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Purification and properties of 6-methylsalicylic acid synthase from *Penicillium patulum*

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6-Methylsalicylic acid synthase has been isolated in homogeneous form from *Penicillium patulum* grown in liquid culture from a spore inoculum. The enzyme is highly susceptible to proteolytic degradation *in vivo* and *in vitro*, but may be stabilized during purification by incorporating proteinase inhibitors in the buffers. The enzyme exists as a homotetramer of M_r 750000, with a subunit M_r of 180000. 6-Methylsalicyclic acid synthase also accepts acetoacetyl-CoA as an alternative starter molecule to acetyl-CoA. The enzyme also catalyses the formation of small amounts of triacetic acid lactone as an oligatory by-product of the reaction. In the absence of NADPH, triacetic acid lactone is the exclusive enzymic product, being formed at 10% of the rate of 6-methylsalicylic acid. The enzyme is inactivated by 1,3-dibromopropan-2-one, leading to the formation of cross-linked dimers similar to that observed with type I fatty acid synthases. Acetyl-CoA protects the enzyme against the inactivation and inhibits dimer formation. An adaptation of the purification method for 6-methylsalicylic acid synthase may be used for the isolation of fatty acid sythase from *Penicillium patulum*.

INTRODUCTION

6-Methylsalicylic acid synthase from *Penicillium patulum* catalyses the formation of 6-methylsalicylic acid from one molecule of acetyl-CoA and three molecules of malonyl-CoA, as shown in Scheme 1. 6-Methylsalicylic acid is then transformed further into the antibiotic patulin (Martin & Demain, 1978). The reaction requires the reducing agent NADPH, without which the putative enzyme-bound triketide intermediate fails to react with the third malonyl-CoA molecule but cyclizes to form the triacetic acid lactone (Scheme 1). The instability of the polyketide intermediates requires that they remain enzyme-bound throughout the biosynthesis. Consequently polyketides are often synthesized in eukaryotes by multifunctional proteins in which the individual enzyme activities reside in separate protein domains joined together by flexible linkers. In this respect many of the polyketide synthases appear to resemble the animal and fungal type I fatty acid synthases (Hopwood & Sherman, 1990). In contrast, some bacterial polyketide synthases are thought to be composed of separate proteins, more like the type II fatty acid synthase systems, although there is growing evidence to suggest that the tetracenomycin C synthase enzymes may associate in the form of membrane-bound multienzyme complexes (Gramajo *et al.*, 1991) that may be functionally similar to the multidomain proteins of eukaryotes.

The eukaryote enzymes involved in polyketide biosynthesis have not been investigated as extensively as the fatty acid synthases. Only 6-methylsalicylic acid synthase from *Penicillium patulum* (Dimroth *et al.*, 1970), chalcone synthase from parsley cell cultures (Kreuzaler & Hahlbrook, 1975) and resveratrol synthase from peanut cell cultures (Schöppner & Kindl, 1984) have been studied to any degree. Several groups have reported the isolation of 6-methylsalicylic acid synthase to various states of purity (Dimroth *et al.*, 1970; Scott *et al.*, 1974; Beck *et al.*, 1990), but little information is available about the detailed properties or structure of the protein.

The recent determination of the nucleotide sequences encoding several polyketide synthases has revealed interesting features about the derived protein structures that have an important bearing on the nature of the polyketide assembly process (for a review see Hopwood & Sherman, 1990). Thus the predicted primary structure of 6-methylsalicylic acid synthase from *Penicillium patulum* (Beck *et al.*, 1990) appears to be related to the type I fatty acid synthases found in rat liver (Amy *et al.*, 1989) and other vertebrates. Protein domains specifying acyltransferase, β-oxoacyl synthase, β-oxoacyl reductase, β-hydroxyacyl dehydratase and acyl-carrier protein (ACP) (Beck *et al.*, 1990; Bevitt *et al.*, 1992) have been predicted with varying degrees of certainty. No sequence, however, has been assigned to an enoyl reductase or a thioesterase, although a domain(s) involved in catalysing the cyclization and aromatization reactions is expected to occupy part of the unassigned sequence.

One of the most interesting aspects of polyketide synthases is the precise means by which the multistep pathway is programmed to yield the desired product. For the fatty acid synthase-catalysed reactions, in which exactly the same cycle of reactions is repeated with each succeeding addition of malonyl-CoA, it is possible to envisage how each ACP-bound intermediate is recognized during the catalytic cycle. Intermediate programming can also be explained for polyketide synthases such as 6-deoxyerythronolide B synthase, which catalyses the initial stages of erythromycin biosynthesis (Donadio *et al.*, 1991; Bevitt *et al.*, 1992). This enzyme appears to possess a protein domain specifying each stage of the assembly process. However, with 6-methylsalicylic acid synthase, which appears to comprise one set of enzyme activities (Beck *et al.*, 1990) the addition of each malonyl-CoA is followed by a completely different cycle of events. For instance, the reduction with NADPH occurs exclusively at the C_6 polyketide intermediate stage, and not at either the C_4 or C_8 stages, suggesting that the details of the molecular programming are even more highly sophisticated in this enzyme. The mechanism of release of 6-methylsalicyclic acid from the enzyme is also obscure, since the predicted amino acid sequence does not indicate a thioesterase-like sequence as found in mammalian type I fatty acid synthases.

Abbreviations used: ACP, acyl-carrier protein; PMSF, phenylmethanesulphonyl fluoride.

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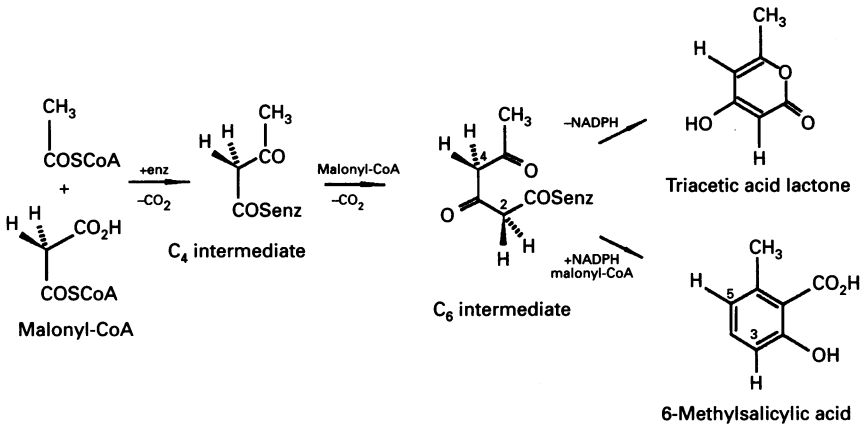


Scheme 1. Biosynthesis of 6-methylsalicylic acid and triacetic acid lactone from acetyl-CoA and malonyl-CoA with 6-methylsalicylic acid synthase from *Penicillium patulum*

Abbreviation: enz, enzyme.

The relationship between molecular programming and the conformation of enzyme-bound intermediates during polyketide biosynthesis has been investigated recently with experiments on the mechanism and stereochemical course of the reaction catalysed by 6-methylsalicylic acid synthase. Incorporation of (*R*)- and (*S*)-[1-^{13}C,2-^{2}H]malonic acids, as their malonyl-CoA derivatives, into 6-methylsalicylic acid has established that the methylene hydrogen atoms in the polyketide intermediate are manipulated by highly stereospecific processes, the hydrogen atoms eliminated from the 2- and 4-positions of the C_6 intermediate (Scheme 1) arising from H_{St} and H_{Re} of malonyl-CoA respectively (Spencer & Jordan, 1990; Jordan & Spencer, 1991; Spencer & Jordan, 1992). These experiments indicate that the conformation of the polyketide intermediates is precisely ordered throughout the enzymic reaction. In addition to stereochemical information, a detailed study of the protein structure of the synthase is also essential to build a picture of the overall biosynthetic assembly process. This paper reports a greatly improved method for the growth of *Penicillium patulum*, the isolation of 6-methylsalicylic acid synthase in homogeneous form and investigations on its molecular properties. Studies on the formation of the triacetic acid lactone and its relationship to 6-methylsalicylic acid synthesis are also described. These experiments represent our first attempts to unravel the process of molecular recognition and intermediate programming which occur during the biosynthesis of 6-methylsalicylic acid.

EXPERIMENTAL

Materials

Penicillium patulum NRRL 2159A (also called *Penicillium urticae*) was obtained from the Deutsche Sammlung für Mikroorganismen (Göttingen, Germany). 6-Methylsalicylic acid synthase was isolated from this strain as described below. Czapek solution agar, Bacto-Agar and yeast extract were from Difco Laboratories. Mono Q anion-exchange columns, Sephacryl S400, DEAE-Sepharose (fast flow) and PD10 pre-packed gel-filtration columns for use during the enzyme purification were from Pharmacia Fine Chemicals. Acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, NADPH and molecular-mass markers (MW-SDS-20) were from Sigma Chemical Co., Poole, Dorset, U.K. Triacetic acid lactone was purchased from Aldrich, Gillingham, Dorset, U.K. [2-^{14}C]Malonyl-CoA was purchased from Amersham International, Amersham, Bucks, U.K. All other chemicals were supplied by BDH, Poole, Dorset, U.K., and were of analytical grade.

Growth of *Penicillium patulum*

Mycelia of *Pencillium patulum* were grown from spores on three Petri dishes [diameter 12.6 cm (5 in)] containing 49 g of Difco Czapek solution agar and 5 g of Difco Bacto-Agar per litre. The plates were incubated at 25 °C until maximum spore formation had occurred (usually 7–10 days). The agar layers were removed from the dishes and carefully washed in 40 ml of Nonidet P40 detergent (0.4 ml/litre) in 1% (w/v) NaCl to remove the spores from the mycelia. The suspension containing the spores was pooled (120 ml) and used to inoculate six 2-litre conical flasks, with baffles, each containing 600 ml of medium. The interior of the flasks had been coated previously with dimethyldichlorosilane (BDH) to prevent aggregation of the mycelia on the flask walls. The medium contained yeast extract (5 g), KH_2PO_4 (2.5 g), K_2HPO_4 (2.5 g), glucose (40 g), $MgSO_4$, $7H_2O$ (0.5 g) and KCl (0.1 g) in a final volume of 1 litre. The KH_2PO_4 and K_2HPO_4 were dissolved in 20 ml of distilled water and autoclaved separately. The flasks were incubated at 28 °C for up to 26 h on a rotary shaker with a 5 cm stroke at a speed of 120 rotations/min.

Assay of culture growth

A 5 ml portion was removed from one of the flasks and suction-filtered to separate the mycelia from the medium. The mycelia were washed with 20 ml of 1 % NaCl solution. The mycelial mat was frozen and freeze-dried for 12 h. The dry weight of the mycelia per millilitre of culture medium was determined.

Detection of 6-methylsalicylic acid in the culture medium

T.l.c. was used to indicate qualitatively the presence of 6-methylsalicylic acid in the medium at various stages of cultivation, as follows. A 5 ml portion of filtered medium was removed and adjusted to pH 1.5 with HCl. The 6-methylsalicylic acid was extracted with 4 × 5 ml of diethyl ether. The extract was dried over anhydrous Na_2SO_4, and the solvent was reduced by evaporation. 6-Methylsalicylic acid was purified by chromatography on a silica-gel t.l.c. plate (5 cm × 10 cm) developed in benzene/dioxan/acetic acid (90:25:4, by vol.) and identified as a fluorescent blue spot under u.v. light. Quantitative measurement

of 6-methylsalicylic acid was performed by g.l.c. of the silylated residue by the procedure of Ehman & Gaucher (1977).

Radiochemical assay for 6-methylsalicylic acid synthase activity

The activity of 6-methylsalicylic acid synthase was determined by measuring the incorporation of [2-^{14}C]malonyl-CoA into 6-methylsalicylic acid (Dimroth *et al.*, 1970). The 6-methylsalicylic acid was separated from any fatty acids by t.l.c. before radioactivity analysis.

Fluorimetric assay for 6-methylsalicylic acid synthase activity

A more rapid assay for 6-methylsalicylic acid synthase is the fluorimetric determination of 6-methylsalicyclic acid: excitation wavelength (λ_{ex}) 308 nm, emission wavelength (λ_{em}) 410 nm (Vögel & Lynen, 1976). The interference by NADPH (λ_{ex} 360 nm, λ_{em} 465 nm) is eliminated by using λ_{ex} 310 nm and λ_{em} 390 nm for the determinations. The reaction mixture contains, in a total volume of 2 ml: Tris/sulphate buffer, pH 7.6, 160 μmol; acetyl-CoA, 0.4 μmol; NADPH, 0.4 μmol; BSA, 2.5 mg; 0.2–1.0 m-unit of 6-methylsalicylic acid synthase. Assays were performed at 25 °C for up to 10 min with stirring. The reaction was started by addition of malonyl-CoA (0.4 μmol), and the increase in fluorescence associated with 6-methylsalicylic acid formation was measured. The extent of fluorescence change is related to that obtained with a standard solution of 6-methylsalicylic acid. One unit is defined as that amount of enzyme catalysing the formation of 1 μmol of 6-methylsalicylic acid/min at 25 °C. The fluorescence assay was used for the study of the reaction kinetics of 6-methylsalicylic acid synthase.

Assay for fatty acid synthase activity

Fatty acid synthase, which co-purifies with 6-methylsalicylic acid synthase up to the hydroxyapatite stage, may be assayed by monitoring the rate of NADPH oxidation at 340 nm (Linn, 1981). The assay is only accurate if fatty acid synthase is separated from 6-methylsalicylic acid synthase, since the latter enzyme also uses NADPH. A good approximation to the fatty acid synthase activity may be made in extracts that also contain 6-methylsalicylic acid synthase by deducting the contribution of 6-methylsalicyclic acid synthase from the overall rate of NADPH oxidation. The latter may be calculated from the fluorimetric determination of 6-methylsalicylic acid formation, described above.

Procedure for purification of 6-methylsalicylic acid synthase from *Penicillium patulum*

Cell disruption. The mycelia from six flasks were collected by suction filtration and washed with 1% NaCl solution, yielding approx. 100 g wet wt. The mycelia were suspended immediately in 200 ml of 0.1 M-Tris/sulphate buffer, pH 7.6, containing 15% (v/v) glycerol, 1 mM-EDTA, 5 mM-2-mercaptoethanol, 0.3 g of phenylmethanesulphonyl fluoride (PMSF)/l and 0.34 g of benzamidine/l. The suspension was homogenized in a 'Bead-Beater' bead mill (Biospec), by using 0.5 mm-diameter glass beads. The homogenizing chamber (350 ml) was surrounded by a water jacket containing a salt/ice/water mixture at −8 °C. The mycelia were then disrupted by operating the bead mill for 3 × 90 s, with 2 min intervening cooling periods. The contents of the chamber were mixed with 300 ml of buffer, and the suspension was filtered through a cheesecloth to remove the glass beads. The unbroken cells and cell fragments were removed by centrifugation in a MSE-21 centrifuge for 20 min at 11000 *g*. All subsequent stages were carried out at 4 °C.

$(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ (12.8 g/100 ml) was added with stirring to give 22% saturation. After 10 min, the precipitated protein was collected by centrifugation for 15 min as above. The supernatant was retained and adjusted to 40% saturation by addition of further solid $(NH_4)_2SO_4$. The suspension was stirred for 15 min and then centrifuged in a MSE-21 centrifuge for 30 min at 5000 *g*.

Precipitation with poly(ethylene glycol). The precipitate from the previous stage was resuspended in 200 ml of 100 mM-Tris/sulphate buffer, pH 7.6, containing 6% (w/v) poly(ethylene glycol) 6000, 5 mM-mercaptoethanol, 1 mM-EDTA and 0.34 g of benzamidine/litre. The solution was stirred for 15 min and centrifuged at 30000 *g* in 50 ml Oakridge tubes. The supernatant was adjusted to 200 ml with buffer, and 75 ml of 50% (w/v) poly(ethylene glycol) 6000 solution was added slowly with stirring. The solution was stirred for a further 20 min and then centrifuged for 20 min at 4000 *g*. The supernatant fraction was discarded and the pellet was redissolved in 50 ml of 50 mM-potassium phosphate buffer, pH 7.6, containing 15% glycerol, 10 mM-mercaptoethanol, 1 mM-EDTA and 0.34 g of benzamidine/l.

DEAE-Sepharose ion-exchange chromatography. The extract from the previous stage was loaded on to a DEAE-Sepharose column (6.4 cm × 12 cm) pre-equilibrated with the above buffer. 6-Methylsalicylic acid synthase was eluted by a linear phosphate gradient (50–400 mM; total volume 500 ml). The fractions containing 6-methylsalicylic acid synthase activity were pooled and concentrated by precipitation with 450 ml of poly(ethylene glycol) 6000 (50%, w/v).

Hydroxyapatite chromatography. The pellet from the previous stage was dissolved in 15 ml of 50 mM-potassium phosphate buffer, pH 7.6, containing 15% glycerol, 2 mM-dithiothreitol, 1 mM-EDTA and 0.34 g of benzamidine/l. This protein solution was applied to a column of hydroxyapatite (3 cm × 10 cm) which had been pre-equilibrated with the same buffer. The 6-methylsalicylic acid synthase was eluted by addition of 100 ml of 200 mM-potassium phosphate buffer, pH 7.6, containing 15% glycerol, 2 mM-dithiothreitol, 1 mM-EDTA and 0.34 g of benzamidine/l. The fractions that contained 6-methylsalicylic acid synthase were pooled and precipitated with $(NH_4)_2SO_4$ (24.3 g/100 ml).

Ion-exchange chromatography. The $(NH_4)_2SO_4$ precipitate containing the 6-methylsalicylic acid synthase was dissolved in 2 ml of 40 mM-Tris/sulphate buffer, pH 7.0, containing 5 mM-dithiothreitol but no glycerol. The solution was desalted by using a Pharmacia PD10 gel-filtration column that has been pre-equilibrated with the same buffer. The eluate was filtered and then loaded on to a Pharmacia Mono Q 5HR column. The enzyme was eluted from the column over a period of 20 min with a linear gradient (0–350 mM-NaCl in a total volume of 20 ml) at a flow rate of 1 ml/min. 6-Methylsalicylic acid synthase appears as a sharp protein peak when the gradient has reached approx. 230 mM-NaCl. Glycerol (40%, v/v) was added immediately to each fraction to restore the level to 15% (v/v). The 6-methylsalicylic acid synthase was precipitated with $(NH_4)_2SO_4$ (24.3 g/100 ml). The purification affords approx. 8 mg of 6-methylsalicylic acid synthase. The 6-methylsalicylic acid synthase gave a single band on SDS/PAGE (Fig. 3*a*). A summary of the purification is given in Table 1.

The purified enzyme was found to be most stable when stored as an $(NH_4)_2SO_4$ precipitate at −70 °C, although even under these conditions it lost approx. 5% of its activity per month. When required, the $(NH_4)_2SO_4$ precipitate is dissolved in 100 mM-Tris/sulphate buffer containing 15% glycerol, 10 mM-dithiothreitol and 1 mM-EDTA. The solution is incubated at 20 °C for 15 min before use to ensure that all the thiol groups of 6-methylsalicyclic acid synthase are reduced.

Table 1. Purification of 6-methylsalicylic acid synthase from *Penicillium patulum*

Step and fraction	Protein (mg)	Total activity (units)	Specific activity (m-units/mg)	Recovery (%)
1. Centrifuged extract	4700	10.4	2.2	100
2. $(NH_4)_2SO_4$ (22–40% saturation)	1595	9.25	5.8	89
3. Poly(ethylene glycol) 6000 precipitation	710	7.69	11.0	74
4. DEAE-Sepharose eluate	80	5.41	67.0	52
5. Hydroxyapatite eluate	29	3.95	135.0	38
6. F.p.l.c. Mono Q eluate	8.8	2.18	245.0	21

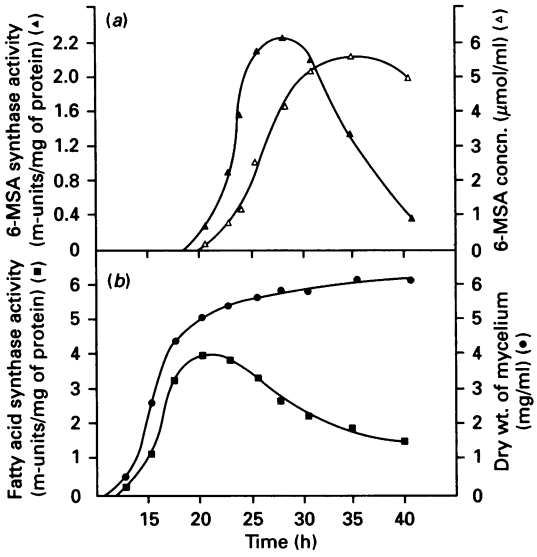


Fig. 1. Growth characteristics and time course for the appearance of 6-methylsalicylic acid synthase, its product 6-methylsalicylic acid and fatty acid synthase in shaker-flask cultures of *Penicillium patulum*

(*a*) △, Formation of 6-methylsalicylic acid (MSA); ▲, development of 6-methylsalicylic acid synthase. (*b*) ●, Mycelial growth; ■, development of fatty acid synthase activity.

Co-purification of fatty acid synthase from *Penicillium patulum*

The fatty acid synthase from *Penicillium patulum* co-purified with the 6-methylsalicyclic acid synthase up to the hydroxyapatite step. To effect separation of the two synthases, the 6-methylsalicylic acid synthase was first eluted from the hydroxapatite column as described above. The fatty acid synthase was then eluted by addition of 100 ml of 400 mM-potassium phosphate buffer, pH 7.6, containing 15% glycerol, 2 mM-dithiothreitol, 1 mM-EDTA and 0.34 g of benzamidine/l. The fractions that contained the fatty acid synthase were pooled and precipitated with $(NH_4)_2SO_4$ (24.3 g/100 ml). There is slight contamination of the first fatty acid synthase fractions with 6-methylsalicylic acid synthase; however, this can be avoided by using the later fractions. The fatty acid synthase may be purified to homogeneity by ion-exchange chromatography on a Mono Q f.p.l.c. column as described above for 6-methylsalicylic acid synthase. Approx. 1 mg of fatty acid synthase was obtained from 100 g wet wt. of cells, but this could be improved if the mycelia were harvested during late exponential growth (18 h) rather than after 26 h (see Fig. 1). The fatty acid synthase was stored at −70 °C as an $(NH_4)_2SO_4$ precipitate and lost less than 10% of its activity after 6 months.

M_r determinations

(i) PAGE. Electrophoresis of 6-methylsalicylic acid synthase was performed in 5%-polyacrylamide gels in the presence of SDS as described by Laemmli (1970). Further details are shown in Fig. 3.

(ii) Gel filtration. Protein samples were dissolved in 1 ml of Tris/sulphate buffer, pH 7.6, containing 5 mM-dithiothreitol and 1 mM-EDTA. Protein standards used were catalyse (1 mg), urease (2 mg), ferritin (2 mg) and glutamate dehydrogenase (3 mg). M_r values for protein standards were: catalase 235000; urease 483000; ferritin 540000; glutamate dehydrogenase 2200000. The samples were applied to a Sephacryl-S400 column (2.5 cm × 100 cm) which had been packed according to the manufacturer's instructions (Pharmacia). The column was pre-equilibrated with the above buffer and developed at a flow rate of 1 ml/min. The elution volumes of the proteins were determined.

Inactivation and cross-linking studies with 1,3-dibromopropan-2-one

6-Methylsalicylic acid synthase (1 mg; 1.4 nmol of tetramer) was treated with a stoichiometric amount of 1,3-dibromopropan-2-one (5.5 nmol) in 1 ml of 100 mM-potassium phosphate, pH 7.0, and the enzyme activity was determined over a period of 4 min by removing samples (10 μl) at timed intervals by dilution into 2 ml of Tris/sulphate assay buffer containing 10 mM-dithiothreitol. Samples were assayed for enzyme activity by the fluorimetric method described above. Alternatively, the inactivated protein samples were passed through a gel-filtration column to remove traces of the inhibitor. The column had been equilibrated previously with 100 mM-Tris/HCl, pH 7.6, containing 0.5 mM-EDTA, 10 mM-dithiothreitol and 15% glycerol. The activity of the 6-methylsalicylic acid synthase in the eluate was approximately the same as before it was passed through the gel-filtration column. The activity of the gel-filtered synthase was monitored for a further 30 min, during which time it remained constant. The enzyme was subjected to SDS/PAGE (Laemmli, 1970).

Detection of triacetic acid lactone.

Incubations were extracted with diethyl ether (4 × 5 ml); the extract was dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was treated with bis-trimethylsilyltrifluoracetamide (20 μl) and pyridine (10 μl), and the solution was heated at 60 °C for 30 min in a sealed tube. The trimethylsilyl derivative was analysed by g.l.c. as described by Ehman & Gaucher (1977) and quantified by using standard amounts of the triacetic acid lactone.

Protein determination.

Protein concentrations were determined by the method of Bradford (1976), with BSA as a standard.

RESULTS AND DISCUSSION

Optimization of growth conditions for *Penicillium patulum* which maximize levels of 6-methylsalicylic acid synthase

Penicillium patulum for the purification of 6-methylsalicylic acid synthase was grown from a spore inoculum, by a method similar to that of Grootwassink & Gaucher (1980), ensuring that the cells were all approximately of similar age and morphology. The organism was harvested approx. 26 h after inoculation, when

the mycelium had reached a dry cell weight of 6 g/litre and when the enzyme level was maximum (Fig. 1). 6-Methylsalicylic acid reached a peak some 10 h later (Fig. 1). The level of fatty acid synthase was also determined in the cultures of *P. patulum* (Fig. 1). The level of this enzyme parallels the increase in mycelial dry weight during the replicatory growth phase, as expected. After the growth had ceased, the levels of fatty acid synthase declined as the organism switched from primary to secondary metabolism. The optimum time to harvest the mycelium to maximize levels of fatty acid synthase is just before the end of the exponential growth phase, at approx. 18 h.

Studies by Lam *et al.* (1988) have indicated that 6-methylsalicylic acid synthase is susceptible to proteolysis *in vivo* and *in vitro*. If growth was extended for more than 26 h, the 6-methylsalicylic acid synthase became very unstable during subsequent column chromatography, suggesting that proteolytic 'nicking' had occurred. PMSF and benzamidine were used in the disruption buffer, extending the half-life of the enzyme from 20 min to 6 h (Fig. 2). The addition of 15% (v/v) glycerol together with proteinase inhibitors improved further the stability of the enzyme (Fig. 2). The purification was therefore carried out with benzamidine and PMSF in the presence of 15% glycerol. The combination of the above precautions and adaptations to the growth conditions resulted in 6-methylsalicylic acid synthase being about 0.75% of the soluble protein of the cell, thus greatly facilitating its purification (see the Experimental section).

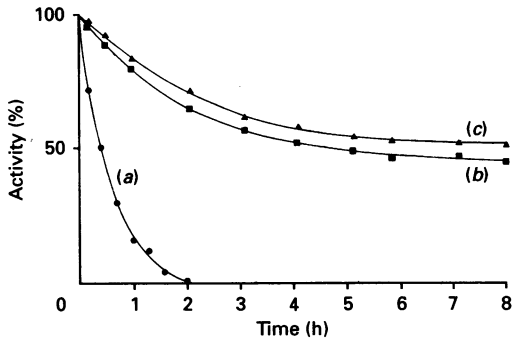


Fig. 2. Stability of 6-methylsalicylic acid synthase in cell-free extracts of *Penicillium patulum* under various conditions

(*a*) ●, Tris/sulphate buffer (90 mM), pH 7.6, containing 5 mM-2-mercaptoethanol and 1 mM-EDTA; (*b*) ■, the same buffer as (*a*), but containing the proteinase inhibitors PMSF and benzamidine; (*c*) ▲, the same buffer as (*a*), but containing PMSF, benzamidine and 15% glycerol.

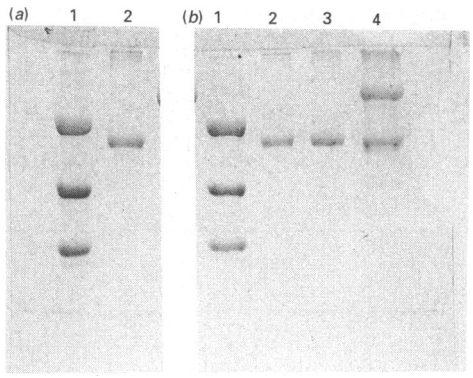


Fig. 3. Electrophoresis of 6-methylsalicylic acid synthase in 5%-polyacrylamide gels containing SDS (Laemmli, 1970), (*a*) before and (*b*) after treatment with 1,3-dibromopropan-2-one

(*a*) Track 1, M_r standards: myosin (205000), β-galactosidase (116000) and BSA (66000); track 2, purified 6-methylsalicylic acid synthase. (*b*) Track 1, M_r standards as in (*a*); track 2, 6-methylsalicylic acid synthase treated with 1,3-dibromopropan-2-one (5 nmol) in the presence of acetyl-CoA; track 3, 6-methylsalicylic acid synthase treated with 1,3-dibromopropan-2-one (5 nmol) in the presence of malonyl-CoA; track 4, 6-methylsalicylic acid synthase treated with 1,3-dibromopropan-2-one (5 nmol).

Molecular properties of 6-methysalicylic acid synthase

Subunit size of 6-methylsalicylic acid synthase. The purified 6-methylsalicylic acid synthase was subjected to SDS/PAGE. The enzyme appeared as a single protein band of M_r 180000 (see Fig. 3*a*), suggesting that it is composed of only a single type of subunit. This is in agreement with the DNA sequence data, which also predict a protein of subunit M_r 180000 (Beck *et al.*, 1990) and contrasts with the fatty acid synthase of *Penicillium patulum*, which is made up of two different types of subunit, with M_r values of 200000 and 192000 (Wiesner *et al.*, 1988).

M_r of native 6-methylsalicylic acid synthase. The M_r of the purified native 6-methylsalicylic acid synthase was determined by gel filtration using a Sephacryl S400 column (2.5 cm × 100 cm). The enzyme (1 mg) was chromatographed together with protein standards at a flow rate of 1 ml/min, and the volumes at which the proteins were eluted were determined (Fig. 4). The M_r of the native 6-methylsalicylic acid synthase was found to be about 750000, suggesting that the enzyme exists as a tetramer of identical subunits. This is somewhat lower than the value of 1.2×10^6 determined previously (Vögel & Lynen, 1976).

Optimum pH and substrate specificity of 6-methylsalicylic acid synthase

The activity of 6-methylsalicyclic acid synthase at different pH values is shown in Fig. 5. The pH optimum for the enzyme is 7.6. The enzyme is more stable at basic pH values, and the presence of malonyl-CoA, acetyl-CoA and NADPH greatly improve the stability. The 6-methylsalicylic acid synthase was assayed at various concentrations of either acetyl-CoA or malonyl-CoA over the range 2–1000 μM while all other substrate concentrations were held constant, in accordance with Cleland (1977). The apparent K_m values were found to be 10 μM and 7 μM for acetyl-CoA and malonyl-CoA respectively, close to those of 20 μM obtained for both substrates previously (Dimroth *et al.*, 1970; Vögel & Lynen, 1976). Concentrations of each substrate were varied. Acetoacetyl-CoA was also found to be a substrate, with an apparent K_m of 65 μM. The $V_{max.}$ for acetoacetyl-CoA (0.012 μmol/min per mg) was much lower than that observed with acetyl-CoA (0.25 μmol/min per mg) under the same conditions. Despite this value, this latter observation was important for examining the stereochemical aspects of the enzyme mechanism (Spencer & Jordan, 1990, 1992; Jordan & Spencer, 1991). The apparent K_m for NADPH was found to be approx. 12 μM.

Formation of triacetic acid lactone

When purified 6-methylsalicylic acid synthase was incubated with acetyl-CoA and malonyl-CoA in the absence of NADPH, the exclusive product was found to be triacetic acid lactone. This has been noted previously (Dimroth *et al.*, 1970; Scott *et al.*, 1974). Triacetic acid lactone is synthesized from 1 molecule of acetyl-CoA and 2 molecules of malonyl-CoA (Scheme 1). The triacetic acid lactone was produced at 10% of the rate of 6-methylsalicylic acid. Addition of NADPH in increasing quantities led to the progressive formation of 6-methylsalicylic acid (results not shown), so that when NADPH was at 0.5 μM the ratio

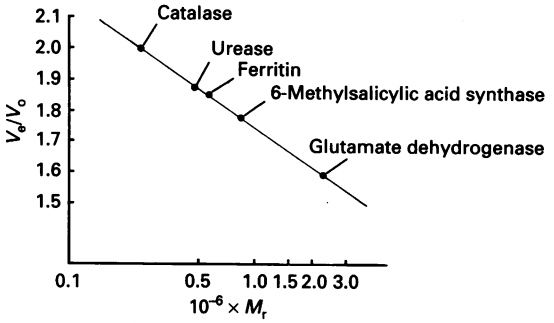


Fig. 4. Determination of the native M_r of 6-methylsalicylic acid synthase from *Penicillium patulum* by gel filtration through Sephacryl S400

Protein standards included catalase (235000), urease (483000), ferritin (540000) and glutamate dehydrogenase (2200000). V_e, elution volume; V_o, void volume.

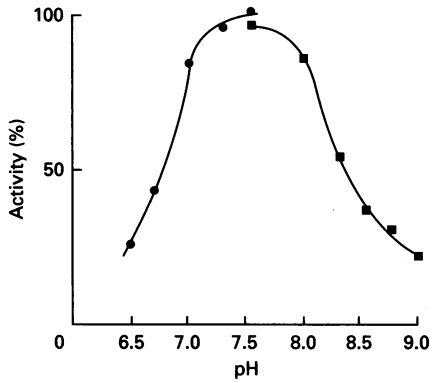


Fig. 5. Profile of 6-methylsalicylic acid synthase acid synthase activity with varying pH

●, Potassium phosphate buffer; ■, Tris/sulphate buffer.

of 6-methylsalicylic acid to triacetic acid lactone was 5:1. At the K_m of NADPH (12 μM) the proportion of triacetic acid lactone had fallen to less than 1%. This exemplifies the importance of NADPH in molecular programming of the overall reaction course, since the enzyme-bound C_6 polyketide (Scheme 1) does not appear to be able to react with a further molecule of malonyl-CoA unless the NADPH-dependent reduction reaction has occurred. Thus the C_6 intermediate is long-lived enough to cyclize and dissociate from the enzyme, as the triacetic acid lactone. This is consistent with the 'progressive' mechanism of polyketide formation, where processing of the keto groups occurs during the assembly of the polyketide chain rather than after all the acyl derivatives have been incorporated.

Release of 6-methylsalicylic acid from the enzyme

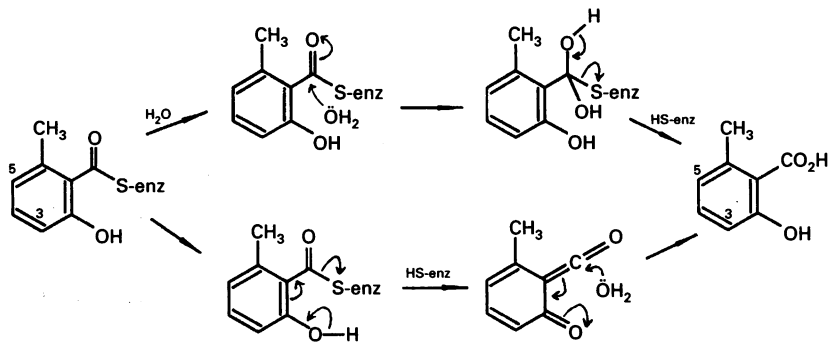
The final release of the completed aromatic ring from the 6-methylsalicylic acid synthase enzyme does not appear to follow the serine-dependent thioesterase-catalysed hydrolysis mechanism that operates in the mammalian fatty acid synthases (Scheme 2). Two pieces of evidence suggest that an alternative mechanism may operate.

Firstly, treatment of the 6-methylsalicylic acid synthase with the serine-proteinase inhibitor PMSF did not inactivate the enzyme. PMSF rapidly and irreversibly inactivates mammalian fatty acid synthases, the thioesterases of which are related to the serine proteinases (Stoops & Wakil, 1981) and esterases (Moss & Fahrney, 1978; Turini *et al.*, 1969). Thus, once the insensitivity of the 6-methylsalicylic acid synthase to this inhibitor had been established, it was routinely included in the buffers during purification to decrease proteolysis and to increase the half-life of the enzyme (see Fig. 2).

Secondly, no evidence for the formation of a product-CoA ester was obtained at any time in the above studies. Furthermore, the 6-methylsalicylic acid synthase cDNA sequence from *Penicillium patulum* (Beck *et al.*, 1990; Hopwood & Sherman, 1990) does not appear to contain an open reading frame specifying a typical thioesterase domain. It is thus possible that an alternative mechanism for the release of the 6-methylsalicylic acid from the enzyme may operate. Polyketide intermediates with an aromatic ring adjacent to the thioester bond are good candidates for a carbanion ($E1_{cb}$) mechanism (Casanova *et al.*, 1967; Holmquist & Bruice, 1969) of the type shown in Scheme 2 (lower path). The carbanion can collapse spontaneously to form a reactive ketene, which is rapidly hydrated to the carboxylic acid. It should not be overlooked, however, that a thioesterase enzyme of a different class from that used in fatty acid synthases may be responsible for the hydrolysis via a tetrahedral intermediate (Scheme 2, upper path).

Inhibition of 6-methylsalicylic acid synthase by 1,3-dibromopropan-2-one

The amino acid sequence of 6-methylsalicylic acid synthase, derived from the cDNA sequence specifying the enzyme, indicates a substantial similarity to type I fatty acid synthases (Beck *et al.*, 1990). Since 1,3-dibromopropan-2-one is a powerful inhibitor of type I fatty acid synthases (Stoops & Wakil, 1981), the effect of



Scheme 2. Release of 6-methylsalicylic acid from the enzyme by thioesterase (upper path) and ketene (lower path) mechanisms

Abbreviation: enz, enzyme.

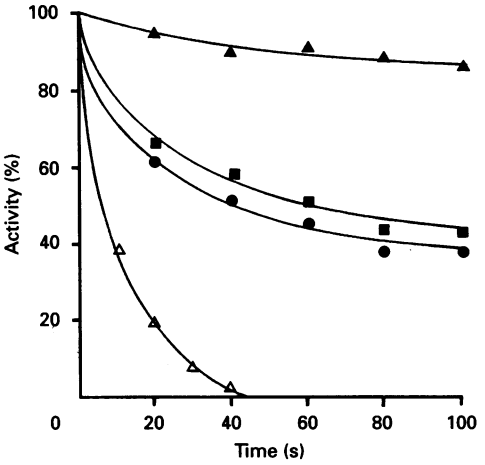
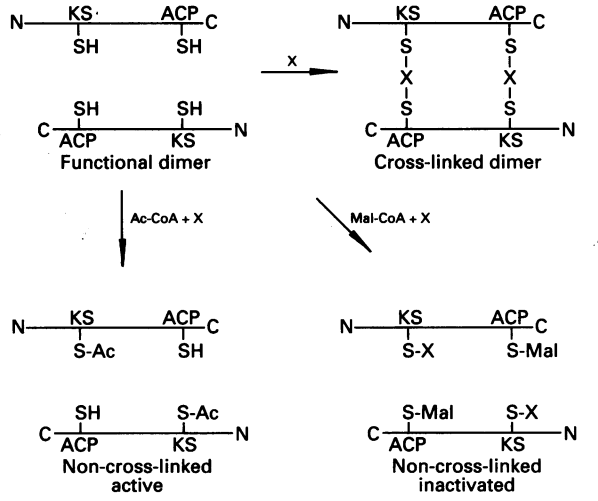


Fig. 6. Inactivation of 6-methylsalicylic acid synthase by 1,3-dibromopropan-2-one

6-Methylsalicylic acid synthase (1.25 nmol) was treated with 1,3-dibromopropan-2-one (5 nmol) in the absence (●) or presence of acetyl-CoA (▲) or malonyl-CoA (■). The enzyme was also treated with 20 nmol of 1,3-dibromopropan-2-one (△).



N— C represents the 6-methylsalicylic acid synthase monomer

Scheme 3. Hypothetical mechanism for the cross-linking of 6-methylsalicylic acid synthase with 1,3-dibromopropan-2-one

Abbreviations: KS, β-oxoacyl synthase; Mal-CoA, malonyl-CoA; Ac-CoA, acetyl-CoA; X, 1,3-dibromopropan-2-one.

this inhibitor on 6-methylsalicylic acid synthase was investigated. The results shown in Fig. 6 indicate that a stoichiometric amount of 1,3-dibromopropan-2-one has a rapid inhibitory effect on the 6-methylsalicylic acid synthase. Addition of higher amounts of 1,3-dibromopropan-2-one (3 mol excess) had a dramatic effect on the enzyme, leading to complete inactivation within a few seconds (Fig. 6).

To determine the effect of 1,3-dibromopropan-2-one on 6-methylsalicylic acid synthase in the presence of either acetyl-CoA (0.2 mM) or malonyl-CoA (0.2 mM), a similar procedure was followed, except that the enzyme was preincubated for 1 min with the acyl-CoA derivative before addition of the inhibitor. Fig. 6 shows that malonyl-CoA had little ability to protect the enzyme from inhibition, whereas, in contrast, acetyl-CoA protected the enzyme substantially. This suggests that acetyl-CoA is binding to the more reactive thiol of the β-oxoacyl synthase domain. Such behaviour has also been observed with the type I fatty acid synthases, suggesting that the 6-methylsalicylic acid synthase may also be functionally, as well as structurally, related.

Cross-linking of 6-methylsalicylic acid by 1,3-dibromopropan-2-one

In order to determine the effects of 1,3-dibromopropan-2-one on the structure of the enzyme, the inactivation described above was allowed to proceed for 4 min, after which time the enzyme samples were assayed and then immediately passed down a PD10 gel-filtration column to separate the synthase from any remaining 1,3-dibromopropan-2-one. The samples were then subjected to SDS/PAGE. The formation of a new protein band of lower mobility indicates that a substantial degree of cross-linking has occurred (Fig. 3*b*, track 4). Although the cross-linked protein band falls just outside the range of the molecular-mass markers, extrapolation of the standard curve (results not shown) gives a value of M_r 350000, close to that expected from a cross-linked dimer of the M_r-180000 monomeric species. The presence of acetyl-CoA or malonyl-CoA during the reaction with 1,3-dibromopropan-2-one totally prevents the cross-linking (Fig. 3b, tracks 2 and 3 respectively), suggesting that the reagent is reacting specifically with the active-site cysteine of the β-oxoacyl synthase and the cysteamine of the ACP in a similar way to that predicted for mammalian fatty acid synthase (Stoops & Wakil, 1981). Acetyl-CoA prevents 1,3-dibromopropan-2-one from reacting with both of these thiols, and thus protects the enzyme from the effect of the inactivator (Fig. 6). Malonyl-CoA prevents cross-linking of the subunits of 6-methylsalicylic acid synthase, but does not protect against the inactivation (Fig. 6). From these studies it is suggested that acetyl-CoA protects the highly reactive thiol group of the β-oxoacyl synthase and that malonyl-CoA protects the phosphopantetheine thiol group of the ACP. The results suggest that, during cross-linking, 1,3-dibromopropan-2-one reacts initially with the thiol group of the β-oxoacyl synthase, followed by reaction with the phosphopantetheine thiol group of ACP belonging to an adjacent subunit in a functional dimer. A hypothetical mechanism is summarized in Scheme 3.

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REFERENCES

Amy, C. M., Witkowsky, A., Naggert, J., Williams, B., Randhawra, Z. & Smith, S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3114–3118
Beck, J., Ripka, S., Siegner, A., Schiltz, E. & Schweizer, E. (1990) Eur. J. Biochem. **192**, 487–498
Bevitt, D. J., Cortes, J., Haydock, S. F. & Leadley, P. F. (1992) Eur. J. Biochem. **193**, 39–49
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Casanova, J., Werner, N. D. & Keifer, E. F. (1967) J. Am. Chem. Soc. **89**, 2411–2416
Cleland, W. W. (1977) Adv. Enzymol. **45**, 273–387
Dimroth, P., Walter, H. & Lynen, F. (1970) Eur. J. Biochem. **13**, 98–110
Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J. & Katz, L. (1991) Science **252**, 675–679
Ehman, R. & Gaucher, G. M. (1977) J. Chromatogr. **132**, 17–30
Gramajo, H. C., White, J., Hutchinson, C. R. & Bibb, M. J. (1991) J. Bacteriol. **173**, 6475–6483
Grootwassink, J. W. D. & Gaucher, G. M. (1980) J. Bacteriol. **141**, 443–455
Holmquist, B. & Bruice, T. C. (1969) J. Am. Chem. Soc. **91**, 2993–2999
Hopwood, D. A. & Sherman, D. H. (1990) Annu. Rev. Genet. **24**, 37–66

Jordan, P. M. & Spencer, J. B. (1991) Tetrahedron **47**, 6015–6028
Kreuzaler, F. & Hahlbrock, K. (1975) Eur. J. Biochem. **56**, 205–213
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lam, K. S., Neway, J. O. & Gaucher, G. M. (1988) Can. J. Microbiol. **34**, 30–37
Linn, T. C. (1981) Arch. Biochem. Biophys. **209**, 613–619
Martin, J. F. & Demain, A. L., (1978) in The Filamentous Fungi, vol. 3. Developmental Mycology (Smith, J. E. & Berry, D. R., eds.), pp. 426–450, Edward Arnold, London
Moss, D. E. & Fahrney, D. (1978) Biochem. Pharmacol. **27**, 2693–2698
Schöppner, A. & Kindl, H. (1984) J. Biol. Chem. **259**, 6806–6811
Scott, A. I., Beadling, L. C., Georgopapadakou, N. H. & Subbarayan, C. R. (1974) Bioorg. Chem. **3**, 238–248
Spencer, J. B. & Jordan, P. M. (1990) J. Chem. Soc. Chem. Commun. 1704–1706
Spencer, J. B. & Jordan, P. M. (1992) Biochemistry, in the press
Stoops, J. K. & Wakil, S. J. (1981) J. Biol. Chem. **256**, 5128–5133
Turini, P., Kurooka, S., Steer, M., Corbascio, A. N. & Singer, T. P. (1969) J. Pharmacol. Exp. Ther. **167**, 98–104
Vögel, G. & Lynen, F. (1976) Methods Enzymol. **43**, 520–530
Wiesner, P., Beck, J., Beck, K.-F., Ripka, S., Müller, G., Lücke, S. & Schweizer, E. (1988) Eur. J. Biochem. **177**, 69–79

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