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CHARACTERIZATION OF AN ENZYME FROM *PHASEOLUS VULGARIS* SEEDS WHICH HYDROXYLATES GA_1 TO GA_8

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; snap bean; $GA_1[^3H]$; $GA_8[^3H]$; pseudo-GA_1; 16-keto-GA_1; mixed function oxidase; hydroxylating enzyme.

Abstract—Hydroxylation of gibberellin-$[^3H]$ A_1 (GA_1-$[^3H]$) to GA_8-$[^3H]$ by the 95000 *g* supernatant fluid from imbibed bean seeds required Fe^{2+} or Fe^{3+} and O_2 but was insensitive to CO. The hydroxylating enzyme has a sedimentation coefficient of 4·5 *S*, and was precipitated by $(NH_4)_2SO_4$ at 35–60% saturation. This hydroxylase was specific for GA_1 and did not hydroxylate either pseudo-GA_1-$[^3H]$ or 16-ketoGA_1-$[^3H]$. Virtually all hydroxylase activity was localized in the cotyledons.

INTRODUCTION

Hydroxylation of gibberellin A_1 (GA_1) to GA_8 appears to be a common feature of GA_1 metabolism in higher plants [1]. Since GA_8 is biologically inactive compared to GA_1 [2,3], this reaction indicates that conversion of GA_1 to GA_8 could have a role in regulating GA mediated processes. The enzyme which catalyzes the hydroxylation of GA_1 to GA_8 was revealed by the release of ^3H to H_2O [4]. This reaction requires a reduced cosubstrate and is inhibited by EDTA. Therefore, the enzyme appeared to be a mixed function oxidase [5] with a cation requirement. In this paper further characterization of the enzyme from imbibed seeds of *Phaseolus vulgaris* is presented.

RESULTS

Cation requirement

The cation requirement was studied by inhibiting the reaction with 50 μM EDTA, and adding back various cations (Table 1). Only Fe^{3+} restored activity, with Ca^{2+}, Mg^{2+} and Mn^{2+} having no apparent effect on the reaction. Both Cu^{2+} and Co^{2+} further inhibited the hydroxylation of GA_1. The Fe requirement was also studied further by adding Fe^{2+} and Fe^{3+} to the EDTA inhibited system (Table 2). Fe in both oxidation states restored activity in the presence of ascorbate. However, Fe^{3+} was considerably less effective than Fe^{2+} in the absence of ascorbate. Fe^{2+} was equally effective with or without ascorbate.

O_2 *and* CO *effects*

100% N_2 inhibited the reaction 90%, indicating an O_2 requirement. The reaction was also inhibited by very high levels of CO (90–100%) in both light and darkness. The effects of CO and N_2 were indistinguishable. O_2 levels of *ca* 10% or less drastically reduced enzyme activity.

Effect of mercaptoethanol

Mercaptoethanol reduced enzyme activity and the effect of this compound was studied further by adding it directly to the reaction mixture (Table 3). Hydroxylation was inhibited 63%, and the inhibition was only partially overcome by the addition of Fe^{2+} or Fe^{3+}.

Substrate specificity

The specificity of this enzyme was examined by using the GA_1 derivatives pseudoGA_1 (2α-hydroxy-

Table 1. Effect of cations on hydroxylation of GA_1

	GA_8-[^{3}H] formed	
	Radioactivity (dpm water-[^{3}H] $\times 10^{-4}$)	Relative rate (fmol mg^{-1} hr^{-1})
Complete* – EDTA	11·8	19·6
Complete	3·5	5·8
Complete + Fe^{3+}	13·6	22·4
Complete + Ca^{2+}	5·1	8·5
Complete + Mg^{2+}	3·3	5·4
Complete + Mn^{2+}	2·9	4·8
Complete + Cu^{2+}	0·0	0·1
Complete + Co^{2+}	0·2	0·4
Control	0	0·0

* Complete reaction mixture contained, in a total volume of 2 ml, 625000 dpm GA_1-[^{3}H] (43 Ci/mmol), 1 mM NADPH, 1 mM ascorbate, 50 μM EDTA, 50 μg chloramphenicol/ml and 0·5 ml HSS. Cations were added as chloride salts to give 1 mM final concn.

GA_1) and 16-ketoGA_1 (Wagner–Meerwein rearrangement product) as substrates (Table 4). Neither of these derivatives was hydroxylated in the C-3 β-position by the enzyme preparation. It should be noted that hydroxylation in the 3α-position is a possibility since the assay used only measures the release of tritium from the 2β-position. Additional evidence for specificity of hydroxylation at 3β was obtained by chromatographing the ethanolic extract of the reaction mixture according to procedures described in Ref. 4. The only radioactive peaks detected on the chromatograms were those for pseudoGA_1 and 16-ketoGA_1.

Enzyme purification

GA_1 hydroxylase was partially purified using $(NH_4)_2SO_4$ precipitation and sucrose density gradient centrifugation. A saturated solution of $(NH_4)_2SO_4$, pH 7·4, was used to make 3 cuts (0–35, 35–60 and 60–90%). The resuspended 35–60% precipitate contained 82% of the recovered activity, but this represented only 23% of the original activity. The specific activity in the three precipitates was always less than in the original high speed supernatant (HSS).

To purify the enzyme, HSS and the resuspended 35–60% $(NH_4)_2SO_4$ fractions were layered on sucrose density gradients (7·9–19·6% and 8–20%, w/w,

Table 2. Effect of iron and ascorbate on hydroxylation of GA_1

Reaction mixture	GA_8-[^{3}H] formed	
	Radioactivity (dpm water-[^{3}H] $\times 10^{-4}$)	Relative rate (fmol mg^{-1} hr^{-1})
Complete* – EDTA	9·6	32·6
Complete	1·9	6·5
Complete + Fe^{2+}	6·0	20·6
Complete + Fe^{3+}	6·2	21·0
Complete – ascorbate	0·4	1·5
Complete + Fe^{2+} – ascorbate	6·4	21·8
Complete + Fe^{3+} – ascorbate	2·8	9·7
Control	0·0	0·0

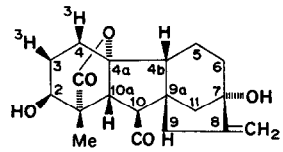
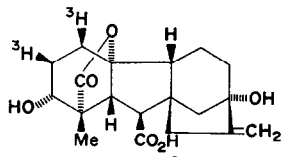
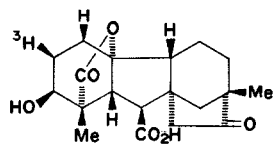
* Complete reaction mixture contained, in a total volume of 2 ml, 625000 dpm GA_1-[^{3}H] (43 Ci/mmol), 1 mM NADPH, 1 mM ascorbate, 50 μM EDTA, 50 μg chloramphenicol/ml and 0·5 ml HSS. Cations were added as sulfate salts to give 1 mM final concn.

Table 3. Effect of iron and mercaptoethanol on hydroxylation of GA_1

Reaction mixture*	GA_8-[^{3}H] formed	
	Radioactivity (dpm water-[^{3}H] $\times 10^{-4}$)	Relative rate (fmol mg^{-1} hr^{-1})
Complete – mercaptoethanol	9·6	32·6
Complete	3·6	12·1
Complete + Fe^{3+}	5·8	19·7
Complete + Fe^{2+}	7·4	21·7
Control	0	0·0

* Complete reaction mixture contained, in a total volume of 2 ml, 625000 dpm GA_1-[^{3}H] (43 Ci/mmol), 1 mM NADPH, 1 mM ascorbate, 10 mM mercaptoethanol, 50 μg chloramphenicol/ml and 0·5 ml HSS. Cations were added as the sulfates to give 1 mM final cation concn.

Table 4. Hydroxylation of GA_1 and related compounds

Substrate*	GA_8-[^{3}H] formed: Radioactivity (dpm water × 10^{-4}-[^{3}H])	GA_8-[^{3}H] formed: Relative rate (fmol mg^{-1} hr^{-1})
 GA_1 [^{3}H]	9·4	19·7
 pseudo GA_1 [^{3}H]	0	0·0
 16-keto GA_1 [^{3}H]	0·03	0·1
Control	0	0·0

* Complete reaction mixture contained, in a total volume of 2 ml, 625000 dpm substrate (43 Ci/mmol), 1 mM NADPH, 1 mM ascorbate, 50 μg chloramphenicol/ml and 0·5 ml HSS.

respectively). The sedimentation coefficient for the HSS (39 hr 25 min) and $(NH_4)_2SO_4$ (40 hr 30 min) fractions was 4·5 *S*. The enzyme specific activity of the peak fraction from the $(NH_4)_2SO_4$ gradient was 1·5-fold greater than that in the layered $(NH_4)_2SO_4$ fraction. However, the specific activity of this peak fraction was 25% less than that of the original HSS.

Table 5. Hydroxylation of GA_1 by extracts from different seed parts

Seed part*	GA_8-[^{3}H] formed: Radioactivity (dpm water-[^{3}H] × 10^{-4})	GA_8-[^{3}H] formed: Relative rate (fmol mg^{-1} hr^{-1})
Whole seed	8·4	11·4
Cotyledon	11·0	15·4
Embryo	0·08	0·5
Control	0	0·0

* HSS fractions were prepared from the indicated seed part and assayed for GA_1 hydroxylase activity. The reaction mixture contained, in a total volume of 2 ml, 625000 dpm GA_1-[^{3}H] (43 Ci/mmol), 1 mM NADPH, 1 mM ascorbate, 50 μg chloramphenicol/ml and 0·5 ml HSS.

Distribution of hydroxylase in seeds

HSS from cotyledon homogenates had higher hydroxylase activity than that from homogenates of whole seeds. Fractions from the embryo axis had virtually no activity (Table 5).

DISCUSSION

Previous studies of GA_1 hydroxylation to GA_8 had revealed a soluble enzyme system which was sensitive to EDTA, required a reduced co-substrate, and produced [^{3}H]-water [4]. These results indicated that this enzyme was probably a mixed function oxidase with a cation requirement. In the present study, Fe was the only cation added that restored hydroxylase activity to an EDTA inhibited system (Table 1). In the absence of ascorbate, Fe^{3+} only partially restored activity whereas Fe^{2+} was as effective as ascorbate (Table 2). Since ascorbate can reduce Fe^{3+} to Fe^{2+}, it is likely that one action of ascorbate in this system is to maintain Fe in the reduced form. These results are consistent

with those obtained with a number of mixed function oxidases that utilize Fe^{2+} [6].

The requirement of this GA_1 hydroxylating system for O_2 and the dependence on ascorbate and Fe^{2+} and Fe^{3+} is similar to that found in a model chemical system, described by Udenfriend *et al.* [7], which hydroxylates aromatic compounds. These similarities raise two important questions. First, does non-enzymatic hydroxylation take place? Results of previous experiments have indicated that heating the HSS for 10 min at 50° completely inactivated the enzyme. Further, hydroxylase activity is precipitable with $(NH_4)_2SO_4$. Finally, sucrose density gradient centrifugation indicated that the hydroxylase activity peak had a sedimentation coefficient of 4·5 *S*. Thus, a single protein or proteins of similar sizes and hydrophobicity catalyze this reaction. The second question arose from the observation of Jones *et al.* [8] that GA_5 was converted to presumptive GA_5 norketone under the oxidizing conditions of Fenton's reagent and Udenfriend's system. By analogy, GA_1 norketone must be considered a possible product here. However, since GA_1-[^{3}H] released ^{3}H from the C-3 position to H_2O and the GA_8-[^{3}H] produced co-chromatographed with standard GA_8 in GLC and TLC systems [4], GA_1 norketone is not likely to be a product of the system described here.

Restoration of hydroxylase activity by addition of Fe indicates that the cation is not covalently bound to the enzyme but is coordinated in some other way. Enzyme inhibition by mercaptoethanol (Table 3) supports the involvement of sulfhydryl groups in coordination of the metal with the enzyme. Since Fe addition can partially overcome inhibition by mercaptoethanol, inhibition may result from removal of Fe by the formation of a salt with mercaptoethanol. Whether sulfhydryl groups are involved in cation coordination will require further study.

The requirement of GA_1 hydroxylase for Fe is different from the requirements of other oxidative steps in GA biosynthesis. West *et al.* [9, 10] have shown that Mg^{2+} is required by microsomal mixed function oxidases that catalyze the sequence from *ent*-kaurene to *ent*-7α-hydroxykaurenoic acid. According to Graebe [11, 12], oxidative reactions in the GA biosynthetic pathway are specifically affected by certain cations. In the presence of Mn^{2+} and Mg^{2+}, GA_{12} and GA_{12}-aldehyde were synthesized from mevalonate. However, in the absence of Mn^{2+}, GA_{12}-aldehyde was metabolized to several more highly oxidized products.

The mixed function oxidase mechanism requires molecular oxygen [5]. Atmospheres with very little or no O_2 (containing either N_2 or CO) effectively inhibited GA_1 hydroxylase activity, indicating that this enzyme has an O_2 requirement which is consistent with the proposed mechanism. Incorporation of $^{18}O_2$ into the molecule would conclusively demonstrate this requirement.

Cytochrome P450 is known to be involved in many mixed function oxidases including those which catalyze the steps from *ent*-kaurene to *ent*-7α-hydroxykaurenoic acid [10]. Carbon monoxide competes with O_2 reversibly for binding to P450 and the CO binding itself is reversed by light. Our results indicate that CO and N_2 inhibited GA_1 hydroxylation by reducing O_2 levels. Thus, cytochrome is not involved in this hydroxylation.

Patterson and Rappaport [4] proposed that the enzyme which hydroxylates GA_1 to GA_8 is a mixed function oxidase. This proposal is based principally on water-[^{3}H] labeling, the requirements for a reduced cosubstrate, NADPH, and a cation. The fulfilment of the cation requirement by Fe^{2+}, and the O_2 dependence of the reaction, further support the proposed mixed function oxidase mechanism:

$$GA_1\text{-}[^3H_{1,2}] + NADPH + H^+ + O_2 \xrightarrow{Fe^{2+}} GA_8\text{-}[^3H_1] + NADP^+ + \text{water-}[^3H].$$

Microsomal mixed function oxidases are reputed to have little substrate specificity compared to other mixed function oxidases [5, 13]. The enzyme which catalyzes C-3 β-hydroxylation of GA_1 apparently does have substrate specificity. The GA_1 concentration (3·3 nM, 6·5 pmol) in the incubations was relatively low, whereas, at the same concentration, the GA_1 derivatives pseudoGA_1-[^{3}H] and 16-ketoGA_1-[^{3}H] were not hydroxylated (Table 4). Evidently, both β configuration of the C-2 hydroxyl and the native C/D ring conformation are critical for C-3 β-hydroxylation by this enzyme. This specificity is interesting as these GA_1 derivatives have little bioactivity [2, 14]. Thus, only the biologically active GA_1 is hydroxy-

lated in the C-3 position to the inactive GA_8. Stolp *et al.* [14] observed that pseudoGA_1-[^{3}H] was not metabolized by barley aleurones nor did it induce α-amylase synthesis. However, GA_1-[^{3}H] was rapidly metabolized, and did induce α-amylase synthesis. As suggested by those workers, differences in bioactivity and metabolism may be linked.

Dissection of imbibed seeds and subsequent assay for GA_1 hydroxylase activity revealed a localization of activity in the cotyledons, with virtually no activity in the embryo axis (Table 5). If it is assumed that protein from the axis diluted cotyledonary activity, specific activity of the whole seed would be proportionally less than that of the cotyledon. In fact, the embryo axis contained only 1·5% of the protein in the whole seed. However, the specific activity of the whole seed was 25% less than that of the cotyledons. Thus, the difference between specific activity of the cotyledons and that of the whole seed cannot be attributed solely to dilution of the protein, which would result from the absence of enzyme in the embryo axis. Alternatively, these differences in activity could have resulted from higher concentrations of endogenous substrate, or greater competition for cofactors, in the embryo axis. Other regulatory factors such as enzyme inhibitors or allosteric effectors could influence this activity. An explanation of the difference could furnish insight into the metabolic regulation of GA_1 levels and, consequently, the control of GA mediated responses in the two seed parts.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus vulgaris* cv Kentucky Wonder were scratched with a razor blade to facilitate uniform imbibition, surface sterilized with 1% NaOCl for 10 min, and then washed 5× with sterile H_2O. 5 g of seeds were transferred in a sterile hood to a 100 × 15 mm Petri dish containing Whatman No. 1 filter paper moistened with 7 ml H_2O. Imbibition by the seeds was continued for 18 hr at 25° under diffuse room light.

Preparation of enzyme extracts. Decoated imbibed seeds were homogenized at 4° with a Sorvall Omnimixer (3/4 speed for 1 min) in 0·05 M Tris–maleate buffer, pH 6·5, containing 0·2 M sucrose and 0·25 g PVP per g tissue. The ratio of tissue to grinding buffer was 1:4. Homogenates were centrifuged at 1000 *g* for 10 min at 0–4° and the supernatant was then centrifuged at 12000 *g* for 15 min under the same conditions. The resulting supernatant fraction was centrifuged at 95000 *g* in an ultracentrifuge (SW 27 rotor, r_{av} = 11·8 cm) for 2 hr at 0°. Unless otherwise indicated, the 95000 *g* supernatant fractions (HSS) were utilized in these studies. If not used immediately, the HSS was stored in 10% glycerol at −20° for later use.

Incubation in gibberellin A_1-[$^3H_{1,2}$]. The GA_1-[^{3}H] (43 Ci/mmol) and its derivatives, pseudoGA_1-[^{3}H] (2α-hydroxyGA_1) and 16-ketoGA_1-[^{3}H]* (2,β,4aα-dihydroxy-1β,7β-dimethyl-8-keto-4bβ-gibbane-1,10-carboxylic acid 1 → 4a lactone, Wagner–Meerwein rearrangement product), were prepared and purified by methods described by Nadeau and Rappaport [15] and Stolp *et al.* [14]. All incubation and homogenizing buffers were autoclaved or prepared with autoclaved H_2O or buffer in a transfer hood.

The incubation medium contained, in a vol. of 2 ml, 40 μmol Tris–maleate buffer, pH 6·5, 2 μmol each of NADPH and ascorbate, 100 μg chloramphenicol, GA_1-[^{3}H] (625000 dpm), and 0·5 ml HSS. The incubation components were added to 15 × 125 mm culture tubes stoppered with polyurethane foam stoppers. For controls, portions of the HSS were heated to 100° for 10 min before the incubation mixture was added. Incubations were run 6 hr in a reciprocating water bath (60 cycles/min) at 25° under diffuse room light. The reaction was terminated by the addition of 1 ml of 0·1 M EDTA, pH 6·5.

Incubation under modified atmospheres. The desired atmospheric composition was established by use of flowmeters and the atmospheres were metered to Thunberg tubes (45 ml) at the rate of 3 l/hr. The gas composition was monitored by GLC. The standard reaction mixture was placed in a Thunberg tube and purged with the desired atm. for 1 hr. This was followed by addition of 0·5 ml HSS, restoration of the atmosphere, and incubation by standard methods.

Distillation and rate determination. Terminated reaction mixtures were transferred to the tube portion of a Thunberg apparatus which was placed in a 100° H_2O bath with the stopper bulb in an ice bath. The distillate was collected in the stopper bulb and radioactivity of samples was measured by scintillation counting. During GA_8-[^{3}H] formation the 3β-[^{3}H] of GA_1-[^{3}H] was released to H_2O. The ratio of GA_8-[3H]:water-[^{3}H] was 1·9 [4]. This ratio was used in the calculation of GA_8-[^{3}H] production.

$(NH_4)_2SO_4$ *precipitation.* A stepwise precipitation (0–35, 35–60 and 60–90% saturation) of protein was carried out by slowly adding a satd soln of $(NH_4)_2SO_4$ to the HSS which was stirred gently for 10 min. After standing 1 hr at 0° the mixture was centrifuged at 12000 *g* for 10 min. The resulting ppts were resuspended in a minimal vol. of 0·05 M Tris–maleate buffer, pH 6·5, and assayed for GA_1 hydroxylase activity and protein. The enzyme assay mixture included 1 mM $FeCl_3$ with the standard additions.

Sucrose density gradient centrifugation. Homogenates were prepared as described except that sucrose was excluded. 2 ml of this HSS were layered on a 34 ml linear sucrose density gradient (7·9–19·6%, w/w) and centrifuged at 95000 *g* for 39 hr 25 min (SW 27 rotor r_{av} = 11·8 cm). A 2 ml fraction of the resuspended 35–60% $(NH_4)_2SO_4$ precipitate was similarly centrifuged (8–20%, w/w, gradient) for 40 hr 30 min. After displacing the gradients, 1 ml fractions were assayed for GA_1 hydroxylase activity with the standard additions plus 1 mM $FeCl_3$. Parallel gradients were assayed for protein, and the sedimentation coefficient for the activity peaks was calculated.

Dissection of seeds and assay for GA_1 *hydroxylase activity.* Seeds imbibed by the standard method were dissected into cotyledons and embryo axis. These were homogenized by the standard method in 0·1 M potassium phosphate buffer, pH 6·5, which contained 0·01 M mercaptoethanol, 0·2 M sucrose and 0·25 g PVP/g seed. The ratio of buffer to tissue was 3:1. The HSS was assayed for hydroxylase activity and protein.

* The designation 16-keto-GA_1-[^{3}H] conforms to usage in other publications.

Protein determination. Protein was determined by the method of Lowry *et al.* [16] using bovine serum albumen, fraction V as a standard.

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