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SHORT COMMUNICATION

MEVALONATE PHOSPHORYLATION IN *AGAVE AMERICANA*

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Key Word Index—*Agave americana*; Amaryllidaceae; sapogenins; biosynthesis; mevalonic acid phosphorylation; mevalonate and phosphomevalonate kinase.

Abstract—Cell-free extracts and acetone powder preparations from *Agave americana* phosphorylated mevalonic acid (MVA) to phosphomevalonic acid (MVAP) and pyrophosphomevalonic acid (MVAPP) through reactions catalyzed by mevalonate kinase and phosphomevalonate kinase. Both activities are maximal at pH 7·0. Glutathione and mercaptoethanol increase the activity. The formation of MVAP and MVAPP reaches its maximal level after 0·5 hr of incubation. Flowers of the *Agave* show more activity than leaves and scape. The addition of NAF prevents the dephosphorylation of the compounds formed.

INTRODUCTION

MEVALONIC acid (MVA) is the substrate most frequently used in the studies on biosynthesis of terpenoids in plants, although CO_2 and acetate can also be used. These precursors are converted to isopentenyl-pyrophosphate (IPP) through mevalonate-5-phosphate (MVAP) and mevalonate-5-pyrophosphate (MVAPP). This pathway has been found to occur in a very wide variety of organisms[1–6] and its requirements have been established.[7–9] The mechanisms of plant sterol synthesis have been recently reviewed.[10–14]

At least five sapogenins have been isolated from *Agave americana* (Amaryllidaceae), namely: hecogenin,[15,16] chlorogenin,[17] neotigogenin, kammogenin and 9-dehydrohecogenin.[18] Little information is available on the biosynthesis of these compounds in *Agave*.

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Only Ehrhardt *et al.*[19] showed that *Agave toumeyana* incorporated (1-^{14}C) acetate into cycloartenol.

This paper describes the incorporation of (1-^{14}C and 2-^{14}C) MVA by *Agave americana* enzyme extracts into phosphorylated derivatives, as the first steps in the biosynthesis of sapogenins. The conditions, rate and requirements of these reactions have been investigated.

RESULTS AND DISCUSSION

Phosphorylation of Mevalonic Acid

Cell-free extracts from *Agave americana* leaves phosphorylated MVA to MVAP and MVAPP by MVA kinase (E.C. 2.7.1.36) and MVAP kinase (E.C. 2.7.4.2) respectively. Both MVAP and MVAPP were identified by paper chromatography. The R_f values obtained are essentially in good agreement with those previously reported for such compounds (Table 1). The identity of these compounds was further confirmed by acid and enzymatic hydrolysis. When reaction products were heated in 1 N HCl for 7 min the compound corresponding to MVAPP disappeared, and MVAP appeared. Incubation with alkaline phosphatase for 15 hr at room temp. converted both compounds into free MVA. Several solvents were used for chromatography, since phosphorylated derivatives can be hydrolyzed during chromatography in some solvents.

TABLE 1. PAPER CHROMATOGRAPHY OF RADIOACTIVE MVA METABOLITES

	R_f values Solvent					
	1	2	3	4	5	6
MVAP						
Reported	0·15–0·18	0·53–0·61	0·19	0·37	0·01–0·03	0·07–0·14
Found	0·09–0·13	0·56–0·70	0·12–0·20	0·27–0·32	0·05–0·12	0·17–0·25
MVAPP						
Reported	0·03–0·05	0·29–0·35	0·09	0·20–0·25	0–0·01	0–0·08
Found	0·01–0·05	0·29–0·35	—	0·09–0·14	0·01–0·14	0·02–0·10

The formation of MVAP and MVAPP in similar amounts (Table 2) clearly shows that both kinases are active; no other radioactive products were detected. The same results were obtained when acetone powder preparations were used. Higher phosphate incorporation was found when extracts from leaf cortex were employed. The parenchyma is practically inactive.

Valenzuela *et al.*[20] reported a maximal activity at pH 6·0 for MVA kinase from *Pinus radiata* seedlings, whereas our results with the enzyme from *Pinus pinaster* seedlings showed an optimum pH of 7·9. In the case of the *Agave* enzymes, the optimum pH was 7·3–7·5. Other optimal pH reported are 7·3 and 7·5 for the kinase from rat liver[21] and rubber latex.[8]

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TABLE 2. INCORPORATION OF (2-^{14}C) MVA BY EXTRACTS OF *Agave americana* LEAVES

Compound formed	Solvent	Radioactivity (dpm × 10^3) in reaction system
MVAP	2	36·0
	3	36·0
	4	36·0
	5	43·2
	6	43·2
MVAPP	1	38·4

* For solvent key, see Experimental.

Effect of Glutathione and Mercaptoethanol

Glutathione was added to the reaction system just before incubation, whereas mercaptoethanol was added to the buffer as a protector of –SH groups of the enzymes in the extracts. Table 3 shows that mercaptoethanol greatly increases the formation of phosphorylated derivatives of MVA, protecting active groups in the kinases during the preparation of the extracts. These results are generally in good agreement with those found earlier.[7–9]

TABLE 3. EFFECT OF GLUTATHIONE AND MERCAPTOETHANOL ON MVA KINASE ACTIVITY

Compound formed	Solvent	Radioactivity (dpm × 10^3) Glutathione M × 10^{-2}	Mercaptoethanol M × 10^{-2}
MVAP	2	38·4	84·0
	4	40·8	84·0
	5	48·0	51·6
	6	44·4	75·6
MVAPP	1	40·8	120·0

Rate of Incorporation of MVA and Effect of NaF

Phosphorylation of (2-^{14}C) MVA by acetone powder preparations from *Agave americana* leaves, flowers and scape, with and without NaF, has been investigated. The results indicate that formation of MVAP and MVAPP by acetone powders from flowers extracts reaches its maximal value within 0·5 hr, becoming practically level afterwards. The preparations from *A. americana* scape and leaves show a lesser kinase activity, the scope of the curve being less when the scape, i.e. more parenchymal tissues, was used. The same results were obtained with crude extracts. In any case, the incorporation of MVA using *Agave americana* extracts is much slower than when pine is used.[22]

NaF seems to protect the MVA phosphates formed in the reaction. This effect is probably due to inhibition of phosphatase activity present in the medium.[23] According to Gosselin,[24] a clear effect of NaF could be observed only when working with aged preparations. In a previous communication,[22] we reported the influence of NaF on the MVA derivatives either through a direct action or increasing the activating effect of glutathione.

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The results obtained with extracts of different protein concentration suggest the presence of an inhibitor which strongly interferes the reaction when its concentration reaches a definite level. This agrees with the results of George-Nascimento and Cori,[25] working on terpene biosynthesis in cell-free extracts from orange flavedo, who found that the rate of phosphorylation was not strictly proportional to the amount of protein added. Experiments are being carried out to demonstrate if the inhibitor is present in the extract or it is produced as the reaction proceeds.

EXPERIMENTAL

Materials. Agave americana plants, growing locally, were used. (1 and 2-^{14}C) MVA was supplied in the form of lactone by the Radiochemical Centre, Amersham, England; the K salt was prepared by treating the lactone at 36° for 30 min with an excess of a KOH solution. The pH of the solution was *ca.* 10.

Enzyme preparations. Cell-free extracts from *Agave americana* leaves were obtained by homogenizing the cortex in an ice-cold blendor for 15 min with Tris-HCl 0·5 M buffer, pH 7·9, so that the final plant/buffer ratio is 1:1. The homogenate was squeezed through a cloth and centrifuged at 10 000 *g* for 15 min at 4°. The acetone powders were prepared by grinding the leaves, flowers or scape for 15 min in a homogenizer with a volume of acetone at −20° equivalent to 10 × the wt of the plants. The homogenate was filtered through a coarse fritted glass funnel and the residue was washed with several portions of acetone at −20° until the filtrate was colourless. The residue was washed with Et_2O (twice the volume of acetone used for homogenization) at −20° and dried at 4°. Prior to use, the powder was extracted for 10 min with a volume of Tris-HCl buffer pH 7·9 equivalent for 10 × the wt of the powder and centrifuged at 10 000 *g* for 15 min at 4°. Protein content of enzyme extracts was determined by the Lowry method.[26]

Enzyme assays. Enzymatic reactions were carried out incubating the extracts at 37°. The reaction system, in a total volume of 3 ml, contained 24 μmol of ATP, 12 μmol of $MgCl_2$, 30 μmol of glutathione, 30 μmol of NaF, 145 mμmol of MVA-^{14}C (1 μCi), 30 μmol of Tris-HCl buffer pH 7·9 and 2 ml of cell-free extract. Reactions were stopped by heating the reaction tube at 90° for 2 min. Precipitated protein was centrifuged off at 2000 *g* for 5 min. The supernatants were analyzed by PC.

Chromatographic identification of MVA metabolites. Aliquots (25 μl) of supernatants from the reaction mixture were applied to Whatman No. 1 paper strips and developed in: (1) *n*-BuOH–HCO_2H–H_2O (73:13:10)[27] (ascending); (2) *t*-BuOH–HCO_2H–H_2O (20:5:8)[28] (descending); (3) *t*-AmOH–HOAc–H_2O (4:1:2)[3] (ascending); (4) isobutyric acid–NH_4OH–H_2O (22:1:10)[4] (descending); (5) EtOH–NH_4OH–H_2O (8:1:1)[29] (descending); and (6) *n*-PrOH–NH_4OH–H_2O (6:3:1)[30] (ascending). Radioactive spots on the dried strips were detected and measured in a Nuclear-Chicago Actigraph III system. Measurement conditions were previously established.

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