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MEVALONATE KINASE FROM *PINUS PINASTER* SEEDLINGS

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Abstract—Cell-free extracts and acetone powder extracts from *Pinus pinaster* seedlings contain mevalonate kinase which phosphorylates mevalonic acid (MVA) to mevalonate-5-phosphate (MVAP). This enzyme which has a pH optimum of 7·8–8·0 can only utilize ATP as a phosphate donor, GTP, UTP and ITP being inactive. No significant nucleoside diphosphate kinase activity is present in the enzyme preparations. 10 mM Mn^{2+} added to the cell-free extracts strongly decreases the MVA phosphorylation, whereas Mg^{2+} at the same concentration produces only a little inactivation. EDTA at 10 mM inhibits activity almost completely. Dialysis decreases the ability to phosphorylate MVA. The K_ms of MVA kinase from acetone powder extracts were 8×10^{-5} M for MVA and $1{\cdot}4 \times 10^{-4}$ M for ATP. The enzyme has been partially purified by protamine sulfate treatment, ammonium sulfate precipitation and Sephadex G100 fractionation.

INTRODUCTION

MEVALONATE kinase (ATP: mevalonate-5-phosphotransferase, E.C.2.7.1.36) catalyzes the phosphorylation of mevalonic acid (MVA) to mevalonate-5-phosphate (MVAP). This enzyme is present in many organisms[1–5] in which the kinase reaction is the first stage in the formation of several isoprenoid compounds. The incorporation of radioactivity from MVA into α-pinene was demonstrated in *Pinus nigra*[6] and *P. attenuata*,[7] whereas Valenzuela *et al.*[8] only recovered 0·05% of the radioactivity of 2-^{14}C-MVA in the limonene fraction of *P. radiata*. In this source,[9] the phosphorylated intermediates of terpene biosynthesis have been identified as pyrophosphomevalonic acid (MVAPP) and isopentenyl pyrophosphate (IpPP), in addition to MVAP. These intermediates have also been demonstrated in reactions carried out with extracts from orange juice vesicles,[10] acetone powders from callus culture of *Kalanchoë crenata*[11] and cell-free and acetone powder preparations from *Agave americana*.[12]

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Plant MVA kinase has been partially purified from *Cucurbita pepo* seedlings[13] and *Hevea brasiliensis* latex.[14] Its mechanism of action has been studied in the hog liver enzyme,[15] showing that MVA phosphorylation is sequential and that an –SH group is important in the reaction. We have reported the intrachloroplastic location of mevalonate-activating enzymes in *Spinacia oleracea* leaves, *A. americana* leaves and *P. pinaster* seedings.[16]

In this paper, some properties and partial purification of MVA kinase of cell-free extracts and acetone powder extracts from *P. pinaster* seedlings are reported.

RESULTS AND DISCUSSION

Formation of 5-phosphomevalonic acid—optimum pH

MVA kinase activity has been demonstrated in cell-free extracts and acetone powder extracts from *P. pinaster* seedlings. The formation of MVAP from 2-^{14}C-MVA was followed by PC and was identified using several solvents.[12] The acetone powders from *P. pinaster* seedlings preserved their MVA kinase activity for several weeks when stored at 4°. As in *A. americana*,[12] the addition of 10 mM β-mercaptoethanol to the buffer protects the kinase activity during the preparations of the extracts. MVAP formation by acetone powder extracts from *P. pinaster* seedlings was studied over the range pH 4–8 by using the following buffers: 0·15 M citrate buffer (pH 4–7) and 0·1 M Tris-HCl buffer (pH 7·2–8). No phosphorylation of MVA was obtained below pH 7. A pronounced optimum was found at pH 7·8–8. These results differ from those reported by Valenzuela *et al.*[8] working with cell-free extracts from *P. radiata* seedlings where the maximum activity in Tris-succinate buffer was obtained at pH 6. In our case, all attempts to obtain phosphorylation of MVA at this pH value have been unsuccessful. MVA kinase from pig liver,[3] rabbit liver,[4] superovulated rat ovary,[5] *Hevea* latex[14] and *Sarcophaga bullata* larvae[17] has a similar optimum pH value (7–7·5) whereas the yeast[1] and orange[10] enzymes show the optima at more acid pH values. In French bean seedlings, Rogers *et al.*[18,19] demonstrated the presence of two isoenzymes of MVA kinase, one located in the chloroplasts and the other outside them, with an optimum pH 7·5 and 5·5 respectively. Nevertheless, we have reported[20] the separation by Sephadex G100 of two fractions with MVA kinase activity from *P. pinaster* seedlings and *A. americana* leaves, both fractions being optimally active at pH 7·9.

Nucleotide dependence of MVA kinase activity

At 1 and 5 mM neither GTP, UTP nor ITP were effective in replacing ATP at the same concentrations. ADP is also unable to replace ATP as a phosphate donor. In addition, there was no significant nucleoside diphosphate kinase activity, since GTP, UTP and ITP were also inactive in the presence of ADP. All the experiments were carried out in the presence of 4 mM Mg^{2+}. These results indicate that MVA kinase from *P. pinaster* seedlings

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is more specific for ATP than the enzymes from other sources. Thus, in the enzymes from pig liver[3] and *Hevea*[14] ITP is as effective as ATP at similar concentrations. Although a marked preference for ATP characterizes the *S. bullata* larval enzyme[17] this enzyme is also able to use UTP and ITP at low concentrations (1·2 mM). MVA kinase from yeast autolysate[1] can also utilize GTP, UTP or CTP in the place of ATP.

Effect of dialysis and metal ions on MVA kinase activity

The cell-free extracts of *P. pinaster* seedlings are able to phosphorylate MVA without addition of metal ions (Table 1). Supplementation of 10 mM Mn^{2+} strongly decreases the phosphorylation of MVA, whereas Mg^{2+} at the same concentration produces only a little inactivation. Addition of 10 mM EDTA produces an almost complete inhibition. The MVA kinase isolated from *Hevea* latex[14] exhibited activation by Mn^{2+} and Mg^{2+} at low concentrations (1 and 4 mM respectively), being inhibited at higher concentrations. However, in *S. bullata* larvae[17] high concentrations of Mg^{2+} did not significantly affect the MVA kinase activity. Working with cell-free extracts of *P. radiata* seedlings, Valenzuela *et al.*[8] found maximum kinase activity after the addition of 3 mM Mn^{2+}, decreasing with further increase in metal concentration. When the extracts were dialyzed for 30 min, 1 and 2 hr, a reduced ability to phosphorylate MVA was found (Table 1). Addition of 5–10 mM Mg^{2+} slightly increases the formation of MVAP. Similar results were obtained when extracts from acetone powders of *P. pinaster* seedlings were used.

TABLE 1. EFFECT OF METAL IONS AND EDTA ON MVA KINASE ACTIVITY OF *Pinus pinaster*

	MVAP formed (dpm × 10^{-3}/mg protein)			
		Mn^{2+}	Mg^{2+}	EDTA
Undialyzed cell-free extract	48·0	22·8	43·2	5·4
Dialyzed cell-free extract	9·6	4·8	12·0	—

Cofactors (10 mM final concentration) were added to reaction system containing 300 μmol Tris-HCl buffer, pH 7·9, 30 μmol glutathione, 24 μmol ATP and 145 nmol 2-^{14}C-MVA (1 μCi) in a total vol. of 3 ml. 10 mg protein of undialyzed cell-free extract and 5 mg protein of dialyzed cell-free extract were also added.

Influence of incubation time and protein concentration on MVA kinase activity

MVA phosphorylation by acetone powder extracts from *P. pinaster* seedlings increases with time, becoming practically level after 30 min. Extracts of higher protein concentration show a lower specific activity at any incubation time considered, this being independent of buffer concentration. This has been observed several times, with extracts of different protein concentrations.

In order to investigate the influence of protein content on the MVAP formation, different amounts of acetone powder extracts were incubated with the same amounts of substrate and cofactors for 30 min. The MVAP decreased when a definite level of protein (15–20 mg) was exceeded. A concomitant increase of MVA remaining was also observed, suggesting that the MVAP formed was hydrolyzed by a phosphatase present in the enzyme preparations, the activity of which is more pronounced as the extract concentration increases. This interfering activity was eliminated when the MVA kinase was partially purified. The phosphatase was mentioned by Valenzuela *et al.*[8] in cell-free extracts from *P.*

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radiata seedlings and George-Nascimento *et al.*[21] in orange flavedo. Mitchell and Downing[22] have separated a phosphatase activity from mevalonate-activating enzymes in a cell-free homogenate of *Nepeta cataria* by means of a 15–30% sucrose gradient.

Kinetic properties of MVA kinase

As an initial evaluation of the enzyme kinetic properties, the rate of MVA phosphorylation by acetone powder extracts was studied at MVA concentrations varying from 4×10^{-5} M to 5×10^{-4} M in the presence of 8×10^{-3} M ATP. Similar experiments were carried out under conditions in which the ATP concentration was varied between 10^{-5} M to 4×10^{-3} M in the presence of $5{\cdot}7 \times 10^{-5}$ M MVA. The reciprocal plots of 1/V against 1/S provide the following K_m values: for MVA, 8×10^{-5} M and for ATP, $1{\cdot}4 \times 10^{-4}$ M. The K_m for MVA of MVA kinase from *P. pinaster* resembles that of the pig liver enzyme,[3] whereas the K_m for ATP is similar to those of the superovulated rat ovary[5] and hog liver[15] and lower than those of *Hevea* latex[14] and *S. bullata* larvae.[17]

TABLE 2. PURIFICATION OF MVA KINASE FROM *Pinus pinaster*

Purification step	Protein (mg/ml)	Activity (dpm × 10^{-3})	Sp. act. (dpm × 10^{-3}/mg)	Purification
Acetone powder extract	7·1	48	7	1·0
Protamine sulfate supernatant	6·8	69	10	1·5
Ammonium sulfate (30–45%)	6·2	154	25	3·6
Sephadex G100 (1st fraction)	1·8	229	127	18·8
Sephadex G100 (2nd fraction)	1·6	211	132	19·5

The conditions of the assay are given in the text.

Partial purification of MVA kinase

Table 2 shows the results of partial purification of MVA kinase of acetone powder extracts from *P. pinaster* seedlings. In all the experiments, the protamine treatment resulted in only a 1·5-fold increase in the specific activity of the preparation, whereas Levy and Popjak[3] reported a 3·5-fold increase in the pig liver enzyme. After this treatment, the supernatant was subjected to ammonium sulfate fractionation. About 80% of the enzyme precipitated between 30 and 45% ammonium sulfate saturation with a 2·5-fold increase in specific activity. Valenzuela *et al.*[8] reported that the enzyme may be concentrated by precipitation with 55% saturated ammonium sulfate, but no increase in specific activity was obtained by this procedure.

Further purification was carried out by Sephadex gel filtration. Although Sephadex G200 has been successfully used as a purification technique for both *Hevea* latex[14] and *S. bullata* larvae[17] MVA kinase, the enzyme from *P. pinaster* seedlings present in the fraction precipitated between 30 and 45% saturation of ammonium sulfate could not be successfully eluted from Sephadex G200 columns. However, good recovery of enzyme activity was obtained when Sephadex G100 was used. Two fractions with MVA kinase activity were eluted, both with a nearly 20-fold increase in specific activity over the original acetone powder extract. The second fraction loses its activity rapidly at 4° even in the presence of 10 mM β-mercaptoethanol.

The first active fraction from the Sephadex G100 column was applied to a DEAE-Sephadex A50 column. On stepwise elution with the Tris-HCl buffer containing increasing concentration of KCl the enzyme was present in the fraction eluted at 0·2 M KCl. No significant increase in the specific activity was obtained by this procedure.

EXPERIMENTAL

Materials. *P. pinaster* seedlings, 60-days-old, grown from seeds stratified at 5° for 6 weeks were used. 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 37° for 30 min with an excess of KOH sol.

Enzyme preparations. Cell-free extracts from *P. pinaster* seedlings were prepared by homogenizing the seedlings in an ice-cold blendor for 15 min with 0·1 M Tris-HCl buffer, pH 7·9, so that the final plant/buffer ratio was 1:1. The homogenate was squeezed through a cloth and centrifuged at 10000 *g* for 15 min at 4°. Dialysis was carried out in "Visking" tubes against 1 mM Tris-HCl buffer, pH 7·9, changing the buffer × 3 (0·5, 1 and 2 hr). Acetone powders were obtained as described previously for *A. americana* leaves.[12] The extracts were prepared with 0·1 M Tris-HCl buffer, pH 7·9, containing 10 mM β-mercaptoethanol. In some experiments, other buffers were used as reported in the text. All procedures were carried out at 0–4°. Protein content of enzyme preparations was determined by the Lowry method.[23] Protein concentration of column eluates was determined from absorption at 280 nm.

Purification procedures. Protein from the 10000 *g* supernatant sol was treated with a 2% (w/v) solution of protamine sulfate to give a final conc of 0·1 mg of protamine sulfate/mg protein. The mixture was stirred slowly for 2 min, allowed to settle 30 min and then centrifuged at 10000 *g* for 15 min at 4°. The protamine sulfate supernatant was fractionated with solid $(NH_4)_2SO_4$. The fraction precipitating between 30 and 45% saturation was dissolved in 0·1 M Tris-HCl buffer, pH 7·9, containing 10 mM β-mercaptoethanol and filtered upward through a Sephadex G100 column (4·5 × 45 cm) previously equilibrated with the same buffer. Protein was eluted at a flow rate of 40 ml/hr; 10 ml fractions were collected. The first enzymatically active fraction eluted from the Sephadex G100 column was applied to a DEAE-Sephadex A50 column (1·5 × 20 cm) equilibrated with 0·1 M Tris-HCl buffer, pH 7·9, containing 10 mM β-mercaptoethanol and 0·1 M KCl. The first active fraction from Sephadex G100 (18 mg of protein in 10 ml) was applied to the column and eluted with the same buffer with a continuous gradient of KCl 0·1–0·5 M, at a flow rate of 60 ml/hr. Fractions of 10 ml were collected.

Enzyme assays were carried out by incubating the extracts at 37° for 30 min. Unless otherwise specified, the reaction system contained 24 μmol ATP, 12 μmol $MgCl_2$, 30 μmol glutathione or β-mercaptoethanol, 145 nmol 2-^{14}C-MVA (1 μCi), 300 μmol Tris-HCl buffer, pH 7·9 and enzyme preparation in a total vol. of 3 ml. Reactions were stopped by heating the tubes at 90° for 2 min. In concentrated preparations (25–30 mg of protein/ml) longer than 5 min at 90° was needed in order to obtain total inactivation. Precipitated protein was centrifuged off at 2000 *g* for 5 min.

Chromatographic identification of MVAP. Aliquots (25 μl) of supernatants from the reaction mixture were applied to Whatman No. 1 paper strips and developed in several solvents.[12] Radioactive spots on the dried strips were detected and measured in a Nuclear-Chicago Actigraph III system.

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