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Elicitor-induced prolyl hydroxylase from French bean (*Phaseolus vulgaris*)

Localization, purification and properties

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The enzyme prolyl hydroxylase (proline:2-oxoglutarate dioxygenase, EC 1.14.11.12), induced in suspension-cultured cells of *Phaseolus vulgaris* L. (French bean) by treatment with an elicitor preparation from the phytopathogenic fungus *Colletotrichum lindemuthianum*, has been investigated. The enzyme, which catalyses the hydroxylation of poly-L-proline with the stoichiometric decarboxylation of 2-oxoglutarate, has been shown to be localized mainly in smooth endoplasmic reticulum. After solubilization from microsomal membranes, the hydroxylase was purified by ion-exchange chromatography and affinity chromatography on poly-L-proline–Sepharose 4B. The subunit M_r, as assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, was 65000, the subunit apparently being recovered as a doublet; the subunits associate under non-denaturing conditions to give at least a tetramer. The bean hydroxylase has kinetic properties and cofactor requirements similar to those previously reported for the enzyme from other plants. Elicitor treatment of suspension-cultured bean cells leads to a rapid induction of prolyl hydroxylase activity concomitant with induction of a protein:arabinosyl-transferase and increased levels of an arabinosylated hydroxyproline-rich protein.

Hydroxyproline residues, frequently *O*-glycosylated, are found in a number of plant proteins, the bulk of which are located in the cell wall. The range of types of hydroxyproline-rich glycoproteins and their roles in cell structure and function and in disease resistance have now been established (Fincher *et al.*, 1983; Gould & Northcote, 1985; Roberts *et al.*, 1985). Hydroxylation of proline is a post-translational modification for both animal (Kivirikko & Myllylä, 1982) and plant (Chrispeels, 1969) proline-rich precursors. The hydroxylation of peptide proline by prolyl hydroxylase (proline:2-oxoglutarate dioxygenase, EC 1.14.11.12) requires the stoichiometric decarboxylation of oxoglutarate in both animals (Kivirikko *et al.*, 1972) and plants (Tanaka *et al.*, 1980), and is usually assayed by this parameter. Substrate specificity has been analysed for the plant enzyme (Tanaka *et al.*, 1980; Cohen *et al.*, 1983; Erickson *et al.*, 1984) in both crude and partially purified preparations, although poly-L-proline is the substrate routinely used in assays. Although the vertebrate enzyme has been purified to homogeneity from a number of sources (Kivirikko & Myllylä, 1982) and nearly to the same extent in plants (Tanaka *et al.*, 1981), to our knowledge no subunit M_r has yet been cited for the plant enzyme. Furthermore, there are conflicting reports on the subcellular localization of the proline hydroxylation reaction in plants; it is not yet clear whether it is confined to the endoplasmic reticulum or whether there is a significant contribution by Golgi membranes (Karr, 1972; Gardiner & Chrispeels, 1975; Wienecke *et al.*, 1982; Samson *et al.*, 1983; Cohen *et al.*, 1983). There is, in addition, considerable interest in the developmental regulation of prolyl hydroxylase in relation to its endogenous substrates in plants. We have recently demonstrated a striking induction of membrane-bound prolyl hydroxylase activity in suspension-cultured French-bean cells exposed to elicitor macromolecules from cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* (Bolwell *et al.*, 1985). We have now purified the

bean prolyl hydroxylase to homogeneity and examined its subcellular distribution and some aspects of its regulation.

Materials and methods

Poly-L-proline ($M_{r,av.}$ 30000 and 8000), CNBr-activated Sepharose 4B and poly-L-hydroxyproline were obtained from Sigma. [2-^{14}C]Oxoglutarate (2.0GBq/mmol) was obtained from Amersham International. Poly-L-proline ($M_{r,av.}$ 30000) was coupled to CNBr-activated Sepharose 4B (Kivirikko & Myllylä, 1982).

Fungal-elicitor preparation

Colletotrichum lindemuthianum was grown in the dark for 10 days at 25°C in a 9-litre aerated batch fermenter in Mathur (Mathur *et al.*, 1949) medium modified by the addition of 15g of glucose/litre. Preparation of mycelial walls and heat-release of the crude cell-wall elicitor fraction were as previously described (Dixon & Lamb, 1979).

Growth and treatment of plant cell cultures

Cell-suspension cultures of *Phaseolus vulgaris* cultivars Canadian Wonder and Immuna were grown in total darkness in a modified Schenk & Hildebrandt medium as previously described (Dixon *et al.*, 1981). Cell cultures, 6–8 days after subculture, were exposed to *Colletotrichum* elicitor (30μg of glucose equivalents/ml of culture) for 6h, harvested by vacuum filtration, frozen in liquid N_2 and stored at −70°C until required.

Preparation of membranes

Cells were homogenized (1g of tissue/ml of buffer), in a pestle and mortar, in 50mM-Tris/HCl buffer, pH8.0, containing 1mM-dithiothreitol, 0.4M-sucrose, 0.3M-KCl and 20mM-$MgCl_2$ at 4°C. The slurry was filtered through muslin and centrifuged at 1000g for 15min. The supernatant was then centrifuged at 15000g for 10min to sediment the larger organelles. The supernatant was then subjected to centrifugation at 100000g for 90min. The final microsomal pellet was resuspended in 50mM-Tris/HCl buffer, pH7.4, containing 1mM-dithiothreitol, 0.4M-sucrose, 50mM-KCl and 10mM-$MgCl_2$. This microsomal fraction was then subjected to further fractionation or enzyme-purification procedures as described below.

Membrane fractionation

Microsomes were fractionated on discontinuous sucrose gradients into free polysomes, rough endoplasmic reticulum and smooth membranes as described by Bolwell & Northcote (1983*a*,*b*). A microsomal fraction (1ml) was also analysed on a linear gradient of 10–40% (w/w) sucrose (6ml total volume) on a cushion of 55% (w/w) sucrose (1ml), all sucrose solutions containing 50mM-Tris/HCl buffer, pH7.4, 1mM-dithiothreitol, 50mM-KCl and 10mM-$MgCl_2$. After centrifugation at 100000g for 40min, fractions (400μl) were collected by pumping from the bottom of the tube. Fractions were assayed for prolyl hydroxylase (using the standard assay described below), protein (by the method of Read & Northcote, 1981), IDPase (Shore & MacLachlan, 1975) and antimycin-insensitive NADH:cytochrome *c* reductase (Lord *et al.*, 1973). Sucrose concentration in each fraction was determined by refractometry.

Enzyme purification

The resuspended microsomes were sampled for protein content and then made up to 0.1% (v/v) Triton X-100 and thoroughly mixed. Solid $(NH_4)_2SO_4$ was added with stirring to 65% saturation and the mixture equilibrated for 1h before centrifugation at 30000g for 30min. The pellet was taken up in 50mM-Tris/HCl buffer, pH7.4, containing 50mM-KCl, 10mM-$MgCl_2$, 0.5mM-2-mercaptoethanol and 10% (v/v) glycerol, and sonicated until clear. The sample was then dialysed for 16h against several changes of the same buffer at 4°C. The enzyme was subjected to ion-exchange chromatography on a column (1cm × 10cm) of DEAE-cellulose previously equilibrated with 50mM-Tris/HCl, pH7.4, containing 50mM-KCl, 10mM-$MgCl_2$, 0.5mM-2-mercaptoethanol and 10% (v/v) glycerol. The enzyme, which did not bind, was finally subjected to affinity chromatography on a column (1cm × 2cm) of poly-L-proline–Sepharose 4B. After a cycling phase, the column was washed sequentially with (*a*) 10ml of 50mM-Tris/HCl, pH7.4, containing 50mM-KCl, 10mM-$MgCl_2$ and 1mM-2-mercaptoethanol, and (*b*) 10ml of the same buffer but containing 1M-KCl. After all the non-specific proteins had been eluted, the enzyme could be released, with retention of activity, by elution with poly-L-proline ($M_{r,av.}$ 8000) (8mg/ml), or as an inactive protein with 6M-urea, in Tris/HCl buffer, pH7.4, containing 50mM-KCl, 10mM-$MgCl_2$ and 1mM-2-mercaptoethanol. Partially active enzyme could be recovered from the 6M-urea fraction by extensive dialysis against Tris/HCl buffer, pH7.4, containing 50mM-KCl, 10mM-$MgCl_2$ and 10mM-2-mercaptoethanol.

Assay of prolyl hydroxylase

Proline hydroxylation coupled to decarboxylation of oxoglutarate was measured by a micro-scale adaptation of the method of Tanaka *et al.* (1981) described by Bolwell *et al.* (1985).

For hydroxyproline measurements the incubation mixtures were made up to 6M with respect to HCl and hydrolysed at 100°C for 16h. After neutralization the hydroxyproline content was determined by the method of Kivirikko (1963).

For measurements of the stoichiometry of the decarboxylation of oxoglutarate and hydroxyproline formation the incubation mixture consisted of 25 μl of membrane (at least 150 μg of protein), 200 μg of poly-L-proline, 23 KBq of 2-[^{14}C]oxoglutarate (7.5 mM) in 50 mM-Hepes, pH 6.8, containing 1 mM-$FeSO_4$ and 2 mM-L-ascorbate in a total volume of 75 μl. $^{14}CO_2$ evolution and hydroxyproline were measured as in the standard assay.

M_r determinations

The M_r of the intact enzyme was determined by gel filtration on Sephacryl S-300. Solubilized microsomal preparations from elicitor-treated bean cells were dialysed against 50 mM-Tris/HCl, pH 8.0, containing 50 mM-NaCl, 1 mM-2-mercaptoethanol and 10% (v/v) glycerol and applied to a column (40 cm × 1 cm) of Sephacryl S-300 which had been pre-equilibrated with the same buffer, and calibrated over the M_r range 12000–700000 with proteins of known M_r.

The M_r of the enzyme was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 12%-acrylamide slab gels. Samples were prepared by boiling for 2 min in 0.05 M-Tris/HCl buffer, pH 6.8, containing 100 mM-dithiothreitol, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate and 0.006% Bromophenol Blue. After electrophoresis the gel was stained in Coomassie Blue, and tracks were scanned at 638 nm in an MSE densitometer (MPS type 940 800).

Results

Enzyme purification

Purification of prolyl hydroxylase was carried out by a modification of the method of Tanaka *et al.* (1981). The use of an homogenization buffer designed to maintain ribosomal binding and the integrity of endoplasmic reticulum, and probably binding of prolyl hydroxylase, resulted in differences in properties during purification protocol for the bean enzyme as compared with the previously reported *Vinca* (periwinkle) enzyme (Table 1). There was a higher recovery of enzyme in the microsomal fraction and the enzyme was totally particulate. After the addition of $(NH_4)_2SO_4$ the enzyme was precipitated. The timing of the sonication step appears to be critical here. Sonication before $(NH_4)_2SO_4$ fractionation often gave rise to enzyme recovery in both soluble and precipitated fractions. Sonication as employed here solubilizes the prolyl hydroxylase from the pellet. The bulk of the enzyme did not bind to DEAE-cellulose under the conditions employed. The enzyme bound avidly to immobilized poly-L-proline (M_r 30000) but could be eluted with poly-L-proline (M_r 8000). However, the prolyl hydroxylase could not be completely eluted from the column, even with 6M-urea, and enzyme could still be detected bound to the gel if it was solubilized by boiling the gel in sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis sample buffer. This avid binding has been found for the animal enzyme, where recoveries from the affinity column were usually 20–50% (Tuderman *et al.*, 1975) under similar conditions. Ability to recover the enzyme in 6M-urea has the advantage of being less expensive, and some activity can be restored by extensive dialysis against buffer containing 10 mM-2-mercaptoethanol. The recovery of protein showed that the enzyme, which was purified over 3000-fold, was a relatively minor component of the microsomes.

Properties of the purified enzyme

Gel filtration of the solubilized enzyme on Sephacryl S-300 gave an M_r for the multimeric complex in excess of 300000. This may indicate levels of aggregation similar to that exhibited by the tetramer found for the animal enzyme, which was eluted similarly on gel filtration but gave an M_r of 240000 on equilibrium centrifugation (Tuderman *et al.*, 1975). Sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis (Fig. 1) showed that the final product was recovered as a possible

Table 1. *Purification of prolyl hydroxylase from Phaseolus vulgaris (cultivar Canadian Wonder) cell suspension cultures*
The purification of the enzyme from 100g of elicited cells is shown.

Fraction	Total activity (nkat)	Total protein (mg)	Specific activity (μkat·kg^{-1})	Purification (-fold)	Recovery (%)
Crude extract	94.9	225	0.42	1	100
Solubilized microsomes	69.3	8.9	7.79	19	73
DEAE cellulose	52.8	1.2	44.0	104	56
Affinity chromatography:					
(*a*) Poly-L-proline-eluted	24.8	0.017	1458.8	3473	26
(*b*) 6M-Urea-eluted, then dialysed	0.93	0.024	38.8	—	1

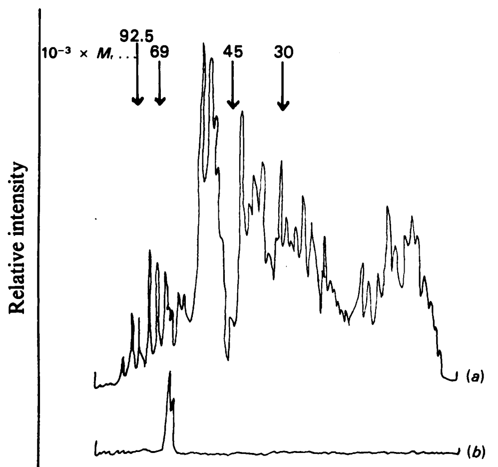

Fig. 1. *Electrophoretic profiles of solubilized bean microsomes and purified prolyl hydroxylase after analysis on a sodium dodecyl sulphate/polyacrylamide gel*

Samples were prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described in the Materials and methods section, and were analysed on a 12% gel. Densitometer profiles of Coomassie Blue-stained gel tracks are shown for (*a*) total solubilized bean microsomal proteins (100 μg) and (*b*) affinity-purified prolyl hydroxylase (10 μg).

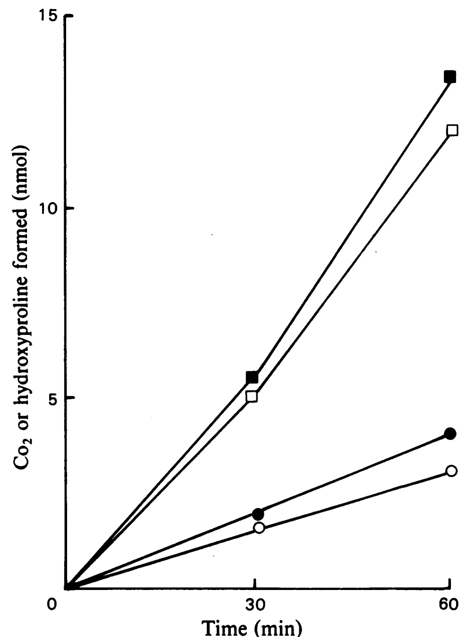

Fig. 2. *Stoichiometric evolution of* $^{14}CO_2$ *and production of hydroxyproline by a solubilized bean membrane preparation.*

Time courses are shown for hydroxyproline production in the presence (□) and absence (○) of poly-L-proline, and for $^{14}CO_2$ evolution from [2-^{14}C]oxoglutarate in the presence (■) and absence (●) of poly-L-proline, by a solubilized bean membrane preparation (192 μg of protein/assay).

doublet with a subunit M_r of 65000, a value very similar to that of one of the animal-enzyme subunits (Kivirikko & Myllylä, 1982).

Requirements for prolyl hydroxylation

The enzyme was characterized and routinely assayed by the coupled decarboxylation of oxoglutarate by microsomal preparations in the presence of poly-L-proline. The enzyme required the presence of Fe^{2+} and O_2 and was activated by the addition of 0.1% (v/v) Triton X-100. Addition of ascorbate had a limited effect, probably indicating the presence of a pool of endogenous reductants. Rates of CO_2 release were approximately linear up to 60 min and were stoichiometrically in agreement with hydroxyproline production in the large-scale assay (Fig. 2). Appreciable hydroxyproline production occurred in controls and probably indicates a substantial pool of endogenous substrate rather than contamination with mitochondrial decarboxylase.

The enzyme was inhibited by the chelators EDTA, αα-bipyridyl and salicylyl hydroxamate, consistent with the requirement for Fe^{2+}. The specific chelator αα-bipyridyl decreased decarboxylation to zero in controls, thus negating the validity of this inhibitor in assays where it is used in control incubations. A possible requirement for Mg^{2+} is indicated by an inhibitory effect of EGTA.

Kinetic analysis

The enzyme exhibited Michaelis–Menten kinetics for poly-L-proline (M_r 30000), with a K_m of 5 μM (Fig. 3). This compares with K_m values with respect to poly-L-proline (M_r 6000–8000) of 4 μM for the *Vinca* enzyme (Tanaka *et al.*, 1980) and 40 μM for the enzyme from *Lolium* (ryegrass) (Cohen *et al.*, 1983).

Subcellular distribution

The distribution of prolyl hydroxylase in microsomal membranes prepared by differential centrifugation was examined in discontinuous (Table 2) and linear (Fig. 4) sucrose gradients with the buffer systems which maximize attachment of ribosomes to endoplasmic reticulum. Under these conditions the bulk of the enzyme (88%) was retained in the microsomal fraction. Analysis of the microsomes on a previously characterized discontinuous system (Bolwell & Northcote, 1983*a*,*b*) showed that the highest activity was associated with smooth

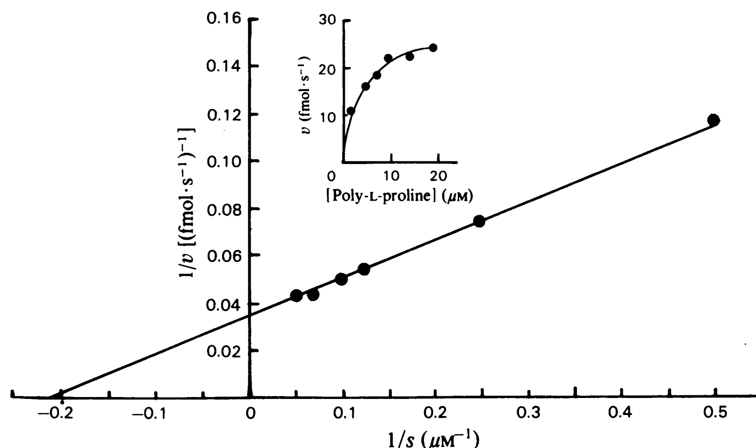

Fig. 3. *Effect of poly-L-proline concentration on the catalytic rate of bean prolyl hydroxylase*
The kinetic data show the effect of various poly-L-proline ($M_{r,av.}$ 30000) concentrations on the poly-L-proline-dependent evolution of $^{14}CO_2$ from [2-^{14}C]oxoglutarate catalysed by a solubilized bean microsomal preparation (78 μg of protein/assay).

Table 2. *Distribution of prolyl hydroxylase in subcellular fractions*
Tissue was homogenized and subjected to differential centrifugation. The microsomal fraction was then fractionated on a discontinuous gradient of 34% (w/w) sucrose and 55% (w/w) sucrose as described previously (Bolwell & Northcote, 1983*b*) to separate smooth and rough membrane. All fractions were resuspended in reaction buffer before measurement of prolyl hydroxylase activity.

	Total protein		Total activity		Specific activity	
Fraction	(mg)	(% of total)	(nkat)	(% of total)	(μkat·kg⁻¹)	(Relative sp. activity)
Homogenate	10.88	(100)	13.0	(100)	1.19	(1)
1000*g* pellet	0.51	(4.7)	2.3	(17.5)	4.50	(3.9)
15000*g* pellet	0.83	(7.6)	2.8	(22.2)	2.30	(2.9)
Microsomal fraction:	1.09	(10.0)	11.4	(87.6)	10.50	(8.8)
Rough membrane	0.66	(6.1)	1.8	(13.6)	2.70	(4.5)
Smooth membrane	0.32	(2.9)	8.9	(68.4)	27.8	(32.0)
Supernatant	7.83	(72)	0	(0)	0	(0)
Recovery (%) ...	94.3		127.3		—	

rather than rough membrane. Fractionation of microsomes on the linear gradients showed a peak of activity clearly associated with antimycin-insensitive NADH: cytochrome *c* reductase activity, which is a well-characterized marker for endoplasmic reticulum in plants (Lord *et al.*, 1973; Nagahashi & Beevers, 1978). In contrast, latent IDPase, a marker for plant Golgi apparatus (Ray *et al.*, 1969; Morré & Buckout, 1979) showed a distinct peak of activity that coincided with a limited amount of prolyl hydroxylase. Bean prolyl hydroxylase appears to be preferentially associated with smooth endoplasmic reticulum.

Discussion

Post-translational hydroxylation of proline residues can occur for a range of protein sequences *in vivo* as borne out by the subset of hydroxyproline-rich glycoproteins produced by the plant cell. Prolyl hydroxylase activity has already been demonstrated *in vitro* against both poly-L-proline peptides with at least five residues and, for the partially purified enzymes, polymers of repeated Pro-Pro-Gly peptides (Tanaka *et al.*, 1980, 1981) and Ser-(Pro)$_n$ peptides (Erickson *et al.*, 1984). In view of this complex substrate requirement, and in the absence of knowledge of the type of repeated sequence within the bean endogenous substrates, the enzyme activity hydroxylating poly-L-proline has been purified in this study.

Elicitation of suspension-cultured bean cells by a cell-wall preparation from *Colletotrichum lindemuthianum* gives rise to a rapid accumulation of hydroxyproline residues in the cell wall (Bolwell *et al.*, 1985), a significant proportion of which may be

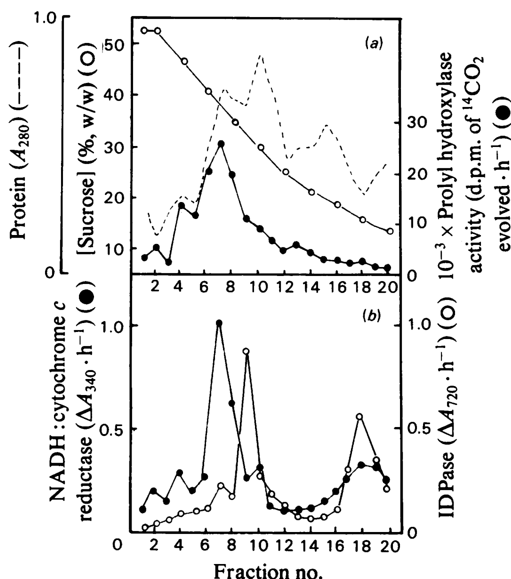

Fig. 4. *Fractionation of bean microsomal membranes on a linear sucrose gradient (10–55%, w/w)*

After centrifugation, fractions were collected and analysed for (*a*) protein, prolyl hydroxylase activity and sucrose concentration, and (*b*) antimycin-insensitive NADH:cytochrome *c* reductase and IDPase activities. Recoveries of enzyme activities on the gradient were: prolyl hydroxylase, 106%; NADH:cytochrome *c* reductase, 82%; IDPase, 88%.

accounted for by a specific arabinosylated glycoprotein of M_r 42500 (Bolwell, 1984). These changes are accompanied by a rapid 20–30-fold increase in prolyl hydroxylase activity as measured by the poly-L-proline-dependent decarboxylation of oxoglutarate and increased protein: arabinosyl-transferase (Bolwell *et al.*, 1985). Induction of this enzyme followed similar kinetics to the rapid induction of phenylalanine ammonia-lyase and chalcone synthase in this system (Robbins *et al.*, 1985), two enzymes whose increased appearance in elicitor-treated cells is apparently a consequence of increased gene expression *de novo* (Bell *et al.*, 1984; Ryder *et al.*, 1984). Further studies on the molecular mechanisms underlying the induction of prolyl hydroxylase by fungal elicitors clearly required the purification of the enzyme before production of a monospecific anti-(prolyl hydroxylase) serum.

The method of purification was modified from that of Tanaka *et al.* (1981). The modifications gave rise to important differences for the bean enzyme, in particular high recovery in a single fraction at each purification stage, which is not observed without these changes. The choice of a homogenization buffer which optimized ribosomal binding to endoplasmic reticulum, and the timing of the sonication step, proved particularly important, otherwise considerable activity can be recovered in soluble fractions during membrane preparation (Sadava & Chrispeels, 1971) or in different fractions from $(NH_4)_2SO_4$ precipitation and the DEAE-cellulose step (Erickson *et al.*, 1984). Recovery of activity in more than one fraction may, however, reflect a possible multiplicity of the enzyme. In the present study the enzyme was recovered in a single fraction at each stage until the affinity step. The enzyme bound avidly to the column and was eluted specifically with poly-L-proline or with 6M-urea. Some enzyme always remained bound to the column. The pure enzyme had a subunit M_r of 65000, a value similar to that of the α-subunit of the animal enzyme (Kivirikko & Myllylä, 1982), and this was possibly recovered as a doublet. Although the recovery of activity from the final step was high, the actual amount of enzyme detected by Coomassie Blue staining in protein assays or on gels was extremely low as compared with absorbance measurements. This probably demonstrates that the enzyme is present in small amounts in the cell or may reflect a further similarity in composition to the animal enzyme, which has a very high proportion of acidic residues and would be expected to stain poorly with Coomassie Blue. The multimeric enzyme had an M_r in excess of 300000 on gel filtration.

The properties of the enzyme with respect to cofactor requirement and K_m for poly-L-proline were similar to those previously published (Tanaka *et al.*, 1980, 1981; Cohen *et al.*, 1983). These requirements confirmed additional similarity to the animal enzyme, which does not, however, hydroxylate poly-L-proline (Kivirikko & Prockop, 1967).

The subcellular localization was investigated by fractionation procedures that give rise to well-defined membrane subsets in bean (Bolwell & Northcote, 1983*a*,*b*) and in linear sucrose gradients. Differential centrifugation showed the enzyme to be totally particulate (associated with microsomes), whereas other procedures (Sadava & Chrispeels, 1971; Tanaka *et al.*, 1980) lead to a significant association with other cell fractions or to solubilization of the enzyme. This indicates a rather loose association of the enzyme with membranes and therefore we have here utilized buffers and salt concentrations which optimize the integrity of the endomembrane system. Under these conditions the bulk of the enzyme is found associated with smooth endomembranes, unlike the vertebrate-animal enzyme, which is predominantly associated with rough endoplasmic reticulum (Grant & Jackson, 1976). Careful fractionation of bean microsomes on linear sucrose gradients indicated an association of the prolyl hydroxylase

with smooth endoplasmic reticulum, suggesting that the hydroxylation of endogenous sequences *in vivo* is completed before transfer to the Golgi apparatus and further processing. This is supported by evidence from experiments *in vitro*, which show that prolyl hydroxylase is inhibited by poly-L-proline (Tanaka *et al.*, 1980), whereas poly-L-hydroxyproline is a potent inhibitor of protein: arabinosyl-transferase (G. P. Bolwell, unpublished work), which is probably localized in the Golgi apparatus (Gardiner & Chrispeels, 1975; Owens & Northcote, 1981; Bolwell & Northcote, 1983*a*). This spatial separation indicates that the processing of putative proline-rich precursors is temporally separated, hydroxylation being completed in the endoplasmic reticulum before the commencement of arabinosylation in the Golgi.

Recent evidence suggests that hydroxyproline-rich glycoproteins will be assigned an increasingly important role in plant cell structure and plant–pathogen interactions. Thus interest in their regulation has grown, one important aspect of this being the need to understand the post-translational modifications that are essential for their functions. In bean, elicitor-modulated prolyl hydroxylase, whose induction correlates with increased hydroxyproline deposition in the wall, is an essential part of this regulation. The use of immobilized poly-L-proline, which can possibly function as a universal substrate, has enabled purification of the elicitor-induced enzyme. The final characterization of this enzyme will probably be dependent on knowledge of the range of sequences of the proline-rich endogenous substrates. Acquisition of these, deduced from the sequence of cDNA clones, has already begun (Showalter *et al.*, 1984).

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