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ECDYSONE 20-MONOOXYGENASE, A CYTOCHROME P450 ENZYME FROM SPINACH, *SPINACIA OLERACEA*

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; enzymology, cytochrome P450; phytoecdysteroids; 20-hydroxyecdysone; ecdysone; monooxygenase.

Abstract—A microsomal preparation isolated from first leaves of 25-day-old spinach catalysed the hydroxylation of ecdysone to produce the insect moulting hormone, 20-hydroxyecdysone. Hydroxylation was dependent on NADPH and molecular oxygen, and was inhibited by carbon monoxide. Carbon monoxide inhibition was partially reversible by white light. Polyclonal antibodies to the Jerusalem artichoke NADPH-cytochrome P450 reductase inhibited the hydroxylation reaction as well as the spinach microsomal NADPH cytochrome *c* reductase. These results taken together establish ecdysone hydroxylation as a cytochrome P450 dependent reaction in spinach, which is known to synthesize large amounts of phytoecdysteroids.

INTRODUCTION

Insect moulting hormones, ecdysteroids, are reported in both arthropods and higher plants [1–6]. The production of ecdysteroids such as 20-hydroxyecdysone by arthropods (e.g. insects and crustaceans) is required for the control of growth, development and reproduction [7–9]. Plants biosynthesize a vast array of phytoecdysteroid structures, which have recently received significant attention due to their potential role as defence compounds against non-adapted herbivorous insects [10–15]. A thorough characterization of key steps in ecdysteroid biosynthesis may reveal similarities and differences in the biochemistry between insects and higher plants.

Insects biosynthesize C_{27} ecdysteroids, ecdysone and 20-hydroxyecdysone, from cholesterol [1, 2, 16]. This involves sequential modifications of the sterol nucleus and side chain resulting in ecdysone (2β, 3β, 14α, 22*R*, 25-pentahydroxy-5β-cholest-7-en-6-one), which, in most insect systems, serves as prohormone to the active moulting hormone, 20-hydroxyecdysone (Fig. 1) [1, 17]. The early steps in the conversion of cholesterol into ecdysone are difficult to address and poorly understood, due in part to the lack of significant quantities of available intermediate ecdysteroid structures needed to characterize enzyme systems *in vitro* and the lack of significant pools of intermediate ecdysteroids during *in vivo* ecdysteroid biosynthesis. However, several enzymes responsible for nuclear and side chain hydroxylations, which occur late in the ecdysteroid biosynthetic pathway have been identified and characterized in locust, all of which satisfy established criteria for cytochrome P450 mixed function oxidases

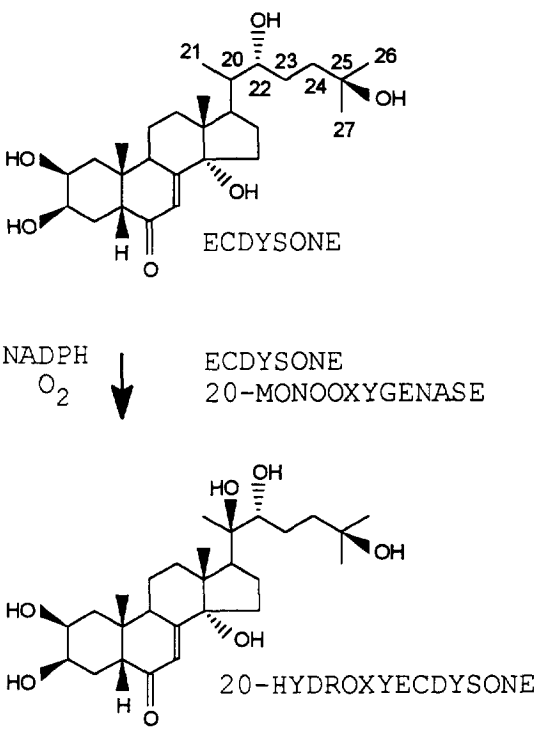

Fig. 1. Biosynthetic conversion of ecdysone into 20-hydroxyecdysone in higher plants.

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(e.g. the C-2, -22 and -25 hydroxylations) [16, 18]. The best understood of the ecdysteroid hydroxylating enzymes is ecdysone 20-monooxygenase. This enzyme is an important regulatory step in invertebrate ecdysteroid biosynthesis and has been extensively examined in several systems, where it has been characterized as a cytochrome P450 monooxygenase [19–23]. The ecdysteroid biosynthetic pathway in invertebrates thus appears to be mediated in large part by cytochrome P450 linked enzymes.

In plants, however, little is known regarding the biochemistry and enzymology of phytoecdysteroid biosynthesis even though higher plants produce ecdysteroids at concentrations that are two to five orders of magnitude greater than those reported in arthropod systems. Recently, *Spinacia oleracea* has been developed as a model system for examination of phytoecdysteroid biosynthesis and distribution during plant ontogeny [24–26]. Investigations into ecdysteroid production in spinach have documented a developmental pattern of ecdysteroid production with a coordinate regulation of overall biosynthesis and the potential regulatory effects of ecdysteroid conjugates on biosynthesis [24–26]. Ecdysteroids, 20-hydroxyecdysone and polypodine B are synthesized from the sterol precursor lathosterol and dynamically transported throughout spinach with development. Mature subtending leaves are active sites of ecdysteroid biosynthesis throughout ontogeny while immature apical organs act primarily as ecdysteroid sinks, accumulating levels of 20-hydroxyecdysone/polypodine B, which can exceed 800 μg g^{-1} fresh weight [24–26].

The later enzymic steps of phytoecdysteroid production are probably similar to those defined for ecdysteroid biosynthesis in arthropods, suggesting that the conversion of ecdysone into 20-hydroxyecdysone in plants may be mediated by a similar cytochrome P450 linked monooxygenase. The spinach system, with defined sites of ecdysteroid biosynthesis throughout ontogeny, appears to be an ideal model for investigating the biochemistry and enzymology of ecdysteroid production in higher plants. The aim of this research is therefore to establish the presence of ecdysone 20-monooxygenase acting in cell-free preparations of *S. oleracea* leaves and to characterize the enzyme as a first step in the molecular and enzymological analysis of ecdysteroid synthesis in plants. We report here the characterization of a cytochrome P450 mediated ecdysone 20-monooxygenase from isolated microsomes of spinach, *S. oleracea*.

RESULTS

Differential centrifugation of a homogenate of 25-day-old first leaves of spinach produced a 27 000 *g* pellet and a 100 000 *g* pellet both of which exhibited ecdysone hydroxylase activity. The reaction substrate (ecdysone) and product (20-hydroxyecdysone) were separated by reverse phase (RP) HPLC. Individual components were identified by comparison of retention times with those of authentic ecdysteroid standards. Ecdysone had a retention value of 1.00, while the retention value of 20-hydroxyecdysone was consistently 0.53. The ecdysteroids were collected from the RP-HPLC system and subjected to TLC. The UV absorbing compound with an R_f value of 0.36 co-migrated with authentic ecdysone and the UV absorbing compound with an R_f value of 0.30 co-migrated with authentic 20-hydroxyecdysone.

The light membrane fraction (microsomal fraction) which sedimented at 100 000 *g* demonstrated a higher ecdysone monooxygenase specific activity than was observed with the 27 000 *g* fraction. The NADPH dependent cytochrome *c* reductase activity, although not strictly parallel to the hydroxylation activity in the different membrane fractions, was also highest in the 100 000 *g* pellet and this activity was used as a marker for microsomal membranes (Table 1).

The 20-monooxygenase activity within the microsomal fraction varied between preparations with an average activity of 200 pmol of 20-hydroxyecdysone produced min^{-1} mg^{-1} microsomal protein. Hydroxylase activity was linear with time over a 10 min incubation period, which we established as the standard assay condition (Fig. 2). Spinach ecdysone 20-mono-

Table 1. Distribution of ecdysone 20-monooxygenase and spinach NADPH cytochrome *c* reductase activities during differential centrifugation of *Spinacia oleracea* leaves

Fraction	Volume (ml)	Protein (mg)	Ecdysone 20-monooxygenase specific activity pmol min^{-1} mg^{-1} protein	NADPH cytochrome *c* reductase* specific activity nmol min^{-1} mg^{-1} protein
Homogenate	15	54	120	n.d.†
2000 *g* supernatant	12	43	180	0.001
27 000 *g* pellet	3‡	9.0	540	0.250
27 000 *g* supernatant	10	35	100	0.007
100 000 *g* pellet	1‡	1.2	2100	2.776

*Cytochrome *c* reductase activity was assayed by methods previously published [35].

†Cytochrome *c* reductase activity was not determined for homogenate.

‡Volume of buffer used to resuspend pellet.

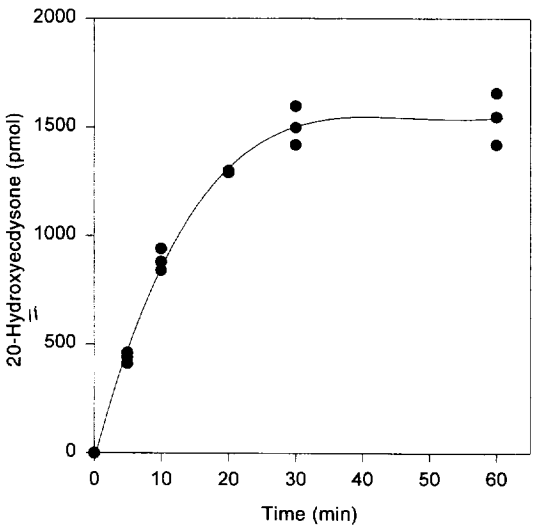

Fig. 2. Hydroxylation of ecdysone by spinach leaf microsomes; 0.1 mg of microsomal protein was incubated with ecdysone (55 μM) for increasing incubation times under assay conditions.

oxygenase is thus less stable than terpenoid hydroxylases, in which P450 monooxygenases have linear activities from 15 to 60 min [27–30]. The spinach microsome ecdysone 20-monooxygenase was moderately unstable as it could not be stored for prolonged periods either at 4° or −20° without an appreciable loss of activity. However, quick frozen membranes maintained in 15% glycerol retained full activity when stored at −80° for at least a month.

The ecdysone 20-monooxygenase activity was dependent upon oxygen and NADPH (Table 2). In the absence of NADPH, activity was found to be *ca* 17% of controls, suggesting the presence of some residual endogenous NADPH in the microsomal preparation. In the presence of NADPH, but in the absence of a NADPH regenerating system under standard assay conditions, activity was diminished by one-third of control values. Neither NADPH nor ascorbic acid were able to support enzyme activity (Table 2). Enzyme activity was inhibited by 50% after purging with 500 ml of N_2, but no inhibition was observed with a 50% N_2-air mixture, implying that the enzyme was O_2 dependent. Carbon monoxide, when gassed as a 50% mixture with air and thus under conditions which were not anoxic, strongly inhibited enzyme activity. We established that inhibition by carbon monoxide was reversible by white light illumination (Table 2).

The kinetics of the ecdysone 20-monooxygenase were determined under standard assay conditions. Plots of enzyme activity versus substrate concentration gave rise to a characteristic saturation curve which yielded a linear Lineweaver–Burk plot (Fig. 3). The apparent K_M for ecdysone was 25 μM, which is similar to that obtained for the terpenoid hydroxylases [27, 29, 30].

Incubation of spinach microsomes with rabbit antiserum to NADPH-cytochrome P450 reductase from

Table 2. Characteristics of the *S. oleracea* ecdysone 20-monooxygenase

Conditions	Activity (%)*
Cofactor dependence:	
0.5 mM NADPH + regenerating system†	100
No cofactors, no regenerating system	17
0.5 mM NADPH, no regenerating system	66
0.5 mM NADH	17
0.5 mM Ascorbate	17
Oxygen dependence	
0.5 mM NADPH + regenerating system + 100% N_2‡	50
0.5 mM NADPH + regenerating system + 50% N_2	100
0.5 mM NADPH + regenerating system + 50% CO§	49
Carbon monoxide inhibition and photoreversibility	
0.5 mM NADPH + regenerating system + light‖	100
0.5 mM NADPH + regenerating system + dark	100
0.5 mM NADPH + regenerating system + 50% CO + dark	49
0.5 mM NADPH + regenerating system + 50% CO + light	100

*Percentage conversion based on the standard assay condition, as average of 3 replicates.

†Standard assay conditions of 0.1 mg microsomal protein incubated for 1 min at 30° with 1 mM DTT and NADPH regenerating system consisting of 15 mM glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase.

‡The reaction mixture was gassed with 500 ml of N_2 gas.

§The reaction mixture was gassed with 500 ml of CO gas.

‖Illumination was supplied by a tungsten lamp placed directly adjacent to the reaction mixture at a distance of 15 cm.

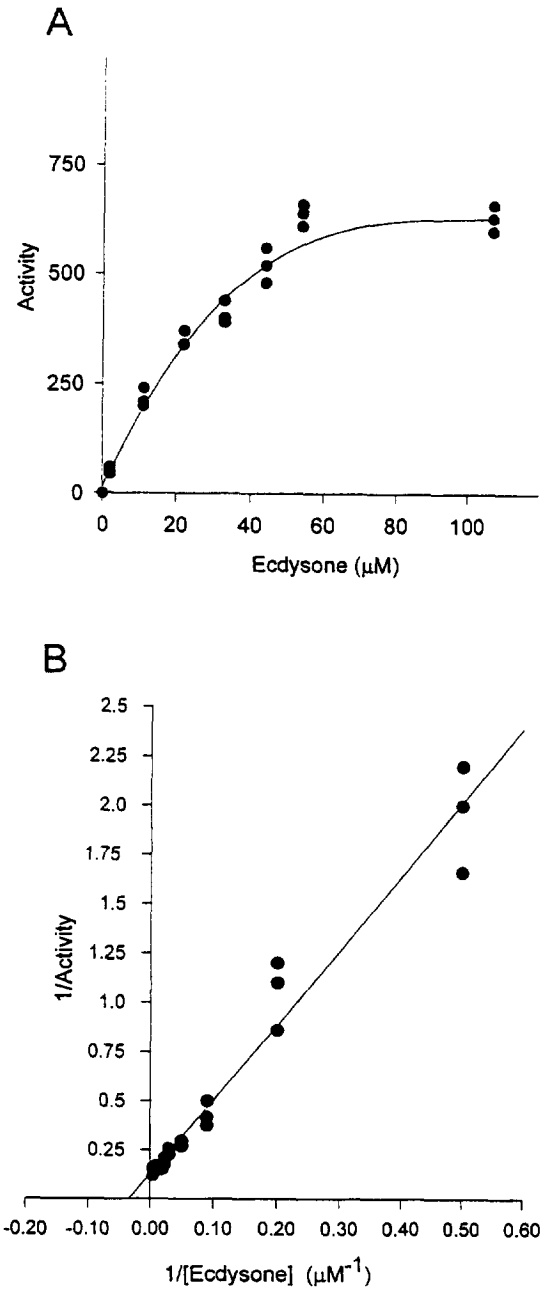

Fig. 3. Dependence of spinach ecdysone 20-monooxygenase activity on substrate concentration. A: 0.1 mg of microsomal protein was incubated under standard assay conditions with varying concentrations of ecdysone; activity is defined as pmol of 20-hydroxyecdysone produced min^{-1} mg^{-1} protein. B: a double reciprocal plot of the data.

Jerusalem artichoke [31] resulted in the inhibition of both NADPH cytochrome *c* reductase activity and ecdysone 20-monooxygenase activity. The ecdysone 20-monooxygenase present in the spinach microsomal preparation was inhibited half maximally by incubation with 0.50 mg antiserum/mg^{-1} microsomal protein. Pre-immune serum at 1 mg mg^{-1} microsomal protein had no effect on enzyme activity (Fig. 4).

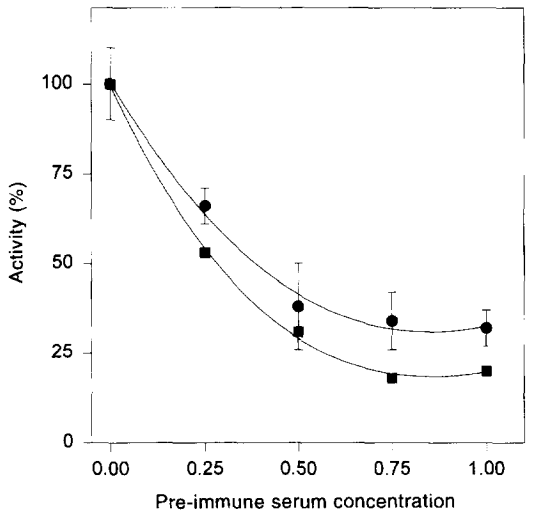

Fig. 4. Inhibition of the spinach ecdysone 20-monooxygenase and NADPH cytochrome *c* reductase by a polyclonal antibody to the NADPH-cytochrome P450 reductase of Jerusalem artichoke. (●) The ecdysone 20-monooxygenase assay was run under standard assay conditions with serum protein incubated for 1 min at 30° prior to initiation of the monooxygenase reaction. The data reported for each serum concentration is the average of the three replicates. (■) The cytochrome *c* reductase activity was assayed by the methods of ref. [35]. The spinach microsomal protein was incubated with post-immune serum containing polyclonal antibodies to the NADPH-cytochrome P450 reductase of Jerusalem artichoke at 0, 0.25, 0.50, 0.75 and 1.0 mg mg^{-1} spinach microsomal protein, with the total concentration of serum protein kept constant at 1 mg mg^{-1} spinach microsomal protein by adjusting the concentration of pre-immune serum from 1 mg mg^{-1} spinach microsomal protein to 0.75, 0.50, 0.25 and 0, respectively. Thus, the 0.0 value represents the control for 1 mg spinach microsomal protein mg^{-1} pre-immune serum protein.

Spectral studies of enzymes isolated from the spinach leaves proved difficult due to the fragile nature of the isolated enzyme and pigments associated with both isolated membrane pellets. The 27 000 *g* pellet was dark green in colour, while the 100 000 *g* pellet appeared to be a pale yellow. These pigments are known to interfere with the spectral properties of membrane enzymes [28, 30]. Attempts to purify further the enzyme by continuous or discontinuous sucrose density gradients were unsuccessful due to the relative instability of the enzyme. CO-difference spectra of the reduced enzyme produced a major 420 nm absorbance peak, suggesting significant inactivation of cytochrome P450. The pigmentation of the microsomal pellet coupled with the enzyme instability precluded inhibitor and type I spectral analysis of the enzyme.

DISCUSSION

We have studied the conversion of ecdysone into 20-hydroxyecdysone by subcellular fractions of spinach leaves known to biosynthesize the latter ecdysteroid at

a high rate [24–26]. The enzyme was found to be primarily microsomal as its activity was highest in the subcellular fraction which had maximal NADPH cytochrome *c* reductase activity, a marker enzyme for endoplasmic reticulum. We used microsomal fractions to characterize the enzyme responsible for this hydroxylation.

The ecdysone 20-monooxygenase activity from the microsomes of spinach has several attributes that lead us to conclude that the enzyme is a cytochrome P450 monooxygenase. First, we showed that the enzyme activity is localized in microsomal membranes. This agrees with the localization of other cytochrome P450 hydroxylases from leaf material involved in terpenoid biosynthesis in *Nepeta mussinii* and *Catharanthus roseus* [30, 32]. Second, we showed that the monooxygenase activity is dependent on oxygen and NADPH and is moderately sensitive to inhibition by carbon monoxide (Table 2). Similar results are reported for cytochrome P450s involved in terpenoid biosynthesis [27–30]. The carbon monoxide inhibition is reversible by white light, as would be expected for a cytochrome P450 enzyme (Table 2). Cytochrome P450s previously isolated from other higher plant systems could be inhibited by carbon monoxide, but photoreversibility of the inhibition in some cases was not demonstrated. The apparent absence of photoreversibility in the microsomal preparation isolated from *N. musinii* was ascribed to the presence of green pigmentation within the isolated membrane fraction [30]. The microsomal preparation isolated from spinach lacked green pigmentation, displaying a pale yellow appearance. Third, we showed that the enzyme activity was very sensitive to inhibition by antibodies to NADPH cytochrome P450 reductase. The combination of these criteria convincingly establish the ecdysone 20-monooxygenase activity of spinach leaves as a cytochrome P450 activity.

A detailed characterization of a microsomal ecdysone 20-monooxygenase from the Malpighian tubules of the African migratory locust, *Locusta migratoria*, was previously described [20]. This enzyme is a cytochrome P450 dependent monooxygenase with a K_M of 0.22 μM. The K_M of the plant derived ecdysone 20-monooxygenase is *ca* 25 μM, suggesting that the enzyme activity of the respective ecdysone 20-monooxygenases reflects the ecdysteroid concentration in each system. In insects, 20-hydroxyecdysone production is tightly regulated throughout ontogeny and is typically reported to be found in nanomolar quantities [7]. The submicromolar K_M for the insect ecdysone 20-monooxygenase is consistent with the observation of a low level of 20-hydroxyecdysone. In plants, however, 20-hydroxyecdysone is observed to accumulate to intracellular levels which can reach micromolar concentrations (e.g. spinach at 2 μM ecdysteroid concentration in apical levels) [4]. This level is considerably greater than those found in insects. The high K_M for the spinach ecdysone 20-monooxygenase is consistent with the high carbon throughput required for the accumulation of the elevated levels of 20-hydroxyecdysone observed. The high concentration of ecdysteroids in new leaves is consistent with the hypothesis that they serve as a defence compound against insect herbivory.

Factors regulating ecdysteroid biosynthesis in plants have recently been addressed in the spinach system. Phytoecdysteroids, both end product and intermediate structures (e.g. polypodine B, ecdysone, 2-deoxy-20-hydroxyecdysone and 22,25-dideoxyecdysone), demonstrated an inhibition of endogenous ecdysteroid production and the simultaneous production of end product and intermediate ecdysteroid polyphosphates when infused into excised organs. The presence of elevated levels of phosphorylated ecdysteroids is correlated with an inhibition of incorporation of mevalonic acid into lathosterol and into 20-hydroxyecdysone [25]. The inhibition of carbon flux into lathosterol and 20-hydroxyecdysone by ecdysteroid polyphosphates suggests that a primary regulatory point in the phytoecdysteroid pathway involves carbon allocation into the pathway, and suggests that the 20-monooxygenase may not serve as a primary site for pathway regulation. However, the characterization of this enzyme as a P450 monooxygenase suggests that other enzymes involved in ecdysteroid production in plants may also be P450 enzymes.

EXPERIMENTAL

Spinach (*S. oleracea* L.) var. Avon, Burpee # 51334AK, used as a source for microsomal membranes, was grown in a greenhouse from seed under natural illumination for the autumn and winter seasons of Tucson, Arizona, latitude 32°N. Plants used for experimental purposes were 25 days old, post planting, and contained 3 true leaf sets; the second leaf set was used as a source for microsomal membranes. Microsomal membranes were isolated from freshly harvested leaves (5 g) using slight modifications of the methods of ref. [30]. Under standard fractionation conditions, 5 g tissue was homogenized in 50 ml 0.1 M KPi (pH 7.) extraction buffer containing 0.2 M sucrose, 50 mM Na metabisulphite, 50 mM ascorbate and 10 mM DTT. The homogenate was slurried with 5 g PVP (M_μ 40 000) for 5 min at 4°. The mixt. was filtered through a single layer of miracloth (Calbiochem) and centrifuged at 2000 *g* for 30 min at 4° using a IEC Centra-7R centrifuge. The supernatant was then centrifuged at 27 000 *g* for 30 min at 4° using a Beckman TL-100 ultracentrifuge and TLA 100.3 rotor. The 27 000-*g* supernatant was centrifuged at 100 000 *g* for 1 hr at 4° using a Beckman TL-100 ultracentrifuge and TLA 100.3 rotor to yield a microsomal fr. The microsomal pellet was resuspended in 1 ml of 0.1 M K-Pi (pH 7.5) containing 10 mM DTT. The microsomal prepn was stored on ice and used as quickly as possible for assays or stored with 15% glycerol at −80°. The Lowry proteins assay was used to determine protein concns.

The ecdysone 20-monooxygenase activity was routinely assayed using 100 μg microsomal protein

incubated with 0.5 mM NADPH, 10 mM DTT, 15 mM glucose-6-phosphate and 5 units glucose-6-phosphate dehydrogenase in 0.1 M K-Pi (pH 7.5) in a final reaction vol. of 1 ml in Eppendorf tubes. The monooxygenase reaction was initiated by addition of (62 500 dpm) of [22, 23 – ^{3}H] ecdysone (New England Nuclear NET-621), diluted with unlabelled ecdysone in 10 μl MeOH–H_2O (1:1) to a final concn of 55 μM. All enzyme reactions were terminated by addition of 0.8 ml MeOH. Cold carrier 20-hydroxyecdysone (10 μg) was added to each assay prior to evapn of the MeOH under a stream of N_2. The residue was resuspended and ecdysteroids removed by liquid–liquid partitions as previously described [33]. The final ecdysteroid sample was resuspended in 100 μl of MeOH, half of which was analysed by RP-HPLC on a C18, 4.6 mm × 25 cm, 5 μm particle size column eluted with MeOH–H_2O (9:11) at 1 ml min^{-1} and 33°. Ecdysteroid frs were collected and assayed for radioactivity by liquid scintillation counting, using a Beckman LS liquid scintillation counter, with Omnisolve as the scintillant. Secondary identification of ecdysteroids was accomplished by TLC on silica gel G (Sigma) of 0.3 mm thickness. TLC was used to identify the presence of ecdysteroids in various RP-HPLC column frs. A solvent system of $CHCl_3$–EtOAc–EtOH–HOA_2 (10:5:4:1) was used, providing R_f values for authentic standards as previously reported [33]. Structural identifications of 20-hydroxyecdysone and ecdysone were in agreement with authentic standards as previously reported [33, 34].

For experiments carried out under different atmospheres, CO, air and N_2 were bubbled through incubation solns via a 18.5 gauge needle attached by rubber tubing to a graduated gas container. A 500 ml vol. of gas was bubbled through the solns prior to addition of substrate. The reaction vessels were borosilicate glass sample vials (2 ml vol.) with screw-cap lids. The vials were closed after perfusion with each gas and sealed with parafilm. White light illumination was achieved by use of a 300 W tungsten lamp which was placed *ca* 20 cm from the transparent constant temp. water bath.

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