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STEROL BIOSYNTHESIS BY A CELL FREE EXTRACT OF *DIOSCOREA FLORIBUNDA*

Y. SELVARAJ and M. SUBHAS CHANDER

Indian Institute of Horticultural Research, 255 Upper Palace Orchards, Bangalore 560080, India

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Key Word Index—*Dioscorea floribunda*; Dioscoreaceae; homogenates; sterol biosynthesis.

Abstract—The biosynthesis of sterols from mevalonate by a cell free extract prepared from actively growing tuber portions and leader shoots with young leaves of *Dioscorea floribunda* has been demonstrated. The preparation was capable of synthesizing 86.4 μg and 34.0 μg of sterols from leader shoots with young leaves and actively growing tuber portions respectively. The cofactor requirement for the above system was also studied.

INTRODUCTION

Dioscorea tubers contain reasonably large quantities of diosgenin, which is ideally suited for conversion to 16-dehydropregnenolone. Because of its commercial value, the question of getting maximum diosgenin yield assumes greater importance. Incubation studies have indicated that fresh *D. floribunda* tuber homogenates when incubated at 37° for 24 hr gave an increased diosgenin yield of up to 16.2% over the control. In the same experiment when 500 ppm squalene was incorporated the diosgenin yield increased by 30.9% over the control. Incubation of oven-dried material does not result in an increase. However, dried tuber homogenates when incubated with crude enzyme extract prepared from *D. floribunda* leader shoots and young leaves registered an increased diosgenin yield ranging from 3.13 to 57.5% over the control [1–3]. The reason for such increased diosgenin yields could be the action of enzymes related to diosgenin biosynthesis. In the present investigation, evidence is obtained for the *in vitro* conversion of mevalonate to sterols by the cell free extracts prepared from *D. floribunda*.

RESULTS

The sterols from incubations of *D. floribunda* homogenates were obtained by alumina chromatography of the non-saponifiable lipid and was subjected to TLC which showed spots corresponding to lanosterol, sitosterol and cholesterol in all enzyme concentrations used. In the solvent system used cholesterol and sitosterol were not separated. The quantitative yields of sterol formed are therefore expressed in terms of sitosterol. Quantitative estimation of sterols showed that increasing amounts of sterol were formed from a constant quantity of mevalonate (2 mM mevalonate 2 ml) when increasing concentrations of enzyme extracts (1–3 ml) were used. The isolated multi-enzyme system from leader shoots and young leaves of *D. floribunda* was capable of synthesizing 16.2–86.4 μg of sterols in 3 hr depending upon the volume of enzyme extract (protein concentration 10.53–31.59 mg) used for assay. Compared to this, the multi-enzyme system isolated from actively growing tuber portions synthesized 13.5–34.0 μg sterols in 3 hr (protein concentration 7.40–22.33 mg).

The results of investigations on the cofactor requirements for the isolated enzyme system are given in Table 1. Sterol was formed only when the incubation mixture contained ATP, NADPH, glucose-6-phosphate and the cations Mg^{2+} and Mn^{2+}. In the absence of Mn^{2+} there was no sterol synthesis. However, in the absence of Mg^{2+} but with Mn^{2+} present sterol synthesis was reduced to 31.9–43.8% of the control value.

DISCUSSION

The demonstration of a multi-enzyme system capable of synthesizing sterol from mevalonate in *Dioscorea floribunda* homogenates supports the suggestions that the increased diosgenin yield obtained in previous incubation experiments [1–3] is the result of either the liberation of endogenous enzymes (as in a fresh tuber incubation) or the action of enzymes added exogenously on available substrates (precursors of diosgenin). The enzyme system isolated from leader shoots and young leaves was 1.8× more active biosynthetically than the system isolated from actively growing tuber portions. This may also explain the results obtained in our earlier incubation experiments [2] when 18.2% increased diosgenin yield was obtained in a fresh tuber incubation compared to 57.5% higher yield in dry tuber homogenate incubations after the addition of enzyme preparations from leader shoots and young leaves of *D. floribunda* plants.

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Table 1. Cofactor requirement for sterol synthesis from mevalonate by a cell free preparation from *D. floribunda*

Cofactor deleted	Sterol formed in leader shoots and young leaves (μg)	Sterol formed in actively growing tuber portions (μg)
Control*	86.4	34.0
− ATP	—	—
− NADPH	—	—
− Glucose-6-phospahte	—	—
− Mn^{2+} + Mg^{2+}	—	—
− Mg^{2+} + Mn^{2+}	27.6(31.9)†	14.9(43.8)
− Mg^{2+} − Mn^{2+}	0	0

*Homogenate prepared in the presence of Mn^{2+} and Mg^{2+} and incubated with all cofactors.
†Values in parenthesis are the percentage activity improved to the center.

EXPERIMENTAL

Plant material. Leader shoots, young leaves and actively growing tuber material of *Dioscorea floribunda* obtained immediately after harvest were used for this study.

Preparation of homogenates and enzyme assay. The cleaned plant material was homogenized in a precooled (0–4°) mortar and pestle with 0.1 M Pi buffer, pH 7.5, containing 5 mM $MgCl_2$, 2 mM $MnCl_2$ and 30 mM nicotinamide. The homogenates were squeezed through four layers of muslin cloth and centrifuged at 10200 g for 30 min at 0–4° in a refrigerated centrifuge. The clear supernatant was used as the enzyme extract for sterol biosynthesis studied.

The enzyme assays were carried out in 25 ml stopped flasks. The assay mixture consisted of 2 mM mevalonate (2 ml) 3 mM ATP (1 ml), 1 mM NADPH (1.0 ml), 3 mM glucose-6-phosphate (0.5 ml) and enzyme extract (1–3 ml). The incubation was carried out at 37° for 3 hr in a shaking incubator. Suitable enzyme and substrate blanks were run along with the assays as controls. Additions of 2 mM ATP (1.0 ml), 1 mM NADPH (1.0 ml) and 3 mM glucose-6-phosphate were repeated at hourly intervals. The reaction was stopped by addition of ethanolic KOH and the mixture saponified at 70° for 3 hr.

Estimation of sterols. The non-saponifiable lipid was extracted with Et_2O, dried over Na_2SO_4, filtered and freed from Et_2O. The residue was taken-up in petrol (40–60°), applied to an alumina column and eluted with petrol. The adsorbed sterol was eluted with EtOH–Et_2O (3:1). The solvent was evaporated and the residue dissolved in $CHCl_3$. The sterol content in the $CHCl_3$ soln was estimated by the Lieberman–Burchard reaction [4]. A portion of the $CHCl_3$ soln was subjected to TLC Si gel G. Samples and authentic lanosterol, sitosterol and cholesterol standards were applied and the TLC plates were developed in the solvent systems C_6H_6–EtOH (95:5), C_6H_6–EtOAc (5:1) or $CHCl_3$–Me_2CO (95:5) respectively. The plates were dried and sterols were identified by spraying with Ac_2O–H_2SO_4 (20:1) reagent.

Protein estimation. Protein was measured in the enzyme extracts by the method of Lowry *et al.* [5] using bovine serum albumin as a standard.

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