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HYDROLYSIS OF ALLYLIC PHOSPHATES BY ENZYMES FROM THE FLAVEDO OF *CITRUS SINENSIS*

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Key Word Index—*Citrus sinensis*; Rutaceae; orange; enzymic hydrolysis; phosphatases; allyl phosphates; nerol; geraniol.

Abstract—Acid phosphatase activities have been partially purified from an aqueous extract of an acetone powder from orange flavedo. the use of a gel filtration step with an ionic gradient allowed a dissociation of proteins from pigments, thus facilitating purification and stabilization of the enzymes. The enzymes do not require metals for full activity, and they hydrolysed a wide spectrum of phosphorylated substrates. C_{10}–C_{20} allylic pyrophosphates and monophosphates were hydrolysed sequentially by these 'prenylphosphatases'. The final product was the corresponding unrearranged prenyl alcohol. This demonstrated the absence of *E*–*Z* isomerization and suggested an O–P bond cleavage. Prenylphosphatases exhibited a certain degree of chain length specificity. Although the *E* or *Z* conformation of the C-2 double bond was not important, its presence was required for full activity. Excess prenylpyrophosphate inhibited the rate of formation of alcohols, most likely through the inhibition of phosphomonoesterase activity. These prenylphosphatases generated the alcoholic components of essential oils from the corresponding pyrophosphates and removed them from the chain lengthening process.

INTRODUCTION

Essential oils from higher plants contain among other components terpenoid hydrocarbons, alcohols, aldehydes and esters [1]. Mono-, sesqui- and higher terpenoids are formed by the condensation of isopentenyl pyrophosphate with C_5, C_{10} and longer chain allylic pyrophosphates [2].

In the course of *in vitro* studies of terpenoids from mevalonic acid by cell-free systems from plants, allylic alcohols have been found as final products [3, 4]. In cell-free systems from *Citrus sinensis* flavedo, the amount of alcohols formed was usually decreased by the addition of fluoride [5]. Thus, it has been concluded that primary allylic alcohols were formed by the action of one or more phosphatases on pyrophosphorylated substrates such as GPP*, NPP, FPP, etc.

There are several reports on the properties of acid phosphatases (EC 3.1.3.2.) from *Citrus* juice or rind [6, 7], but these authors did not assay allylic substrates. Phosphatases from *Tanacetum vulgare* do not split allylic pyrophosphates [8].

While studying the chain lengthening process in terpene biosynthesis by an enzyme preparation from the flavedo of *Citrus sinensis* [9, 10], we found that significant amounts of geraniol, nerol and farnesols were formed from ^{14}C-isopentenyl pyrophosphate plus dimethylallyl pyrophosphate. Thus, it was thought advisable to study directly the effect of enzyme preparations from this tissue on the allylic pyrophosphates formed as products or used as substrates. This will give support to the widely accepted assumption [3, 4] that prenols are the products of phosphatase activity† upon prenylphosphate and pyrophosphate esters, and also provide information on the origin of derivatives of prenyl alcohols such as aldehydes [11], esters or glucosides [12].

RESULTS AND DISCUSSION

Aqueous extracts from fresh flavedo of *Citrus sinensis* contain several phosphatase activities. These phosphatases are quite unstable and cannot be conveniently stored at this stage. This is probably the case

* *Abbreviations*: GPP: geranyl pyrophosphate, GP: geranyl monophosphate, NPP: neryl pyrophosphate, NP: neryl monophosphate, FPP: farnesyl pyrophosphate, CiPP: citronellyl pyrophosphate, GGPP: geranyl geranyl pyrophosphate, CoPP: copalyl pyrophosphate, P-cellulose: phosphocellulose, DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid).

† The term 'prenylphosphatase' will be used throughout this communication to designate an enzyme activity which splits pyrophosphate or monophosphate esters of prenyl alcohols. For further distinction we will use the terms 'phosphomonoesterase' or 'pyrophosphohydrolase' to indicate hydrolysis of mono- or pyrophosphate esters. Unless specifically stated, no assumptions will be made as to the number of individual enzymes.

Table 1. Phosphatase activities in an enzyme preparation from orange flavedo

(a) Substrate 0.12 mM	Product measured	Specific activity (nkat/mg) Extract	P-cellulose	Purification
IPP-[^{14}C]	Isopentenol-[^{14}C]	0.02	—	—
GPP-[^{3}H]	Geraniol-[^{3}H]	0.23	6.05	25
NPP-[^{3}H]	Nerol-[^{3}H]	0.27	7.26	27
FPP-[^{3}H]	Farnesol-[^{3}H]	1.12	1.75	1.6
GP-[^{3}H]	Geraniol-[^{3}H]	1.42	11.25	7.9
NP-[^{3}H]	Nerol-[^{3}H]	1.51	11.43	7.6
CiPP-[^{3}H]	Citronellol-[^{3}H]	—	2.42	—
GGPP-[^{3}H]	Geranylgeraniol-[^{3}H]	—	1.15	—
CoPP-[^{3}H]	Copalol-[^{3}H]	—	2.23	—

(b) Substrate 5 mM	Product measured	Specific activity (nkat/mg) Extract	P-cellulose	Purification
ATP	Pi	11.22	79.9	7.1
PPi	Pi	7.14	122.4	17.0
P-nitrophenyl-phosphate	Pi	10.8	163.2	15.0
AMPi	Pi	6.8	120.7	18.0

with Schormuller's preparation [7], although not explicitely stated by the author. For this reason we decided to start with an acetone powder which was also our starting material for the study of prenylsynthetase [10]. The phosphatase activity described in this communication differs from the *Tanacetum* enzyme [8] which does not split allylic pyrophosphates.

An aqueous extract prepared from an acetone powder of orange flavedo contained a number of phosphohydrolytic activities which hydrolyse several phosphomonoesters as well as pyrophosphate bonds.

Prenylphosphatase purification

Table 1 shows the specific activity of this extract on several substrates at concentrations that were saturating but not inhibitory. There was a certain chain length specificity for the allylic pyrophosphates, and their rates of hydrolysis were lower than those of other substrates (Table 1b). Inhibition by higher concentrations of several substrates makes the report of K_m values irrelevant.

Table 2 shows a 27-fold purification of the prenyl phosphatase activity. There was practically no difference between the hydrolysis of *E*- and *Z*-isomers at all stages of purification. Table 1 shows the increase in specific activity for different substrates. Pyrophosphohydrolase activity was purified by a factor of 25–27, while phosphomonoesterase was purified only 7–8 times. This could be due to the fact that most of the phosphomonoesterase emerged with the wash of the P-cellulose column used for purification. On the other hand, ATPase and AMPase were not dissociated. The difference observed in the purification patterns for the substrates tested suggest the presence of several phosphohydrolytic enzymes of different specificity for prenylphosphates and other substrates.

It is worth mentioning that a 6- to 8-fold purification of prenylphosphatase achieved in the heating step required vigorous stirring which probably denatured other proteins. The use of an ionic gradient in conjunction with gel filtration is a modification of intervent dilution chromatography [13, 14] described for ionic exchangers. A dissociation of proteins was achieved from ligands such as pigments. Maximum absorbancy at 310 nm was decreased by a factor of 120 by this procedure, and the ratio of absorbancies at 310/280 nm tended to zero. Monitoring the column effluent for absorbancy at 310 nm and enzyme activity showed a neat separation between prenylphosphatase activity and pigments of at least 1.5 column volumes. Elimination of pigments made prenylphosphatase more stable than in the previous steps. After the last stage of purification, the enzyme retained its full activity for up to one year when stored as a freeze-dried powder at −20°. As an aqueous solution in 50 mM Tris–HCl at pH 7.0 it had a half-life of 21 days at 4°. It should be pointed out that if enzyme purity is referred to as absorbancy at 310 nm instead of the customary protein concentration, a 3000-fold purification was achieved by this procedure.

Properties of the purified fraction from P-cellulose

The pH optimum for *p*-nitrophenylphosphate or AMP hydrolysis was 6.0 with half maximum activities at pH 5.0 and 7.0. For all other substrates half maximum values were observed at pH 4.3 and 6.5 with a maximum at 5.5. However all assays were performed at pH 6.0 because of the abrupt increase in non-enzymic hydrolysis of allylic phosphate esters below this pH. Half maximum reaction rates ($S_{0.5}$) were obtained at 50 μM GPP and 80 μM NPP. The $V_{max}/S_{0.5}$

Table 2. Purification procedure of prenylphosphatase from orange flavedo

	Volume (ml)	Proteins (mg/ml)	Specific activity (nkat/mg)		Total units (nkat)	NPP/GPP ratio	Purification*	Yield* (%)
Aqueous extract from acetone powder	165	3.84	GPP	0.23	146	1.2	—	—
			NPP	0.27	168.4			
Aqueous extract heated at 40°	152	1.0	GPP	1.52	232	1.4	6.6	159
			NPP	2.12	323		7.9	191
Ammonium sulphate fraction 35/70	10	12.5	GPP	1.2	150	1.5	5.2	103
			NPP	1.88	233.7		6.9	138
Sephadex G-25 dissociative filtr.	27.4	2.7	GPP	2.94	218.12	1.2	12.7	149
			NPP	3.6	265.8		13.4	157
Phosphocellulose fraction	41	0.2	GPP	6.04	49.6	1.2	26.0	34
			NPP	7.26	59.6		27.0	35

* All data refer to the aqueous extract from acetone powder.

were 2.5×10^{-3} and 2.0×10^{-3}, respectively. This ratio was *ca* ten times higher for NP and GP.

The purified fraction (Table 1) exhibited a chain length specificity, V_{max} decreasing from C_{10} to C_{15} to C_{20}. CoPP was a better substrate than GGPP probably due to the fact that the ring may accomodate the active site in a conformation more similar to a C_{10} than a higher homologue. Although there was almost no difference between the rate of hydrolysis of substrates with *E*- or *Z*-conformation around the C-2 double bond, the presence of this double bond seemed to be necessary for catalysis as shown by the lower V_{max} for the saturated analogue CiPP. This compound has been shown to be inactive in the chain lengthening or cyclization processes of isoprenoid biosynthesis. Furthermore, it is an inhibitor of these reactions ([10]; Rojas, M. C., personal communication).

Partially purified phosphatase from *Citrus sinensis* did not require the addition of bivalent metals for maximum activity. Ca^{2+}, Mg^{2+} or Mn^{2+} at concentrations up to 10 mM had no effect on the hydrolysis of all substrates shown in Table 1; at higher concentrations the rate of hydrolysis of GPP, NPP, FPP, GP, PPi, ATP, AMP and *p*-nitrophenylphosphate decreased to values of the order of 20% of the maximum values when Ca^{2+} and Mg^{2+} were used. With 12 mM Mn^{2+} the activity was completely suppressed. However, the addition of EDTA inhibited NPP and GPP hydrolysis with a $I_{0.5}$ of 5 mM. This may be either due to a metal requirement fulfilled by very low amounts of contaminating metals in the enzyme preparation, or a direct effect of the EDTA molecule on the enzyme as described for phosphoglucomutase [15].

Inorganic pyrophosphate, ATP and Pi inhibited NPP and GPP hydrolysis with $I_{0.5}$ values of 7, 0.5 and 0.4 mM. This effect has been used to improve the yield of higher prenylphosphates in the study of prenylsynthetases [10].

Incubation of purified prenylphosphatase for 15 min at 37° (pH 6.0) with 100 μM DTNB inactivated it by 50%.

Reaction products and course of the prenylphosphatase reaction

Analysis of alcohols and paper chromatography of the remaining polar substrates and products showed that the hydrolysis of GPP or NPP is a sequential process. In the presence of 10.3 n units of prenylphosphatase, *ca* 10% of the substrate disappeared in 10 min, being transformed into the corresponding prenylmonophosphate plus alcohol. Monophosphate concentration reached a maximum value in 15 min and then slowly decreased until all the substrate was transformed into the corresponding primary alcohol (Fig. 1). No tertiary alcohols were found. Thus the reaction sequence is Prenyl-PP → Prenyl-P → Prenol.

As previously demonstrated, these enzyme preparations do not transform an allylic pyrophosphate, monophosphate or alcohol into its diastereomer, since only nerol is formed from NPP and only geraniol from GPP [5]. If an *E*–*Z* isomerization of the pyrophosphates or monophosphates had occurred, then traces of the opposite diastereomeric prenol would have been found. This issue is important since *E*–*Z* isomerization of pyrophosphates has been advocated as a mechanism to explain cyclization of monoterpenoids [12, 16]. No aldehydes were found and this

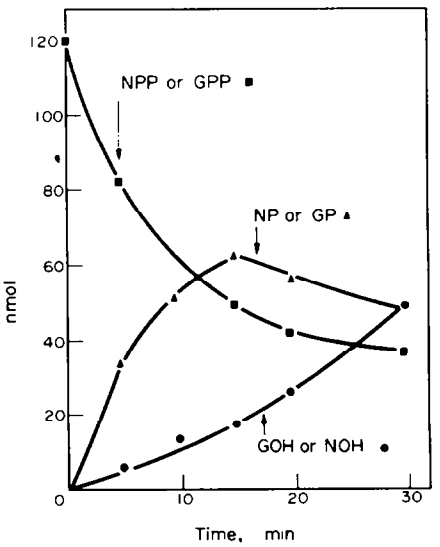

Fig. 1. Time course of substrate and product concentration in the enzymic hydrolysis of GPP and NPP. Substrate concentration: 120 μM. For other conditions see Experimental.

also excludes redox isomerization of allyl alcohols in this system [11].

Alcohols are formed from their phosphorylated precursors, probably by O—P and not C—O fission. The latter would have led to the formation of the rearranged tertiary alcohols linalool and α-terpineol, as in the metal-catalysed hydrolysis of these substrates [17].

Assay of prenylphosphatase with pyrophosphates or monophosphates showed that the latter were hydrolysed faster (Table 1). This would not be consistent with the time course depicted in Fig. 1 since no GPP or NP would be expected to accumulate. An inflection of the time-course curve at 8–15% of substrate hydrolysis may be seen in Figs. 1 and 2. The insert in Fig. 2 shows an increase in reaction rate which then decreased as substrate was utilized. This could be due to an inhibitory effect of GPP or NPP on the hydrolysis of the monophosphates. This was proved by the fact that simultaneous hydrolysis of prenylmonophosphate plus prenylpyrophosphate was not additive, and that the rate of alcohol-[^{3}H] liberated from the monophosphate was decreased by the addition of GPP-[^{3}H] or NPP-[^{3}H]. This phenomenon makes the determination of kinetic parameters somewhat uncertain since concentrations of GPP or NPP above 160 μM resulted in a net decrease in the rate of reaction. No substrate inhibition was observed by GP or NP. A similar change in reaction rate was observed in time curves of the complete hydrolysis of ATP to adenosine by this enzyme preparation.

The different purification ratio, as well as the behaviour in the time-course curve, indicates that hydrolysis of allylic mono- and pyrophosphates may be due to different enzymes. Inhibition patterns, however, point to a single enzyme splitting prenyl mono- and pyrophosphates at different rates. Chain length specificity does not follow a clear enough pattern to postulate one enzyme with broad substrate specificity as described for phosphatases from testicle microsomes [18], or several specific enzymes.

Prenyl alcohols formed from mevalonic acid-[^{14}C] by crude cell-free enzyme systems from plant tissues may now be attributed to the effect of these prenylphosphatases, a fact that had been previously assumed [3, 4].

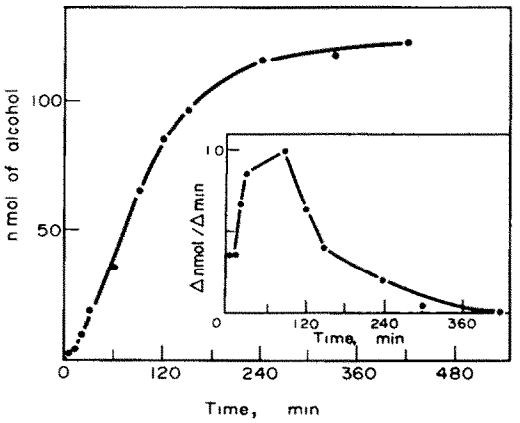

Fig. 2. Kinetics of hydrolysis of GPP or NPP. Assay conditions as in Experimental.

Since terpenoid alcohols are quantitatively important components of essential oils, or precursors of other components such as glucosides, aldehydes or esters, one could visualize a biological function for phosphatases, specific either for the allylic structure or for its chain length. These phosphatases would cleave allylic pyrophosphates, and thus divert them from the pathway to higher homologues or hydrocarbons, allowing the formation of compounds with free or substituted hydroxylic functions.

EXPERIMENTAL

Substrates. GPP-[1-^{3}H], NPP-[1-^{3}H] and FPP-[1-^{3}H] were prepared as described before [19].

Enzyme preparation. Oranges of the 'Chilean variety' [5] collected in winter (July–August) from a single tree were cleaned with running tap water then distilled water. The flavedo (exocarpium) was peeled off and collected at 0°. It was homogenized in a Waring blendor at −40° with 2 l. Me_2CO per kg of flavedo. The suspension was filtered through Whatman No. 1 filter paper in a Buchner funnel and the residue was washed with *ca* 15 l. Me_2CO/kg at −40° and 6 l. Et_2O/kg at −40°. The powder obtained was dried *in vacuo* and stored at 4°.

The acetone powder was homogenized for 3 min at 0° in an Omnimixer with 10 ml 100 mM Na-succinate buffer, pH 6.0 per g of powder. The homogenate thus obtained was strained through cheesecloth and centrifuged for 30 min at 17 000 g and 0°. The ppt. was discarded and the supernatant was heated at 40° with vigorous agitation in an Omnimixer for 5 min. Then it was centrifuged for 45 min at 25 000 g and 0°. The supernatant obtained was precipitated between 35 and 70% saturation of $(NH_4)_2SO_4$ [20]. The temp. must not exceed 4° during this procedure. The ppt. obtained was dissolved in a vol. of 100 mM Na-succinate buffer, pH 6.0 containing 2 mM EDTA and 10 mM 2-mercaptoethanol equivalent to 0.06 of the vol. of the extract. The ammonium sulphate fraction was applied to a 2×35 cm Sephadex G-25 column equilibrated with 100 mM Na-succinate buffer, pH 6.0, containing 2 mM EDTA and 10 mM 2-mercaptoethanol. The sample was allowed to enter *ca* 1 cm into the gel before eluting with 100 mM Na-succinate buffer, pH 6.0, containing 2 mM EDTA, 10 mM 2-mercaptoethanol and 2.37 M $(NH_4)_2SO_4$. The enzyme emerged with all the proteins in the void vol. of the column and this fraction was practically free of the pigments present in the extract.

The fraction containing phosphatase activity was concd by ultrafiltration through a Diaflo membrane PM 30, and dialysed exhaustively against 10 mM Tris–HCl, pH 7.0, containing 10 mM 2-mercaptoethanol. It was then applied to a 1.1×17 cm column of P-cellulose previously equilibrated in 10 mM Tris–HCl, pH 7.0, containing 10 mM 2-mercaptoethanol, and washed with 1 column vol. of the same buffer. Phosphatase activity was eluted with 10 mM Tris–HCl, pH 7.0, containing 10 mM 2-mercaptoethanol and 500 mM KCl. The fraction thus obtained was freeze-dried and stored at −20°. Proteins were determined by turbidimetry [21].

Enzyme assay. Standard assay conditions, unless stated otherwise, were the following: Incubations were performed at pH 6.0 for 10 min at 37° in glass-stoppered conical tubes in a final vol. of 1 ml 50 mM Na-succinate buffer, 140 μM GPP-[1-^{3}H] or NPP-[1-^{3}H] (sp. act. 2×10^7 dpm/μmol) and 15 μg proteins. The reaction was stopped by cooling the tubes to 0° and the aq. phase was extracted with 2 ml hexane. This

hexane phase was either assayed directly for radioactivity by conventional scintillation spectrometry [19] with 35% efficiency, or further analysed for products. When ATP, PPi, AMP or *p*-nitrophenyl-phosphate were used as substrates, the assay was performed for 10 min at 37° and pH 6.0 in a final vol. of 1 ml 50 mM succinate buffer, 5–10 mM substrate and 15 μg proteins. The reaction was stopped by adding 1 ml molybdic acid, and Pi was measured [22]. All results presented have been corrected for non-enzymic controls performed with boiled enzyme. One unit of phosphatase was defined as the amount of enzyme which liberated 1 mol of prenol or of Pi per sec at 37°, as described. Results are expressed in nkat [23].

Analysis of products. Radioactive alcohols were identified by GLC after addition of 0.5–1 mg of authentic carriers. GLC was performed on stainless steel columns of 0.635 o.d. × 300 cm packed with Chromosorb W-HMDS, 60–80 mesh. Liquid phase was 2% ethylene glycol adipate. Gas flow was 40 ml He per min. Temps. were 250° for the detector, 200° for the injector and 120° isothermal for the column oven. Effluents from GLC were either introduced directly into a heated proportional radioactivity counter or collected in scintillation fluid at −70° and counted.

Pyrophosphates and intermediate monophosphates were identified by TLC [24] by scraping the bands from the plates into vials containing 5 ml of scintillation fluid [19].

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