J Plant Growth Regul (1984) 2:251–264

Journal of
Plant
Growth
Regulation

Purification and Partial Characterization of a Gibberellin 2β-Hydroxylase from *Phaseolus vulgaris*

Valerie A. Smith and Jake MacMillan

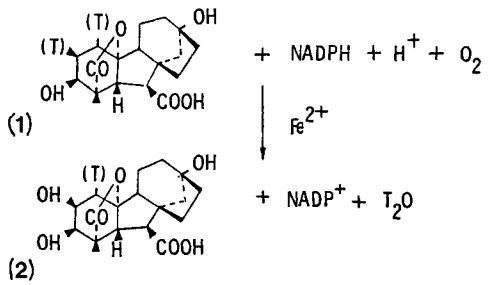
Agricultural Research Council Research Group, School of Chemistry, The University of Bristol, BS8 1TS, UK

Received June 7, 1983; accepted September 13, 1983

Abstract. A gibberellin 2β-hydroxylase has been purified from mature seeds of *Phaseolus vulgaris*. The enzyme is of molecular weight 36,000 and has the characteristics of a dioxygenase; the cofactors are α-ketoglutarate, Fe^{2+} and ascorbate, and activity is stimulated by catalase. The V_{max} of the enzyme is 6.86 nmole h^{-1} mg^{-1}, and the Km values for [1,2-$^3H_2$]GA_1 and α-ketoglutarate are 0.085 μM and 21 μM, respectively. The purified enzyme preparation catalyzes hydroxylation of GA_1, GA_4, GA_9, and GA_{20} but exhibits a marked preference for the 3-hydroxylated gibberellins as substrate.

The mature seed of *Phaseolus vulgaris* contains GA_1 and its biologically inactive metabolite, GA_8 (see Scheme 1, compounds 1 and 2 respectively). An enzyme that catalyzes the conversion of GA_1 to GA_8 has been located in the soluble protein fraction of an homogenate of the imbibed bean seeds by Patterson and Rappaport (1974). Since the GA_1-hydroxylating activity of this preparation required oxygen and was stimulated by the addition of exogenous Fe^{2+}, ascorbate, and NADPH, these authors propose that the enzyme responsible is a mixed function oxygenase operating as shown in Scheme 1.

The present investigation was carried out to corroborate and expand the preliminary work on 2β-hydroxylation of GAs and, in particular, to attempt to answer the question of substrate specificity of the hydroxylating enzyme present in the mature seeds of *Phaseolus vulgaris*. A preliminary account of this work has been presented (Hoad et al. 1982).



Scheme 1

Materials and Methods

Gibberellin Substrates

[1,2-$^3H_2$]GA_1 of specific activity 1.57×10^{15} Bq mole^{-1} (Nadeau and Rappaport 1974) was a gift from Professor L. Rappaport. [1,2-$^3H_2$]GA_4 of specific activity 1.66×10^{14} Bq mole^{-1} (Durley and Pharis 1973) was a gift from Professor R. P. Pharis. [2,3-$^3H_2$]GA_9 and [2,3-$^3H_2$] GA_{20} with specific activities of 1.72×10^{15} Bq mole^{-1} (Yokota et al. 1976) and 1.21×10^{14} Bq mole^{-1} (Murofushi et al. 1977), respectively, were gifts from Professor R. P. Pharis and Dr. A. Crozier.

Extraction Procedure

Approximately 50 g of mature bean seeds (*Phaseolus vulgaris* var. Canadian Wonder) were surface sterilized in a 5% v/v solution of hypochlorite, extensively washed and imbibed for 18 h at 23°C in sterile Petri dishes lined with Whatman No. 1 filter paper and containing sterile water (7 ml).

After imbibition and the removal of testas and embryos, the cotyledons were grated into liquid nitrogen and pulverized using a mortar and pestle. The meal thus obtained was allowed to warm to 4°C, was hand-homogenized at 4°C in 0.05M tris HCl, pH 7.2, containing 10 mM $MgSO_4$ (approx. 130 ml) and centrifuged for 2 h at 4°C and 18,000 $\times$ *g* in a Sorvall SP5 superspeed centrifuge. Lipid pellicles were then removed from the top of each centrifuge tube, and the supernatants decanted and aliquoted into MSE polycarbonate centrifuge tubes (25 ml). To each tube, a 3-ml underlay of 2 M sucrose in 0.05 M tris HCl, pH 7.2, containing 10 mM $MgSO_4$, was added before centrifugation for 18 h at 4°C and 120,000 $\times$ *g* in the 8 $\times$ 25-ml rotor of the MSE 65 ultracentrifuge. The supernatants above the sucrose cushions were carefully removed and concentrated 3-fold by millipore ultrafiltration. The resultant enzyme preparation was assayed and stored in liquid nitrogen. This extraction procedure could not be scaled up because of the limitation imposed by centrifugation. Combined batches of the preparations stored in liquid nitrogen were used for further purification of the enzyme by column chromatography.

Enzyme Assays

The 2β-hydroxylase activity was measured at 25°C by determining the rate of liberation of tritiated water when [1,2-$^3H_2$]GA_1 was used as substrate. Of the

total specific activity (1.57×10^{15} Bq mole^{-1}), the specific activity at the 2β-position was calculated to be 5.5×10^{14} Bq mole^{-1} by Nadeau and Rappaport (1974).

The enzyme solution (5–50 μl) was assayed in a final volume of 0.2 ml. The reaction mixture contained: ascorbic acid (5 mM); $FeSO_4$ (1 mM); α-ketoglutarate (2.5 mM); catalase (10 μg); and [1,2-$^3H_2$]GA_1 (1.83×10^9 Bq), dissolved in 0.05 M sodium acetate, pH 6.5. Reactions were stopped by the addition of a slurry of activated charcoal in 0.025 M EDTA, pH 7.0 (0.25 ml). The samples were then centrifuged to pellet charcoal-bound gibberellin. Aliquots (0.2 ml) of the supernatants were counted for radioactivity, present as tritiated water, in 16 ml of scintillation fluid (0.5% w/v Butyl B.P.D., 20% v/v methoxyethanol in toluene). All assays were carried out in duplicate.

Product Analysis

The purified enzyme was assayed under standard conditions. For quantitative estimation of tritiated water liberation, samples were removed with time, added to a slurry of activated charcoal, and centrifuged. The resultant supernatants were counted for radioactivity. For determination of GA_8 formation, samples were added to an equal volume of ice-cold ethanol, precipitated for 16 h at −20°C, and centrifuged. The supernatants were decanted, evaporated to dryness under vacuum, and redissolved in methanol-water (45:55) containing tetrabutylammonium hydrogen sulphate (0.05%). These samples were analyzed by reverse-phase HPLC using a Spherisorb 50 DS column (Technicol Ltd.) fitted to an Altex HPLC system. The column was equilibrated and eluted isocratically with methanol-water (45:55) containing the ion-pair complex (0.05%) at a flow rate of 1.2 ml min^{-1}. Authentic samples of GA_1 (retention vol. 6.0 ml) and GA_8 (retention vol. 3.8 ml) were used as references. Fractions were collected and counted for radioactivity.

Protein Determination

The protein content of the enzyme preparations at various stages of purification were estimated by the Biuret method (Gornall et al. 1949), by the Folin method (Lowry et al. 1951) or by measuring peptide absorption at 225 nm (A_{225}^{1cm} 1 mg ml^{-1} = 9.17) (Hall and Hartl 1975).

SDS Polyacrylamide Gel Electrophoresis

The protein samples obtained after various purification procedures were analyzed on 10–20% linear acrylamide gradient slab gels prepared and run using the procedure described by Laemmli (1970).

Estimation of Succinate Generation from α-Ketoglutarate

The purified enzyme was incubated in 0.05 M sodium acetate, pH 5.8, containing GA_1 (0.1 mM), ascorbic acid (5 mM), $FeSO_4$ (1 mM) and 2.0 mM α-

[^{14}C(U)]-ketoglutaric acid (3.26×10^{16} Bq mole^{-1} New England Nuclear) in a final volume of 20 µl. Control experiments were carried out in which enzyme was omitted or incubated in the presence of [1,2-$^{3}H_2$]GA_1 (0.1 nM) and cold α-ketoglutarate (2.0 mM). Aliquots (2 µl) of the reaction mixtures containing α-[^{14}C(U)]-ketoglutarate were removed at time intervals and spotted onto plastic-backed silica gel 60 TLC sheets (Merck Ltd.). Standard solutions of α-ketoglutarate and succinate were also applied as references. The TLC sheets were developed for 5 h in n-butanol-acetic acid-water (120:30:50). They were then dried, scanned to locate areas of radioactivity, and cut into strips. These strips were placed in scintillation vials and counted for radioactivity in Butyl BPD scintillation fluid (10 ml).

Estimation of Molecular Weight

The apparent molecular weight of the GA_1-hydroxylating enzyme was determined by gel filtration on a column (2.2 × 90 cm) of Sephadex G-100, equilibrated and eluted with 0.25 M sodium acetate, pH 6.5, at a flow rate of 4 ml h^{-1}. The column was calibrated with phosphorylase B (MW 98,000), bovine serum albumin (MW 68,000), ovalbumin (MW 45,000), chymotrypsinogen A (MW 25,000), and cytochrome C (MW 12,500).

Enzyme Purification

Unless otherwise stated, all operations were carried out at 4°C.

DEAE-Cellulose Chromatography

A column (35 × 3.2 cm) of DEAE-cellulose (Whatman DE-52) was equilibrated in 0.05 M tris HCl, pH 7.2, containing 10 mM $MgSO_4$. The soluble protein fraction of the bean seed homogenate (80–100 ml) was pumped onto the column and eluted with the tris HCl equilibration buffer at a flow rate of 26 ml h^{-1}. Fractions (26 ml) of the excluded protein were collected and assayed for 2β-hydroxylase activity. The enzyme-containing fractions were pooled, concentrated by millipore ultrafiltration (final volume 50–60 ml) and dialyzed against 0.05 M sodium acetate, pH 5.8, containing 10 mM $MgSO_4$.

CM-Cellulose Chromatography

The DEAE-cellulose enzyme solution (50–60 ml) was loaded onto a column (25 × 2.2 cm) of CM-cellulose (Whatman CM-52) equilibrated with 0.05 M sodium acetate, pH 5.8, containing 10 mM $mgSO_4$. The column was washed with the equilibration buffer for 16 h, at a flow-rate of 27 ml h^{-1}; then a linear salt gradient of 10 mM to 0.15 M $MgSO_4$ in 0.05 M sodium acetate, pH 5.8, (500 ml + 500 ml), generated using a three-channel peristaltic pump, was applied. Fractions (13.5 ml) were collected. The protein elution profile was

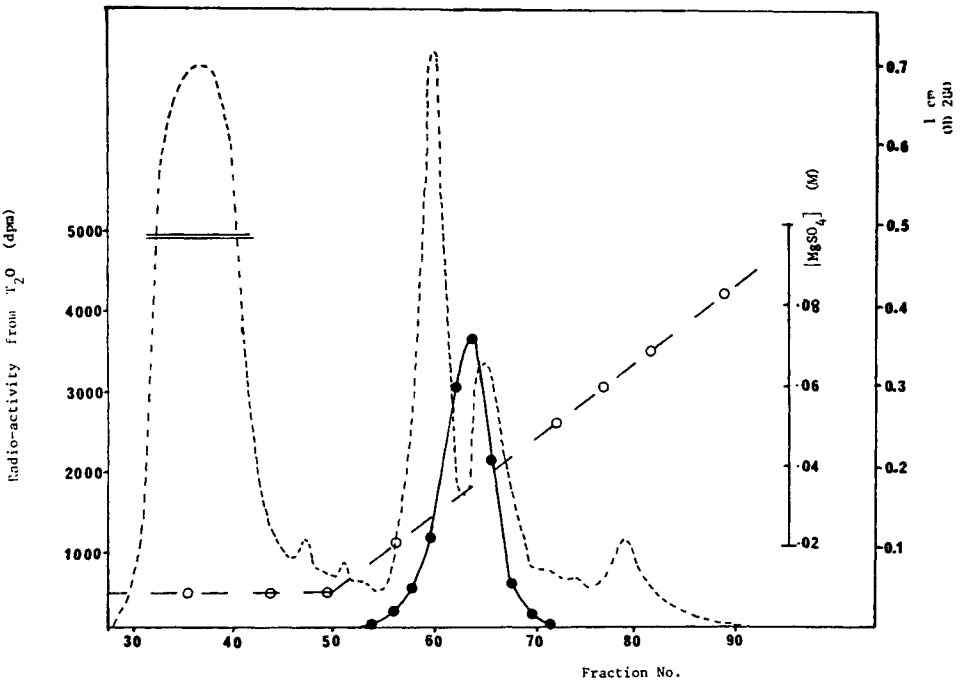


Fig. 1. CM-cellulose elution profile.
●—●—● enzyme activity; ------- protein elution profile; O—O—O $MgSO_4$ gradient.

monitored at 280 nm and the salt gradient determined by conductivity measurement. Fractions containing the 2β-hydroxylase activity (60–67) were pooled and after the addition of glycerol (10% v/v) concentrated to 5–8 ml. A typical CM-cellulose elution profile is shown in Fig. 1.

Gel Filtration

After concentration, the CM-cellulose enzyme solution (5 ml) was applied immediately to a column (90 × 2.2 cm) of Sephadex G-100, equilibrated with 0.25 M sodium acetate, pH 6.5, containing 10% (v/v) glycerol. The column was eluted with the sodium acetate buffer at a flow rate of 4 ml h^{-1}. Fractions (12 ml) were collected and assayed for activity. A typical elution profile is shown in Fig. 2. Enzyme-containing fractions (39–45) were pooled, concentrated by vacuum dialysis, and stored in liquid nitrogen.

Results

Enzyme Purification

As shown in Table 1, the overall purification of the enzyme preparation obtained after gel filtration on Sephadex G-100 was estimated to be 532-fold.

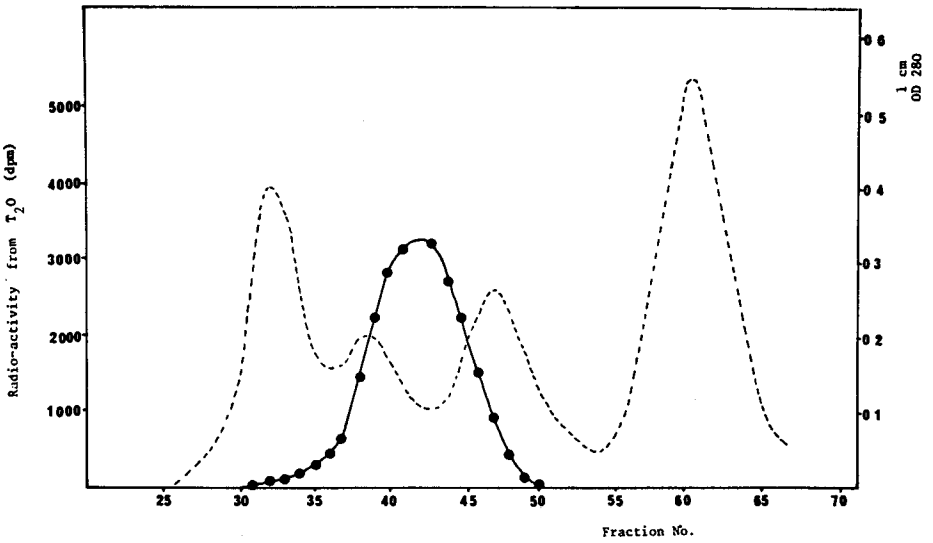


Fig. 2. Sephadex G-100 gel filtration elution profile.
●—●—● enzyme activity; ------- protein elution profile.

Table 1. Enzyme purification.

Fraction	Total units[a]	Specific activity	Purification (fold)	Recovery (%)
Crude homogenate (18,000 × *g* supernatant)	4396	0.36	—	100
Soluble protein fraction (120,000 × *g* supernatant)	786	0.40	1.12	17.9
DEAE-cellulose column	666	0.90	2.51	15.1
CM-cellulose column	415	10.2	28.3	9.44
Sephadex G-100 column	187	191	532	4.25

[a] 1 unit = 1×10^{-12} mole h^{-1}.

Major losses in activity occurred during ultracentrifugation over sucrose cushions. These losses were tolerated because the concomitant removal of much of the viscous material (polysaccharide/nucleic acid) from the preparation permitted further purification by chromatographic procedures.

The molecular weight of the enzyme, as determined by gel filtration on Sephadex G-100, was estimated to be 36,000. The purity of the enzyme preparation thus obtained was examined by SDS-polyacrylamide gel electrophoresis. Although a number of proteins were visualized on a gel of a sample of the purified enzyme, the major protein present in this sample was a very minor component in the proteins chromatographing either side of the peak of enzyme

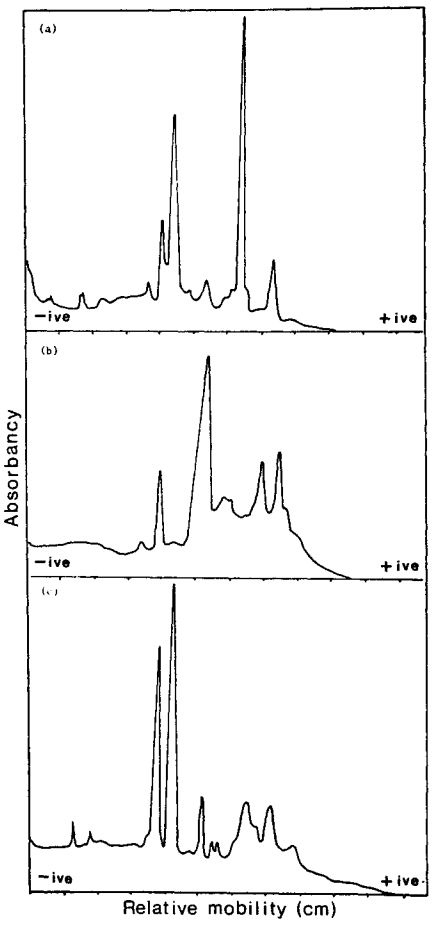


Fig. 3. SDS-polyacrylamide gel electrophoresis of proteins obtained after gel filtration on Sephadex G-100. a: Pre-enzyme protein fractions 32–38 inclusive in the Sephadex G-100 elution profile. b: Fractions 41 and 42 containing maximal enzyme activity. c: Post-enzyme protein fractions 47–49 inclusive.

activity on Sephadex G-100 (Fig. 3). Furthermore, the molecular weight of this protein was estimated to be 35,000 and it may thus represent the 2β-hydroxylase.

Validity of the Enzyme Assay Procedure

Confirmation that the production of tritiated water from [1,2-$^3H_2$]GA_1 resulted from the loss of label from the C-2 position and corresponded to the generation of GA_8 was obtained by reverse-phase HPLC of the reaction products (see *Materials and Methods*). Only two peaks of radioactivity, corresponding to GA_8 and GA_1, were detected in the samples injected onto the HPLC column. The results of this investigation are presented in Table 2 and show that GA_8 and tritiated water are produced in a 1:1 molar ratio. Furthermore, the disap-

Table 2. Quantitative analysis of the enzyme-catalyzed reaction products from 1,2-[^{3}H]GA_1.

Time (min.)	A_1 (pmole)	A_8 (pmole)	T_2O (pmole)
0	48.4	0	0
45	27.5	20.8	20.3
90	24.5	24.2	23.8

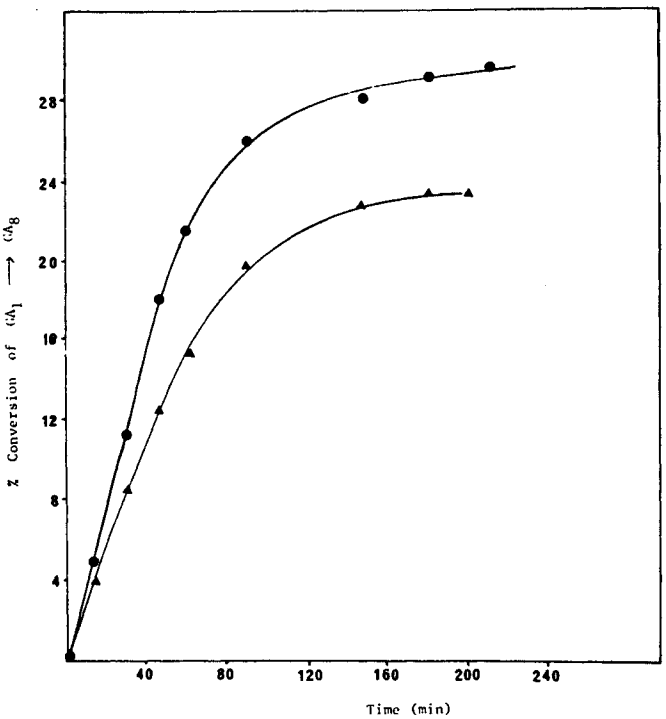


Fig. 4. Kinetics of *Phaseolus vulgaris* GA_1 2β-hydroxylase activity. ●—●—● + catalase; ▲—▲—▲ − catalase.

pearance of [1,2-$^3H_2$]GA_1 is accounted for entirely by the appearance of GA_8 and tritiated water.

Enzyme Cofactor Requirement

In preliminary experiments using the soluble protein fraction of a bean seed homogenate, it was found that the conversion of GA_1 to GA_8 occurred without the addition of exogenous cofactors. However, the GA_1-hydroxylating activity was stimulated markedly by the addition of Fe^{2+} and ascorbate, and further enhanced by the addition of NADPH. Dialysis of the extracted soluble protein resulted in complete loss of activity. This activity could be partially restored

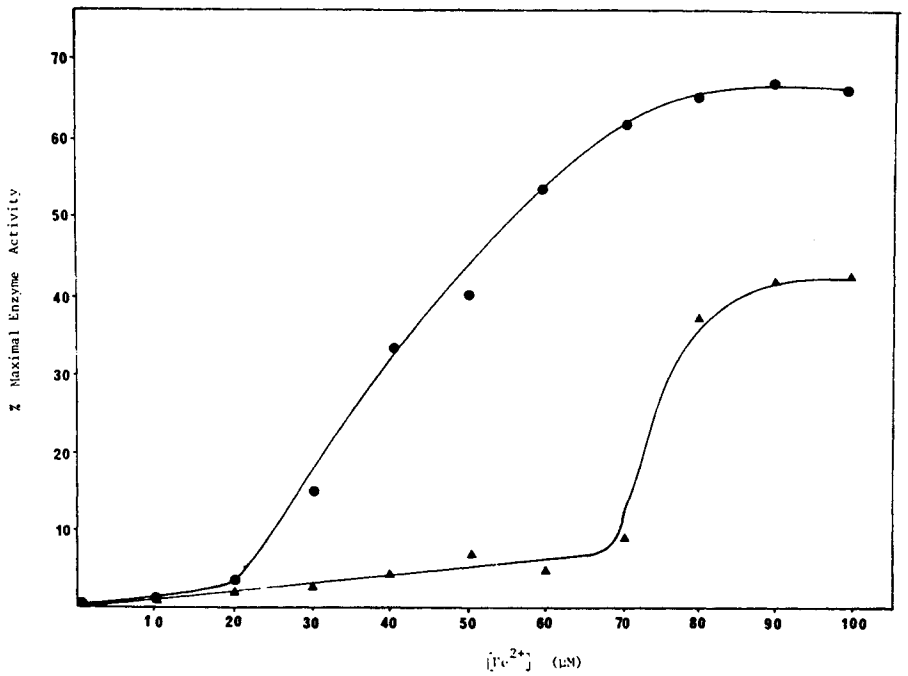


Fig. 5. Reactivation of GA_1 2β-hydroxylase activity by Fe^{2+} after pre-incubation with α,α'-dipyridyl (50 μM).
●—●—● + catalase; ▲—▲—▲ − catalase.

by adding back concentrated dialysis filtrate, but not by a combination of Fe^{2+}, ascorbate, and NADPH. Thus it became apparent that a low molecular weight cofactor other than NADPH was involved in the catalytic mechanism. This cofactor proved to be α-ketoglutarate (Hedden and Graebe 1982). The activity of the purified enzyme is not affected by NADPH. The enzyme does, however, exhibit an absolute requirement for Fe^{2+}, ascorbate as reductant, and α-ketoglutarate. Using 0.05 M sodium acetate, pH 6.5, (one of the few buffers operating around neutrality that will not chelate or precipitate the iron, or form a Schiff base with α-ketoglutarate), maximal enzyme activity is obtained when α-ketoglutarate, ascorbic acid, and $FeSO_4$ are present at concentrations of 2.5 mM, 5 mM and between 0.1 and 1.0 mM, respectively.

Effect of Catalase

Catalase stimulation is an acknowledged property of a number of α-ketoglutarate utilizing dioxygenases. The reason for this, however, remains unresolved (Abbott and Udenfreund 1974). In common with this type of enzyme, the GA_1 2β-hydroxylase activity is enhanced by the addition of catalase (Fig. 4). Since the enzyme activity lost upon storage at −20°C may be completely regained by the addition of catalase to the reaction mixture, catalase appears to reactivate "aged" enzyme. Furthermore, the data presented in Fig. 5 may indicate

that catalase can provide a source of reduced iron; it may also scavenge inhibitors (e.g. iron-binding ligands) from the surface of the GA_1 hydroxylating enzyme.

Enzyme Kinetic Parameters

From the Eadie-Hofstee plot of v *vs.* v/s, the maximal rate (V_{max}) of transformation of [1,2-$^3H_2$]GA_1 to 1-[^{3}H]GA_8 was calculated to be 6.86×10^{-9} mole $h^{-1}mg^{-1}$. Due to presence of the tritium label at the site of catalytic attack, this value is approximately an order of magnitude lower than that expected for unlabeled GA_1 substrate. However, even when this is taken into account, the maximum velocity of the enzyme preparation remains very low.

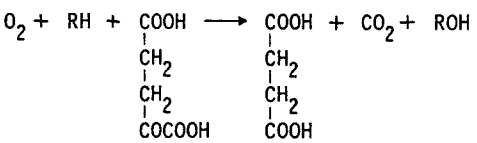
The enzyme has a Km of 0.085 μM and 21 μM for [1,2-$^3H_2$]GA_1 and α-ketoglutarate, respectively. These Km values may reflect the *in vivo* intracellular concentrations of the two substrates.

Role of α-Ketoglutarate

Most of the α-ketoglutarate–dependent oxygenases examined to date have been shown to be dioxygenases; the α-ketoglutarate functions as cosubstrate and is transformed to succinate during the course of the reaction (Scheme 2).

In accord with this mechanism, succinate was found to inhibit 2β-hydroxylation of [1,2-$^3H_2$]GA_1 (Fig. 6). It is a competitive inhibitor with respect to α-ketoglutarate, with Ki = 4 mM. However, it has not been possible to measure the release of $^{14}CO_2$ or ^{14}C-succinic acid from U-^{14}C-α-ketoglutarate (specific activity 9×10^{12} Bq mole^{-1} New England Nuclear). The enzyme requires a relatively high concentration (2 mM) of α-ketoglutarate for maximal activity and, furthermore, α-ketoglutarate is decomposed under the normal assay conditions in the absence of enzyme (Fig. 7). The possibility that α-ketoglutarate is bound to the enzyme but not turned over cannot therefore be excluded.

The observed inhibition of 2β-hydroxylase activity by succinic acid suggested a possible mechanism for the growth retardant, N,N-dimethyl succinic acid hydrazide. However, when this compound was tested over the range 0.01 to 10 mM against various concentrations of α-ketoglutarate (0.01–1.0 mM), no reduction in enzyme activity was observed. Therefore, the physiological effect of this succinate analogue is not related to the conversion of GA_1 to GA_8.



Scheme 2

Substrate Specificity

The data presented in Table 3 were obtained from experiments in which the rate of hydroxylation of [1,2-l$^3H_2$]GA_1 was measured when a variety of possible

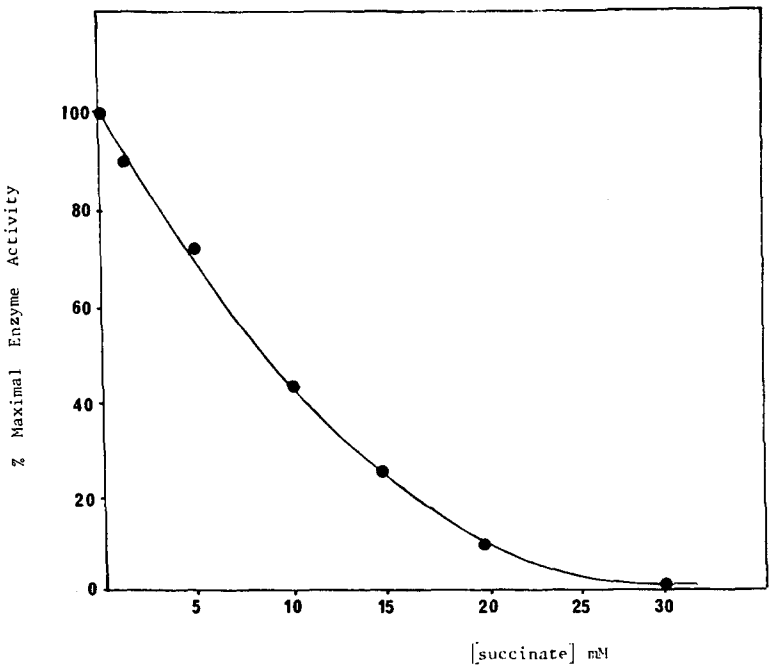


Fig. 6. Succinate inhibition of GA_1 2β-hydroxylase activity.

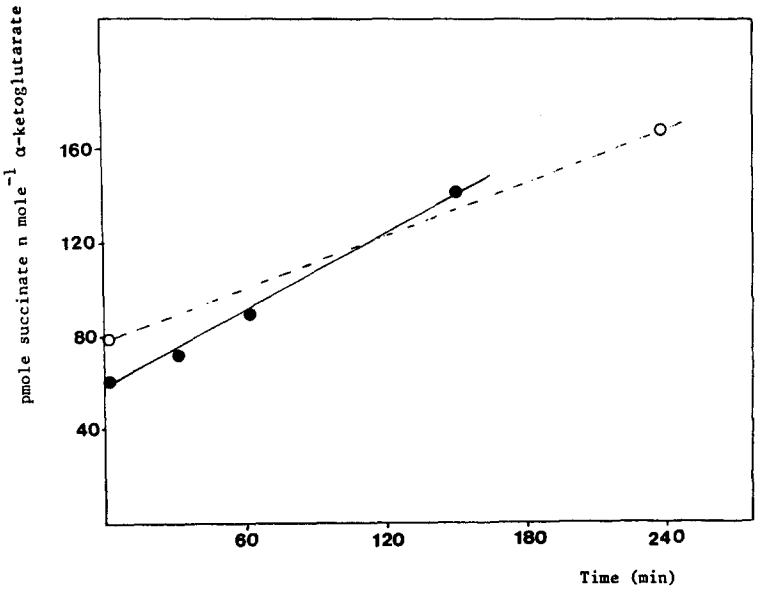


Fig. 7. Kinetics of decomposition of α-ketoglutarate to succinate. From incubations with [1,2-$^3H_2$]GA_1 under identical conditions, the expected rate of enzyme-catalyzed succinate formation was around 2.5–5.0 pmole h^{-1} $nmole^{-1}$ α-ketoglutarate.
●—●—● incubation with enzyme; ○—○—○ incubation without enzyme.

Table 3. Effect of a variety of gibberellins on the rate of hydroxylation of [1,2-$^3H_2$]GA_1.

Gibberellin	I^a (nM)
GA_1	15
GA, methyl ester[b]	600
GA_3	50
GA_4	<<5
epiGA_4	NI[c]
2,2-dimethylGA_4	NI
GA_9	NI
GA_{20}	NI
GA_{29}	NI
GA_8	NI

[a] Inhibitor concentration that reduces the maximum reaction rate by 50%. The concentration of [1,2-$^3H_2$]GA_1 used in these reactions was 3×10^{-10} M.
[b] Inhibition probably results from hydrolysis of methyl group.
[c] NI = No inhibition observed at 1,000 excess of competing gibberellin.

Table 4. Substrate specificity of the 2β-hydroxylase from mature seeds of *P. vulgaris*.

Substrate	Specific activity(pmole h^{-1} mg^{-1})	
	Purified enzyme	120,000 × *g* supernatant fraction
GA_1	950 (1.0)	2.2 (1.0)
GA_4	1300 (1.36)	2.6 (1.18)
GA_{20}	22 (0.02)	0.04 (0.018)
GA_9	59 (0.06)	0.2 (0.09)

Numbers in parentheses are the relative specific activities of each enzyme preparation for the four gibberellin substrates.

GA substrates were included, at various concentrations, in the standard enzyme reaction mixture. These results show that the enzyme is not subject to product inhibition by GA_8 and can discriminate between the various GA structures. The finding that GA_{20}, GA_{29}, GA_9, 2-epiGA_4, and 2,2-dimethylGA_4 have no effect on the rate of hydroxylation of [1,2-$^3H_2$]GA_1 indicates that they are neither competitive inhibitors nor substrates for this particular 2β-hydroxylase. The presence and stereo-configuration of the 3-OH group thus appears to be a primary requirement for enzyme recognition. Substitution of the C-2 protons and blockage of the C-7 carboxyl group also inhibit GA-enzyme interaction. Gibberellin A_4, which differs from GA_1 only by the absence of a hydroxyl group at position 13 of the gibberellin D-ring, is a much more effective inhibitor of [1,2-$^3H_2$]GA_1 hydroxylation than is the unlabeled GA_1. In addition to being an extremely good inhibitor of GA_1 2β-hydroxylation, the results suggested that GA_4 is also a substrate (see below).

The relative ability of the purified enzyme preparation to catalyze hydroxylation of GA_1, GA_4, GA_{20}, and GA_9 was determined directly by comparing

the kinetics of release of tritiated water from each of the 2β-tritiated GAs. Each compound was assayed at a concentration of 0.1 nM. The results presented in Table 4 are based on assumed specific activities of 6.96×10^{13} Bq mole^{-1}, 3.43×10^{13} Bq mole^{-1}, 6.05×10^{13} Bq mole^{-1}, and 8.3×10^{13} Bq mole^{-1} at the 2β-position of diluted [1,2-$^3H_2$]GA_1, [1,2-$^3H_2$]GA_4, [2,3-$^3H_2$]GA_{20}, and diluted [2,3-$^3H_2$]GA_9, respectively.

While the data given in Table 4 confirm that GA_4 is indeed a better substrate for the enzyme than GA_1, they also show that GA_{20} and GA_9 can also be hydroxylated. Furthermore, the relative specific activities of GA_1, GA_4, GA_{20}, and GA_9 hydroxylation are not appreciably altered upon enzyme purification. This observation suggests that the hydroxylase activities reside in the same protein but cannot exclude the copurification of discrete enzymes. By comparison with GA_1 and GA_4, GA_{20} and GA_9 are exceedingly poor substrates, and this again indicates a marked preference for 3-OH gibberellins.

Discussion

The present results confirm the findings by Patterson and Rappaport (1974) and Patterson et al. (1975) that the cotyledons of imbibed seeds of *Phaseolus vulgaris* contain a soluble protein that catalyzes the 2β-hydroxylation of GA_1 to GA_8. However, the catalytic activity of the purified protein does not require a reduced pyridine nucleotide; it has the properties of an α-ketoglutarate-dependent dioxygenase (Abbott and Udenfreund 1974) and not of a P450 oxygenase.

The cofactors for the 2β-hydroxylase purified from *Phaseolus vulgaris* are those required to demonstrate the biosynthetic oxidation of C-20 GAs in soluble protein preparations of endosperm from *Cucurbita maxima* (Hedden and Graebe 1982) and of C-20 to C-19 GAs in the soluble fraction of embryo of *Pisum sativum* (Kamiya and Graebe 1983). In all cases, product formation is strictly dependent upon the presence of α-ketoglutarate and Fe^{2+}. Ascorbic acid is also an absolute requirement for activity of the purified 2β-hydroxylase, but its role is considered to be stimulatory rather than essential in the biosynthetic systems of *C. maxima* and *P. sativum*.

Patterson et al. (1975) assayed GA_1 derivatives in their soluble extract of *P. vulgaris* seeds, demonstrated that the bioinactive 3-epiGA_1 and ring C/D-rearranged GA_1 were not 2β-hydroxylated, and postulated a link between GA bioactivity and ability to serve as enzyme substrate. Our results, using a highly purified enzyme preparation, show that only a limited number of GAs that are structurally similar to GA_1 are recognized. Moreover, the rate of 2β-hydroxylation of these compounds varies according to structure, and the 3β-hydroxylated C-19 GAs are much more effective substrates than non-3-hydroxylated C-19 GAs. Even when present at vast excess, the latter type of GAs do not alter the kinetics of GA_1 2β-hydroxylation.

The results presented here indicate that the 2β-hydroxylase from *P. vulgaris* is a protein of Mr 36,000. Its activity may be regulated *in vivo* by a α-ketoglutarate and succinate concentration, but is not effected by GA_1 metabolic precursors or by the reaction product, GA_8. However, until similar kinetic data

become available for each oxidative step in GA biosynthesis, the possible role of 2β-hydroxylases in the regulation of turnover of bioactive GAs cannot be fully assessed.

Acknowledgment. We acknowledge financial support from the Science and Engineering Research Council and the Agricultural Research Council.

References

Abbott MT, Udenfreund S (1974) α-Ketoglutarate-coupled dioxygenases. In: Hayaishi O (ed) Molecular mechanisms of oxygen activation. Academic Press, New York, London, pp 167–214

Durley RC, Pharis RP (1973) Gibberellin interconversion in dwarf rice. Planta 109:357–361

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the Biuret reaction. J Biol Chem 177:751–766

Hall BG, Hartl DL (1975) Regulation of newly evolved enzymes. II. The *Ebg* repressor. Genetics 81:427–435

Hedden P, Graebe JE (1982) Cofactor requirements for the soluble oxidases in the metabolism of the C_{20}-gibberellins. J Plant Growth Regul 1:105-116

Hoad GV, MacMillan J, Smith VA, Sponsel VM, Taylor DA (1982) Gibberellin 2β-hydroxylases and biological activity of 2β-alkyl gibberellins. In Wareing PF (ed) Plant growth substances. Academic Press, New York, pp 91–100

Kamiya Y, Graebe JE (1983) The biosynthesis of all major pea gibberellins in a cell free system from *Pisum sativum*. Phytochemistry 22:681–689

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Murofushi N, Durley RC, Pharis RP (1977) Preparation of radioactive gibberellins A_{20}, A_5 and A_8. Agric Biol Chem 41:1075–1079

Nadeau R, Rappaport L (1974) The synthesis of [^{3}H]gibberellin A_3 and [^{3}H]gibberellin A_1 by the palladium-catalysed actions of carrier free tritium on gibberellin A_3. Phytochemistry 13:1537–1545

Patterson RJ, Rappaport L (1974) The conversion of gibberellin A_1 to giberellin A_8 by a cell-free enzyme system. Planta 119:183–191

Patterson R, Rappaport L, Breidenbach, RW (1975) Characterisation of an enzyme from *Phaseolus vulgaris* seeds which hydroxylates GA_1 to GA_8. Phytochemistry 14:363–368

Yokota T, Reeve DR, Crozier A (1976) The synthesis of [^{3}H]-gibberellin A_9 with high specific activity. Agric. Biol. Chem. 40:2091–2094