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Purification and characterization of phosphomevalonate kinase from *Catharanthus roseus*

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Abstract

5-Phosphomevalonate kinase activity was partially purified from suspension cultured cells of *Catharanthus roseus* (L.) G. Don. The enzyme had an estimated M_r of 128,000 as determined by size-exclusion chromatography. Kinetic studies indicated that the mechanism of action was sequential with true K_m values of 0.35 and 0.22 mM for 5-phosphomevalonate and ATP, respectively. The enzyme activity was dependent on the presence of divalent ions with a preference for Mg^{2+}; at equimolar concentrations, i.e., 2 mM, the enzyme showed a relative remaining activity of 52, 22 and 4% in the presence of Mn^{2+}, Zn^{2+} and Ni^{2+}, respectively ($Mg^{2+} = 100\%$). The pH optimum for 5-phosphomevalonate kinase activity ranged from 7 to 9, with a maximum at about 8.

Keywords: *Catharanthus roseus*; Apocynaceae; Terpenoid biosynthesis; Mevalonate pathway; 5-Phosphomevalonate kinase

1. Introduction

In higher plants, isoprenoid biosynthesis is one of the major biosynthetic routes yielding many compounds vital for the metabolism and development of the plant. The common precursor of all isoprenoids is isopentenyl diphosphate (IPP). At present, two pathways are known that result in the biosynthesis of IPP, i.e., the mevalonate (MVA) pathway (Bach, 1995; Gray, 1987), and the MEP2 pathway (Lichtenthaler, Rohmer & Schwender, 1997; Rohmer, Seemann, Horbach, Bringer-Meyer & Sahm, 1996). In higher plants both pathways are present; the MVA pathway is supposed to be cytosolic, and the MEP pathway is localized in plant chloroplasts.

Even though the MEP pathway has only been recognised since 1993, high efforts are being undertaken to elucidate this novel pathway searching for enzymes and their corresponding genes (Lange, Wildung, McCaskill & Croteau, 1998; Lois, Rosa Putra, Danielsen, Rohmer & Boronat, 1998; Sprenger et al., 1997; Takahashi, Kuzuyama, Watanabe & Seto, 1998). On the other hand, the MVA pathway (Fig. 1) has been known for decades, and still not all enzymes have been characterised in detail. Only 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (HMGR; EC 1.1.1.34) has been studied extensively, as it is considered to be the major point of regulation of the substrate flux through the pathway (Bach, 1995; Stermer, Bianchini & Korth, 1994; Weissenborn et al., 1995). In contrast, only little information is available on the other enzymes.

As far as 5-phosphomevalonate kinase (PMK; EC 2.7.4.2), a plant PMK has only been partially purified,

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[2] At the 4th European Symposium on Plant Isoprenoids (Barcelona, April 21–23, 1999) it was agreed to use only the names 'Rohmer pathway' (after its discoverer) or 'MEP pathway' (after 2-C-methyl-D-erythritol 4-phosphate, the first committed precursor) for the recently discovered non-mevalonate pathway of IPP biosynthesis.

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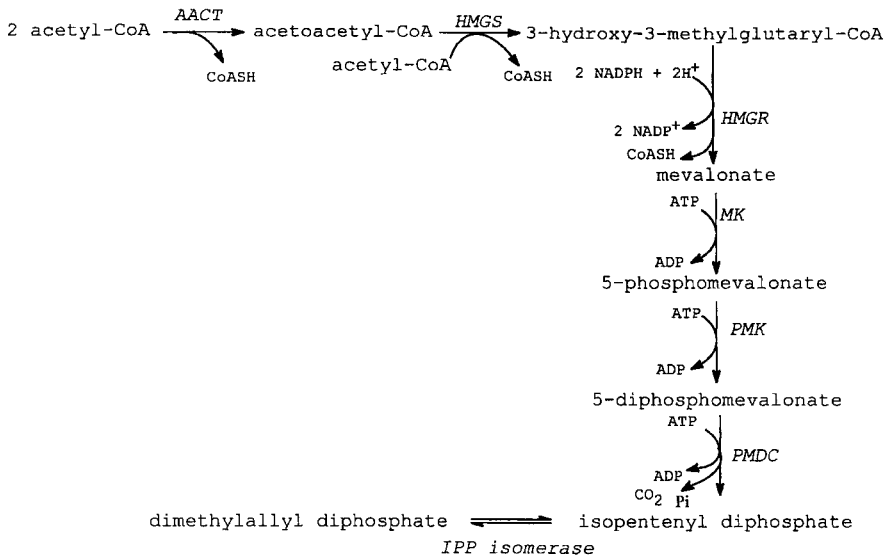

Fig. 1. Biosynthesis of isopentenyl diphosphate. AACT = acetoacetyl-CoA thiolase, HMGS = HMG-CoA synthase, HMGR = HMG-CoA reductase, MK = mevalonate kinase, PMK = 5-phosphomevalonate kinase, PMDC = 5-diphosphomevalonate decarboxylase, IPP = isopentenyl diphosphate.

and characterized from the latex of *Hevea brasiliensis* (Skilleter & Kekwick, 1971). In addition, the enzyme has been purified and characterized from pig liver (Bazaes et al., 1980), and the gene has been isolated from human liver (Chambliss, Slaughter, Schreiner, Hoffmann & Gibson, 1996) and *Saccharomyces cerevisiae* (Tsay & Robinson, 1991).

Based on these few reports, it seems that PMK is quite a unique enzyme, which reveals some remarkable differences when comparing its characteristics among the different sources. For instance, the molecular sizes of PMK have been determined to be between 21 and 22 kDa for the pig (Bazaes et al., 1980) and the human (Chambliss et al., 1996) enzyme, as based on gel filtration of the enzyme and the deduced amino acid sequence, respectively. On the other hand, the expressed yeast cDNA encoding PMK has yielded an active enzyme of 47 kDa, which was in correspondence with the estimated size as based on the deduced amino acid sequence (Tsay & Robinson, 1991). In addition, both the cDNA sequences and the deduced amino acid sequences of the human and the yeast PMK do not have any homologous sequences (Chambliss et al., 1996). Moreover, the consensus amino acid sequence for an ATP-binding pocket present in many kinases has not been recognized in the human PMK cDNA sequence (Chambliss et al., 1996), while it has been recognized in the yeast PMK cDNA sequence (Tsay & Robinson, 1991).

We detected PMK activity in *Catharanthus roseus* cell extracts by the formation of its product, 5-diphosphomevalonate (MVAPP) using a radiochemical assay for MVA kinase (MK) (Schulte, 1998). The quantification of PMK activity in crude extracts may be hampered by the presence of phosphatase activities, which are able to metabolize the substrates, 5-phosphomevalonate (MVAP) and ATP, and the product MVAPP. A recent report discussed the optimization of a radiochemical assay (Sandmann & Albrecht, 1994) and, in addition, we developed a microplate assay (Schulte, Van der Heijden & Verpoorte, 1999) for accurate measurement of MK, and PMK activities.

We study the regulation of isoprenoid biosynthesis in cell suspension cultures of *C. roseus*, in particular the conversion of MVA into IPP (Schulte, 1998; Verpoorte, Van der Heijden & Moreno, 1997). This report presents the partial purification and characterization of PMK from *C. roseus* suspension cultured cells, and shows some new features for a plant PMK.

2. Results

PMK was partially purified from suspension cultured *C. roseus* cells in a sequence of five chromatographic steps. Table 1 summarizes the results of the purification of the enzyme starting with about 400 g cells (fr. wt). Due to high blank rates, PMK activity was not detectable in the concentrated extract, and the concentrated Q Sepharose sample (I). As a consequence, the enzyme activity in the Phenyl Sepharose sample (II) was taken as 100% activity. The MK and PMK activities, which co-eluted from the Q Sepharose column, were separated during the Phenyl Sepharose

Table 1
Partial purification of 5-phosphomevalonate kinase from suspension cultured cells of *C. roseus*. The yield of the Shodex KW 803 step was calculated as if the entire volume after the Mono Q step were applied. S.A. = specific activity, n.d. = not detectable, – not determined

Purification step (sample)	Volume (ml)	Protein (mg)	Activity (nkat)	S.A. (nkat/mg)	Purification factor	Yield (%)
Concentrate	26	–	n.d.	–	–	–
Q Sepharose (I)	7	–	n.d.	–	–	–
Phenyl Sepharose (II)	12	6.8	2.76	0.41	1	100
Sephacryl S200 (III)	4.4	3.0	3.83	1.29	3.2	167
Mono Q (IV)	0.65	0.17	0.364	2.08	5.1	14.5
IV applied to SEC	0.05	0.013	0.028			
Shodex KW803 (V)	0.5	0.002	0.0075	3.57	8.7	3.5

step. The PMK activity was not retained on this column, and eluted with the bulk of the proteins; also all the enzyme activities causing high blank rates in the microplate assay co-eluted with the PMK activity. Due to the presence of, among others, phosphatases in these fractions, the MVAP in the assay may have been converted to MVA, which could give false results when some residual MK activity would still be present after overloading the column. However, no MK activity was detected in these flow-through fractions as determined by a zero activity in presence of MVA as a substrate. The Sephacryl S200 step resulted in the removal of most of these disturbing enzymes. Mono Q chromatography at pH 6 resulted in an increased purification, but, at this low pH, the enzyme was unstable. Therefore, immediately after elution, the buffer was changed to one with pH 7.5. By size-exclusion chromatography (SEC) the specific activity was further increased. The elution pattern showed that the PMK activity eluted in the front of a large protein peak, spread over a number of fractions between 8.4 and 9 ml (Fig. 2). Taking the mid fraction to estimate the size, PMK would correspond to a protein with an M_r of about 128,000. It should be noted that estimations using fresh, crude enzyme preparations yielded M_r values of about 166,000.

SDS-PAGE showed that in the final PMK preparation (sample V) still five polypeptides were detectable (Fig. 3A). Native PAGE gave a similar result (Fig. 3B). Table 2 presents the detected polypeptides in the active fractions (sample V), and the adjacent fractions (samples VI and VII; photographs not shown), after SDS- and native PAGE.

To determine the affinity of PMK towards its sub-

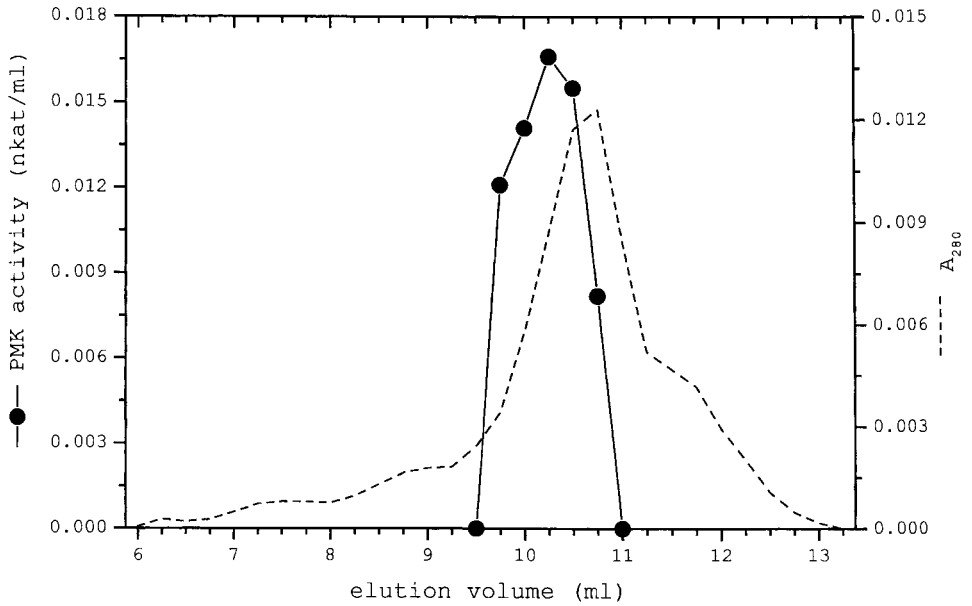

Fig. 2. Purification of 5-phosphomevalonate kinase (PMK) from *C. roseus* suspension cultured cells. Elution profile of PMK activity after size-exclusion chromatography on the Shodex KW 803 column. Indicated are PMK activity (nkat/ml), and absorption at $\lambda = 280$ nm. Details are described in Experimental 4.3.

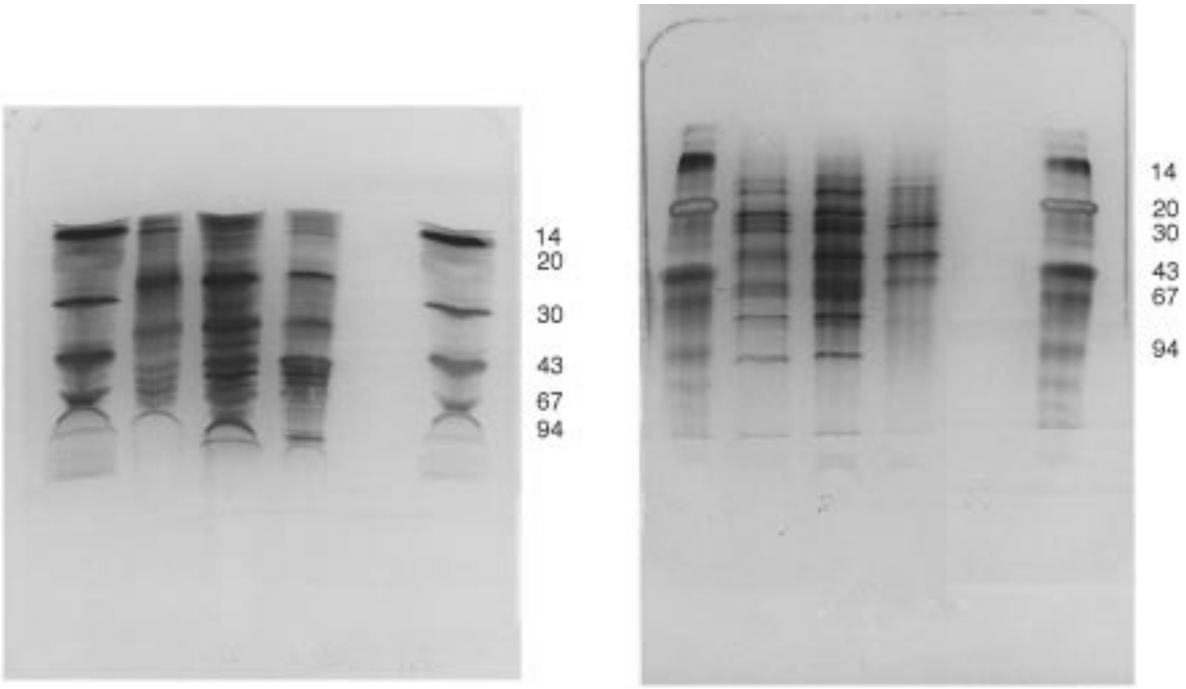

Fig. 3. A) SDS-PAGE (left) and B) native PAGE (right) of partially purified 5-phosphomevalonate kinase from *C. roseus* suspension cultured cells. Samples are from left to right: LMW, II, III, IV, V and LMW. The sample numbers are as indicated in Experimental 4.3 and 4.7, and Table 1. LMW = low molecular weight markers, marker size indicated in kDa.

strates MVAP and ATP, its activity was measured at varied substrate concentrations. Table 3 presents the apparent K_m and V_{max} values for MVAP at different ATP concentrations. Fig. 4 shows the replots of $1/V_{max}$ and K_m/V_{max} against the inverse ATP concentration. The true K_m and V_{max} values were calculated from these replots; V_{max} was determined to be 2.59 ± 0.24 nmol/s/mg, the true K_{mA} value for MVAP was estimated to be 0.35 ± 0.06 mM, the K_{mB} for ATP was 0.22 ± 0.09 mM, and the K_{iA} was calculated to be 0.57 ± 0.36 mM.

PMK required divalent cations for its activity, of which Mg^{2+} was preferred. Table 4 presents the relative PMK activities with some other divalent ions as compared to the activity with Mg^{2+} at equimolar concentrations. Furthermore, PMK showed maximum activities between pH 7 and 9 (Fig. 5). The dip at pH 8.5 is considered to be an artifact, as it was not observed in previous experiments with similar preparations of PMK.

Table 2
Estimated relative masses of detected polypeptides after SDS- and native PAGE in the concentrated samples obtained after size-exclusion chromatography (Shodex KW 803) during the purification of 5-phosphomevalonate kinase from *C. roseus* suspension cultured cells. Presented are the results for the pooled active fractions, sample V, and the pooled adjacent fractions, samples VI and VII (photos not shown). The samples were concentrated ten times by freeze drying

M_r of detected polypeptides: SDS-PAGE			
SDS-PAGE		Native PAGE	
Sample V	Samples VI and VII	Sample V	Samples VI and VII
102,754		55,017	
65,193		44,249	44,249
51,928			
46,345	46,345		
	31,124		
	18,655		
12,529	12,529		

Table 3
The apparent K_m values for 5-phosphomevalonate (MVAP) and the apparent V_{max} values as determined at different ATP concentrations by fitting the kinetic data to the Michaelis–Menten equation $v = V^*_{max}S/(K_m + S)$, in which V_{max} is maximum velocity, S is substrate concentration (MVAP), and K_m is the substrate concentration giving half-maximal velocity. Measurements were performed in duplicate with 1.35 μg of 5-phosphomevalonate kinase partially purified from *C. roseus* suspension cultured cells

[ATP] (mM)	K_m MVAP ± S.D. (mM)	V_{max} ± S.D. (nkat/mg)	χ^2 of fit
0.431	0.427 ± 0.063	1.72 ± 0.07	0.003
2.16	0.365 ± 0.060	2.19 ± 0.10	0.007
4.31	0.364 ± 0.053	2.64 ± 0.11	0.008

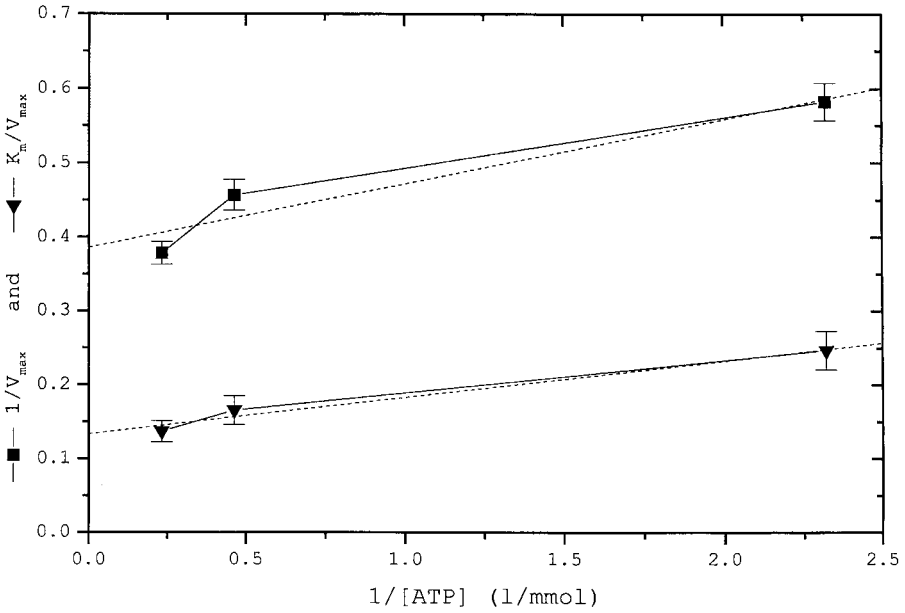

Fig. 4. Replots of $1/V_{max}$ and K_m/V_{max} versus the reciprocal of the ATP concentration. The apparent V_{max} and K_m values were determined towards 5-phosphomevalonate (MVAP) at three different ATP concentrations, i.e. 0.43, 2.15 and 4.3 mM (Table 3). Dotted lines indicate fitted lines; for $1/V_{max}$ the fitted line corresponds to $1/V_{max} = (K_{mB}/V_{max}) \times (1/[B] + 1/V_{max})$, while for K_m/V_{max} the fitted line corresponds to: $K_m/V_{max} = K_{iA} \times (K_{mB}/V_{max}) \times (1/[B] + K_{mA}/V_{max})$. The fitted lines resulted in the functions: $1/V_{max} = (0.086 \pm 0.026) \times (1/[ATP]) + (0.386 \pm 0.035)$, with $r = 0.959$, S.D. = 0.041 and $P = 0.184$, and $K_m/V_{max} = (0.049 \pm 0.007) \times (1/[ATP]) + (0.134 \pm 0.010)$, with $r = 0.989$, S.D. = 0.012, and $P = 0.095$.

In addition, PMK activity was not inhibited by FPP, which was tested in the range of 3.7 to 118 μM FPP (data not shown).

3. Discussion

As part of our studies on the regulation of isoprenoid biosynthesis, we partially purified PMK from *C. roseus* suspension cultured cells. Despite its essential role in the pathway, still little was known about the enzyme.

PMK was purified about 9-fold with a 3.6% yield in a sequence of five chromatographic steps. The efficiency of each purification step was monitored by HPLC SEC. It was noted that during the purification, the M_r of PMK shifted from about 166,000 to 128,000. As the enzyme activity eluted rather broadly from the column, it is difficult to make an exact estimation of the M_r value. Nevertheless, the shift in chromatographic behavior might indicate that the enzyme was partially degraded. A partial breakdown of the protein might have been caused by the low pH, e.g. pH 6, at which the Mono Q step was run. Breakdown of PMK would be an explanation for the extremely low recovery during this purification step (recovery 9.5%). Nevertheless, the purity of the enzyme was increased by a factor 1.6 as deduced from the increase in specific activity. Also the PMK from *Hevea* has been reported to be particularly unstable to pH values below 6 (Skilleter & Kekwick, 1971).

Except for the pH-dependent loss of activity, the PMK activity from *C. roseus* showed to be rather stable. During the first three chromatographic steps at pH 7.5, the enzyme preparations could be stored at 4°C for at least two weeks or stored for longer periods at −80°C without significant loss of activity. As no sulfhydryl reagents were added to buffers, it seemed that these were not essential to protect the activity of the *C. roseus* PMK. In contrast, these were required for the maintenance of the activities of the purified *Hevea* (Skilleter & Kekwick, 1971) and pig liver (Bazaes et al., 1980) enzymes.

As far as the estimated size of PMK, quite some diversity can be noted for the PMK enzymes from

Table 4
5-Phosphomevalonate kinase (PMK) activities (nkat/mg) and relative PMK activities determined with 1.35 μg of partially purified PMK from suspension cultured *C. roseus* cells in the presence of different divalent cations at 2 mM. The enzyme did not show any activity without ions, nor with 2 mM of $CuCl_2$, $CoCl_2$, $CaCl_2$ or $FeSO_4$

Divalent cation	$MgCl_2$	$MnCl_2$	$ZnCl_2$	$NiSO_4$
PMK (nkat/mg)	0.403	0.211	0.089	0.019
Relative PMK (%)	100	52	22	4.7

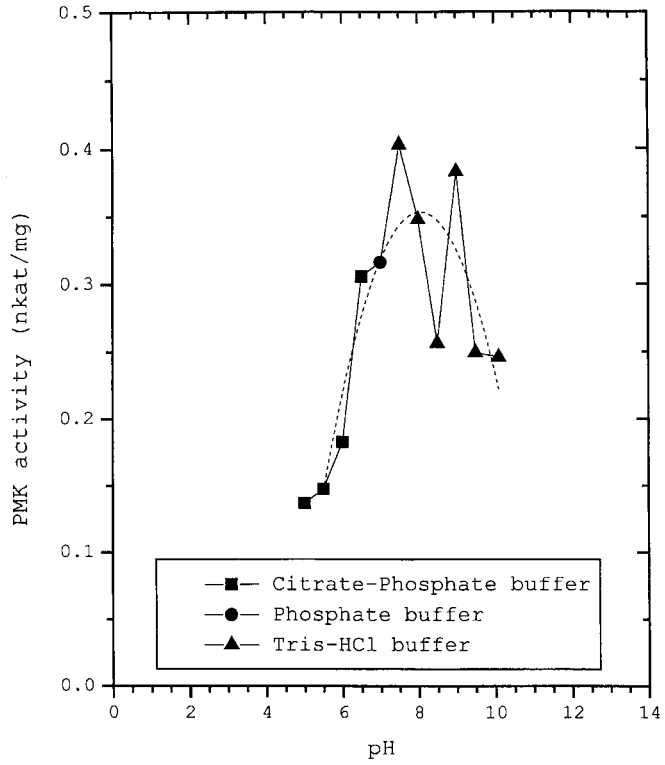

Fig. 5. 5-Phosphomevalonate kinase (PMK) activity partially purified from *C. roseus* suspension cultured cells at different pH values. The PMK activity was determined using 1.35 μg of the partially purified enzyme (sample IV), and using the microplate assay in combination with a stopped assay. For the stopped assay, the buffer of the standard reaction mixture was replaced by a citrate-phosphate buffer, a phosphate buffer or a Tris–HCl buffer to obtain the pH values indicated. Details are described in Experimental 4.5.

different sources (Table 5). The estimated M_r of 128,000 for the *C. roseus* PMK is far larger than those reported for PMK from other sources. The pig liver enzyme has been shown to consist of one polypeptide chain of about 21 kDa as determined by SDS-PAGE and SEC (Bazaes et al., 1980). The predicted size of PMK as calculated from the human liver cDNA has been determined to be 21.9 kDa (Chambliss et al., 1996), corresponding well with the pig liver enzyme. In contrast, the expressed yeast PMK cDNA has yielded a protein of 47.3 kDa (Tsay & Robinson, 1991). Furthermore, the comparison of cDNA sequences and the predicted amino acid sequences for the human and yeast enzymes has indicated that there were no homologies (Chambliss et al., 1996). In addition, the conserved sequence encoding a putative ATP-binding pocket, which was recognized in the yeast PMK (Tsay & Robinson, 1991), was not detected in the human PMK cDNA sequence (Chambliss et al., 1996).

Unfortunately, the results of SEC of the *C. roseus* PMK could not be supported by data from SDS- and native PAGE. SDS-PAGE of sample V revealed three polypeptides as candidates for a PMK monomer, e.g., those of 103, 65 or 52 kDa (Table 2). As far as these results, the 103 kDa polypeptide might correspond with a monomer PMK, while the 52 and 65 kDa polypeptide might indicate a dimer polypeptide corresponding with the estimated native size of 128 kDa. In addition, native PAGE of sample V resulted in one candidate of 55 kDa for PMK (Table 2). However, this value does not correspond to the estimated size of 128 kDa as determined by SEC.

Considering the characteristics of the enzyme, the kinetic data indicated a sequential mechanism of action. Product-inhibition studies should be performed to distinguish between an ordered and a random sequential mechanism. Unfortunately, these could not be performed as the enzyme-coupled microplate assay for PMK does not allow inhibition studies by ADP, and MVAPP was not available at that time.

The *C. roseus* PMK was not affected by FPP (data not shown); the same has been reported for the pig liver PMK (Dorsey & Porter, 1968). The pig liver PMK has been reported to have an ordered sequential mechanism of action (Eyzaguirre & Bazaes, 1985; Tsay & Robinson, 1991). Table 5 gives an overview of characteristics of the PMK enzymes from different sources. As far as the *C. roseus* PMK, the K_m value for ATP, 0.22 mM, is similar to other reported values, while the K_m value for MVAP, 0.35 mM, seems rather

Table 5
Some characteristics of 5-phosphomevalonate kinase from different sources. The sizes for the native enzyme were estimated by gel filtration or deduced from the cDNA sequences. The K_m values for the substrates, 5-phosphomevalonate (MVAP) and ATP, have been obtained under the following conditions: *Catharanthus* true values, pH 7.5 and 30°C; *Hevea* 0.2 mM MVAP, 2.0 mM ATP, pH 7.2 and 40°C; pig liver true values, pH 7.5 and 30°C

Source (*reference*)	Estimated M_r (kDa)	K_m MVAP (mM)	K_m ATP (mM)
Catharanthus roseus	128	0.35	0.22
Hevea brasiliensis (Lange et al., 1998)		0.042	0.2
Yeast (Weissenborn et al., 1995)	47.3		
Pig liver (Takahashi et al., 1998)	21	0.075	0.46
Human liver (Stermer et al., 1994)	21.9		

high in comparison to the K_m values from the *Hevea* enzyme (Skilleter & Kekwick, 1971), and the pig liver PMK (Bazaes et al., 1980).

PMK enzymes have been reported to be dependent on the presence of divalent cations, of which Mg^{2+} has been indicated to be the best activator. The *Hevea* enzyme has been shown to be progressively stimulated by increasing concentrations of Mg^{2+}, Zn^{2+} and Fe^{2+} until 10 mM; Mn^{2+} showed an inhibitory effect above 2 mM (Skilleter & Kekwick, 1971). With Mg^{2+} being the best activator at 5 mM, the pig liver enzyme has given relative activities between 56 and 65% with Zn^{2+}, Co^{2+} and Mn^{2+} (Bazaes et al., 1980). The *C. roseus* PMK had more stringent requirements; compared to the activity with Mg^{2+} at 2 mM, it showed a remaining activity of 52% with Mn^{2+}, and only 22 and 4% with Zn^{2+} and Ni^{2+}, respectively (Table 4).

The pH-activity profile of the *C. roseus* PMK differed from that of the *Hevea* enzyme (Skilleter & Kekwick, 1971), at least to such an extent that the *Hevea* enzyme has shown a sharp decrease in activity at pH values above 7.5, while the *C. roseus* remained active until pH 9. The pH-activity profile for the pig liver enzyme is different as it has an optimum activity plateau between pH 7.5 and 9.5 (Bazaes et al., 1980).

The comparison outlined above between the characteristics of the *C. roseus* PMK and PMK enzymes from other sources has revealed that remarkable differences can be recognized at the gene- and protein levels of these different PMKs, and their individual properties, even though the same reaction is catalyzed. Further research is necessary to reveal how these differences are translated into differences in the in vivo regulation of PMK, and, subsequently, how they influence its function in isoprenoid biosynthesis.

4. Experimental

4.1. Biological materials

Cell suspensions of *C. roseus* Roseus (L.) G. Don were grown on modified MS medium (Murashige & Skoog, 1962), i.e. without growth regulators, and containing 3% sucrose. The cultures were kept under continuous light of 1100 Lux at 100 rpm and 25°C, and were subcultured weekly by weighing 5 g cells (fr. wt) into 50 ml fresh medium. For the purification of the enzyme, *C. roseus* cells grown for 14 days on MS medium were transferred to IM 2 medium (Knobloch & Berlin, 1980) by weighing 20 g cells (fr. wt) into 500 ml medium. The cells were harvested after 8 days, frozen in liquid nitrogen and stored at −80°C.

4.2. Enzyme extraction

A crude extract was prepared by homogenizing about 400 g frozen biomass in a Waring blender precooled with liquid nitrogen. The homogenized material was allowed to thaw at 30°C in the presence of polyvinylpolypyrrolidone (10% wt/fr. wt) and extraction buffer (0.1 M Tris–HCl, pH 7.5, 0.1 M sucrose, 50 mM KCl, 5 mM DTT) in a ratio of 2 ml buffer to 1 g fr. wt. The crude extract was centrifuged at 2600 g at 4°C for 30 min. The supernatant was collected, filtered over glass fiber filter, and concentrated to about 60 ml by means of ultrafiltration in a Provario concentrator equipped with a membrane with a cut-off of 30 kDa. The concentrated extract was frozen in liquid nitrogen and stored at −80°C in fractions of about 8 ml.

4.3. Purification

In two batches, 26 ml of the concentrated extract were applied at 1 ml/min to a Q Sepharose column (2.7 i.d. × 15.5 cm) equilibrated in 10% buffer B in buffer A; buffer A = 50 mM Tris–HCl pH 7.6 and buffer B = buffer A containing 1 M KCl. The column was washed with 90 ml starting buffer at 1 ml/min and a gradient was run from 10 to 100% buffer B at 1.5 ml/min. Fractions containing PMK activity were collected (50 ml) and concentrated to 7 ml (sample I) in an ultrafiltration unit equipped with a membrane with a cut-off of 30 kDa. Sample I (6.5 ml) was applied at 1 ml/min to a Phenyl Sepharose CL-4B column (2.4 i.d. × 17 cm) equilibrated in buffer B. The column was washed with 150 ml buffer B at 1 ml/min, resulting in elution of the majority of the protein, including PMK activity. Fractions containing PMK activity were collected (104 ml), and concentrated to 12 ml (sample II) in an ultrafiltration unit as described above. In two separate runs, 5 ml of sample II were applied at 0.5 ml/min to a Sephacryl S200 column (1.6 i.d. × 40 cm) equilibrated in extraction buffer without DTT. All proteins were eluted in 150 ml. The fractions containing PMK activity from the two runs were combined and concentrated from 15.6 to 4.4 ml (sample III) in an ultrafiltration unit as described above. Four ml of sample III were applied at 0.5 ml/min to a Mono Q column equilibrated in buffer C (50 mM sodium phosphate, pH 6, 50 mM sucrose). The column was washed at 10% buffer D (buffer C containing 1 M NaCl) in C, and a gradient was run at 0.5 ml/min from 10 to 100% D. The fractions containing PMK activity were collected and the activity was brought into extraction buffer without DTT using PD10 columns (11.5 ml). Using centricon units with a cut-off of 30 kDa, 8.5 ml of this solution were concentrated to 650 μl (sample IV). Sample IV was used for all the experiments for the characterization of the enzyme. To estimate the

molecular weight of the enzyme, 50 μl of sample IV was injected into an HPLC system equipped with a Shodex KW 803 size-exclusion column equilibrated with extraction buffer without DTT and run at 0.5 ml/min. The active fractions (fractions 6–9; sample V) and adjacent fractions (fractions 2–4; sample VI, and fractions 11–14; sample VII) were pooled and concentrated using the centricon units as described above.

4.4. Enzyme assay

A microplate assay was used for the determination of PMK activity (Schulte et al., 1999). In short, the conversion of ATP to ADP as catalyzed by PMK in the presence of MVAP was coupled to the oxidation of NADH by means of the auxiliary enzymes PK and LDH. The reaction was followed at 340 nm by measuring the change in absorbance every 30 s for 10 min using a microplate reader with the incubator set at 30°C. Under standard conditions, the substrate concentrations were 3 mM MVAP and 2 mM ATP.

4.5. Characterization of PMK

For the kinetic studies, the PMK activity was determined at 0.43, 2.15 and 4.3 mM ATP in combination with varying MVAP concentrations, e.g., 0.05, 0.2, 0.4, 1.6 and 3.1 mM. The sensitivity of PMK activity towards FPP was determined over a range of 3.7 to 118 μM FPP.

For the determination of the pH optimum of PMK activity, and the divalent-cation-dependency of the PMK activity, the microplate assay was used in combination with a stopped assay (Schulte et al., 1999). In short, PMK was incubated in a reaction mixture giving final concentrations of 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM ATP, 25 mM KF, 1 mM DTT and 10 mM MVAP for 60 min. The reaction was stopped by boiling for 2 min, and the conversion of ATP to ADP was determined using the microplate assay. For the determination of the pH optimum for PMK activity, the standard reaction mixture was prepared in 50 mM: citrate-phosphate buffer pH 5, 5.5, 6 and 6.5, phosphate buffer pH 7 and Tris–HCl pH 7.5, 8, 8.5, 9, 9.5 and 10. For the determination of the dependency of PMK activity on divalent cations, the $MgCl_2$ (2 mM) in the standard reaction mixture was removed or replaced by $ZnCl_2$, $FeSO_4$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $NiSO_4$ and $MnCl_2$.

4.6. Analysis kinetic studies

Kinetic data were fitted to the Michaelis–Menten equation — $v = V_{max} \times S/(K_m + S)$ —, in which V_{max} is maximum velocity, S is substrate concentration, and K_m is the substrate concentration giving half-maximal velocity. True K_m and V_{max} values were calculated from the secondary replots of the apparent values for $1/V_{max}$ and K_m/V_{max} (Segel, 1975).

4.7. Protein analysis

The Shodex KW 803 size-exclusion column was calibrated using as standards the proteins: ribozym A (13.7 kDa), chymozym A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldose (158 kDa) and catalase (232 kDa). Gel electrophoresis was performed on a Phast System (Pharmacia) using microgels (Pharmacia); silver staining was used to visualize the protein bands (Davis, Dibner & Battey, 1986). Low molecular weight markers were used during gel electrophoresis. Protein concentrations of samples were determined using the Bradford micro assay method (Bradford, 1976); BSA was used as a standard.

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