (M_r 6000) on the catalytic rate of prolyl hydroxylase. All points are corrected by no substrate control.

glutarate of the enzyme were determined by double-reciprocal plots of initial velocities vs. poly-L-proline (Fig. 4) and the cosubstrate concentration. The apparent K_m values for poly-L-proline and α-ketoglutarate were 0.23 mM (as proline residues) and 0.09 mM, respectively. The apparent K_m for O_2 was roughly estimated to be 0.06 mM on the basis of the half-maximum rate graphically determined in Fig. 3 and solubilities of oxygen at 30° C.

Effect of some inhibitors. The activity of the enzyme was inhibited by a high concentration of salts. By addition of more than 0.3 M NaCl, no activity was observed. Poly-L-hydroxyproline (M_r 30 000) served as an inhibitor of the reaction and 200 μg and 600 μg poly-L-hydroxyproline per standard assay exhibited 18.7% and 40.9% of inhibition, respectively. Inhibition of succinate at concentrations of 0.5 and 3 mM was 11.5 and 38.3%, respectively.

Substrate specificity. Using synthetic peptides and polypeptides, the substrate specificity of prolyl hydroxylase was examined (Table IV). Three poly-L-prolines prepared from *N*-carboxy-L-proline anhydride (average degree of polymerization of 20, 60 and 120) served as good substrates, and three derivatives of octa-L-prolyl peptides also performed as good substrates. On the other hand, the prolyl hydroxylase from *V. rosea* could not react with either free proline or prolyl peptides whose residual number was four or less. The model peptides of unhydroxylated collagen, $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ served but poorly as substrates for the enzyme. If these peptides were thermally denatured immediately before the reaction, the rate of hydroxylation of the peptides by *V. rosea* prolyl hydroxylase did not increase.

TABLE IV

SUBSTRATE SPECIFICITY OF PROLYL HYDROXYLASE

The prolyl hydroxylase was assayed by determination of $^{14}CO_2$ released from α-[1-^{14}C]ketoglutarate for 30 min using 2.3 units of enzyme under standard conditions, except for the final concentration of α-ketoglutarate of 0.1 mM. All peptides were added to bring about a final concentration of 6.67 mM as proline residues. All values are corrected by no substrate control.

Peptides	$^{14}CO_2$ released (cpm)	Relative activity (%)
Pro	0	0
Pro_2	0	0
Pro_3	56	0.9
Pro_4	28	0.5
Pro_8 *	4979	81.8
t-Butyloxycarbonyl-Pro_8	5562	91.4
Gly-Pro_8	5840	96.0
Poly-L-proline (M_r 2000)	6085	100
Poly-L-proline (M_r 6000)	5901	97.0
Poly-L-proline (M_r 12 000)	5944	97.8
$(Pro\text{-}Pro\text{-}Gly)_5$	1150	18.9
$(Pro\text{-}Pro\text{-}Gly)_{10}$	980	16.1

* Octa-L-proline was prepared from *t*-butyloxycarbonyl-octa-L-proline, the *t*-butyloxycarbonyl group of which was removed by treatment with trifluoroacetic acid at room temperature for 30 min.

Discussion

A hydroxyproline-rich glycoprotein linked covalently to the plant cell wall has not yet been isolated as an intact molecule, and the biosynthesis of the glycoprotein has thus remained obscure. In particular, it has still not been elucidated why the glycoprotein is hydroxylated in preference to many other proteins in a plant cell. Of course, since the complete peptidyl primary structure of the glycoprotein has not yet been determined, no one can speculate whether or not the specificity of plant prolyl hydroxylase can carry out this preferential hydroxylation of the glycoprotein. Two possibilities may be considered as an answer to this question: a plant prolyl hydroxylase has a defined substrate specificity, or the enzyme having no substrate specificity can hydroxylate only a precursor protein of the glycoprotein at a specific cytological site. Our present work suggests that the former possibility is more likely.

As to the plant prolyl hydroxylase, only Sadava and Chrispeels have hitherto reported a prolyl hydroxylation system in aged carrot disks [20]. Their partially purified enzyme reacted not only with ^{3}H-labelled natural plant substrate, prepared by incubating the carrot disks with [3,4-^{3}H]proline in the presence of α,α'-dipyridyl, but also equally with [^{3}H]protocollagen, vertebrate substrate prepared from chick embryo in a similar manner. Based on this observation, they concluded that the enzyme might not recognize a defined peptidyl primary structure. However, their observation does not necessarily indicate that the carrot enzyme lacks substrate specificity, since the tritium-release assay cannot be used, in practice, for comparison of the attributes of two natural substrates with each other. These substrates are labelled in vivo with [3,4-^{3}H]-proline in different tissues, independently. Such preparations are heterogeneous and possibly include certain inhibitors, and show a different content of proline residues to be hydroxylated and different specific radioactivities from each other. Indeed, these authors mentioned that the plant substrate was heterogeneous and could not be easily stored.

Considering the difficulty in isolating a defined natural substrate of a plant prolyl hydroxylase, we chose a poly-L-proline (M_r 6000) as a plant substrate model. This is because hydroxyproline-rich glycoprotein in the cell wall contains oligomeric hydroxyprolyl sequences [22]. A prolyl hydroxylase hydroxylating poly-L-proline was found in *V. rosea* suspension-cultured cells, mainly in both the mitochondrial and microsomal fractions. The enzyme was solubilized from these particulate fractions with 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.3 M NaCl and β-mercaptoethanol. This enzyme may be loosely bound to the membrane or it may have an affinity toward ribosomes; but it does not seem to be integrated with the membranes, since a smaller but still considerable amount of the enzyme could be extracted even when the buffer contained no Triton X-100. Since cycloheximide stopped the hydroxylation of proline residues without any lag time in the protoplasts prepared from *V. rosea* [3], this enzyme is thought to be situated near the site of the protein synthesis in *V. rosea* cells. This leads us to consider that the enzyme may have a defined substrate specificity for hydroxylating only a precursor protein, but that the enzyme is not situated at specific cytological site for doing this.

The substrate specificity of the enzyme from *V. rosea* was examined by using synthetic prolyl peptides and polypeptides (Table IV). Three poly-L-prolines (average degree of polymerization of 20, 60 and 120) served as good substrates, and three derivatives of octa-L-proline also performed as good substrates. On the other hand, free proline and prolyl peptides, each having a residual number of four or less, could not be hydroxylated. These data indicate that the enzyme requires an oligomeric prolyl sequence in the substrate. Both $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$, model substrates of vertebrate prolyl hydroxylase were poor substrates for the enzyme; however, these substrate were hydroxylated at a considerable rate (Table IV). This suggests that *V. rosea* enzyme does not always require an octa-L-prolyl sequence in a polymeric substrate. Considering that hydroxyproline-rich glycoprotein 'extensin' is known to have at least tetra-L-hydroxyprolyl sequences [22], it is interesting as to how long the oligo-L-prolyl peptide-chain is as a minimum substrate for the enzyme.

In addition to prolyl substrate, out enzyme prepared from *V. rosea* cells required O_2, α-ketoglutarate, ascorbate and Fe^{2+}. α-Ketoglutarate was decarboxylated during the hydroxylation of poly-L-proline. Pyruvate and oxaloacetate could not replace α-ketoglutarate as a cosubstrate, in contrast to the carrot enzyme [20]. This suggests that the enzyme hydroxylates poly-L-proline by a mechanism similar to the hydroxylation of prolyl-glycyl-peptide by vertebrate prolyl hydroxylase. This enzyme should thus be classified as an oxygenase acting on paired donors, that is, α-ketoglutarate and peptidyl proline in oligomeric prolyl sequence.

We consider that the prolyl hydroxylase is a novel prolyl hydroxylase, possessing a quite different peptidyl substrate specificity to that of vertebrate prolyl hydroxylase. A further characterization of the plant enzyme and isolation of natural substrate, by using the enzyme as a probe, will reveal the process of hydroxylation in the biosynthesis of 'extensin' at the molecular level.

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