Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 619–626
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Purification and characterization of malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT) from leaves of *Digitalis purpurea* L.

Serge Philibert Kuate, Rodrigo M. Pádua, Wilhelm F. Eisenbeiss, Wolfgang Kreis *

Department of Pharmaceutical Biology, Institute of Biology, Friedrich-Alexander University of Erlangen-Nuremberg, Staudtstr. 5, D-91058 Erlangen, Germany

Received 19 July 2007; received in revised form 15 August 2007
Available online 22 October 2007

Abstract

With respect to the cardenolide pathway and the characterization of enzymes involved in the formation of cardenolides, a malonyltransferase, termed malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT) has been purified. The enzyme catalyses the transfer of the malonyl moiety from malonyl-coenzyme A to 21-hydroxypregnane substrates. Malonyltransferase activity was checked in several potential starting materials including fresh leaves and cell suspension cultures from different plants. Fresh *Digitalis purpurea* L. leaves turned out to be the best enzyme source. The purification protocol included ammonium sulphate precipitation, hydrophobic interaction chromatography on Phenylsepharose 6 FF, ion exchange chromatography on Source 30 Q, affinity chromatography on Cibacron Blue 3GA and gel filtration on Superdex 75. Gel filtration and native SDS–PAGE analysis showed that *Dp*21MaT exists as a monomer with a molecular mass of 27 kDa. Its p*I*, as determined by isoelectric focusing, was 4.66. The enzyme showed maximal activity at pH 6.5 when incubated at 42 °C. The energy of activation was 29.28 kJ mol^{-1}, whereas that of inactivation was 48.57 kJ mol^{-1}. *Dp*21MaT was purified 252-fold with a yield of about 1%. Hanes plots of kinetic data indicated K_m values of 99 μM (V_{max} 47.57 μkat kg^{-1}) and 28.44 μM (V_{max} 39.4 μkat kg^{-1} protein) for 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one and malonyl-CoA, respectively.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: *Digitalis purpurea*; Plantaginaceae; Enzyme purification; Malonyl-coenzyme A; 21-Hydroxypregnane 21-*O*-malonyltransferase; 21-*O*-Hydroxypregnanes; Cardenolides; Cardiac glycosides

1. Introduction

Cardiac insufficiency is still a major concern in developed countries given its significant influence on morbidity and mortality (Jessup and Brozena, 2003). The beneficial effects of foxglove (*Digitalis purpurea* L.) on this condition were already reported as early as 1785 (Withering, 1785). Presently, 23 different cardiac glycosides (Luckner and Wichtl, 2000) have been identified in extracts prepared from *D. purpurea*. The positive inotropic effect of cardenolides is a result of their ability to inhibit sodium–potassium ATPase, allowing calcium to accumulate in the myocytes and thereby leading to an increase in the force of myocardial contraction. Other studies demonstrated that in addition to its inotropic effects, cardenolides have neurohormonal and autonomic actions that may prevent heart failure. Cardenolides proved to be one of the most effective drugs to manage chronic heart failure (Dec, 2003; Pervaiz et al., 2006; Rising et al., 2006). Plants still are the sole source for their acquisition. To improve the production of these important biologically active metabolites it is necessary to further understand the biosynthetic pathway. A malonyltransferase has been reported to play a regulatory role in cardenolide biosynthesis and this step may be

* Corresponding author. Tel.: +49 9131 852 8241; fax: +49 9131 852 8243.
E-mail address: wkreis@biologie.uni-erlangen.de (W. Kreis).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.08.025

rate-limiting. This enzyme catalyses the transfer of a malonyl moiety to the 21-hydroxy group of 21-hydroxypregnanes, most likely initiating butenolide ring formation (Stuhlemmer and Kreis, 1996; Kreis et al., 1998; Luber, 2002).

The purification and the characterization of the malonylCoA: 21-Hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT) from young fresh leaves from *D. purpurea* L. is described here for the first time.

2. Results

2.1. Starting material

Digitalis lanata, *D. purpurea*, *Isoplexis canariensis*, *Isoplexis sceptrum* (Plantaginaceae), *Galphimia glauca* (Malpighiaceae), cell suspension cultures from *D. lanata* strains K3OHD and W.1.4 and *D. purpurea* underwent an enzymatic screening for 21-*O*-malonyltransferase as well as 21-*O*-malonylesterase activity. The esterase is an interfering enzyme suspected to impair the activity of the malonyltransferase catalysing the reverse reaction of the enzyme in question. Considerable 21-*O*-malonyltransferase activity was observed in young fresh leaves from *D. lanata*, *D. purpurea,* and in *I. canariensis,* whereas hardly any activity was detectable in *I. sceptrum* (Table 1). Equally, no activity was recorded in cell suspension cultures regardless of the source. Strangely, the young leaves from *G. glauca* chosen as negative control for malonyltransferase showed the highest 21-*O*-malonylesterase activity but no 21-*O*-malonyltransferase activity. Finally, young *D. purpurea* leaves were used as the starting material, since high 21-*O*-malonyltransferase activity and only low interfering 21-*O*-malonlyesterase were detected in this source.

2.2. Purification of the Dp21MaT

Five purification steps have been employed for the purification of malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT) from *D. purpurea* leaves. Most of the *Dp*21MaT activity was pelleted between 20% and 60% $(NH_4)_2SO_4$. After re-dissolving the protein, the solution was passed through a phenylsepharose column.

Table 1
Relative activities of malonyltransferase and malonylesterase in potential starting materials

Plant material	Relative activity[a] (%)	
	Malonyltransferase	Malonylesterase
D. purpurea young leaves	100	11
D. lanata young leaves	107	13
I. canariensis young leaves	80	21
I. sceptrum young leaves	t	16
D. lanata W.1.4 cell suspension	t	39
D. lanata K3OHD cell suspension	t	25
D. purpurea cell suspension	0	35
G. glauca young leaves	0	100

t: trace.

[a] The activities of *D. purpurea* young leaves (malonyltransferase) and *G. glauca* (malonylesterase) are taken as 100%.

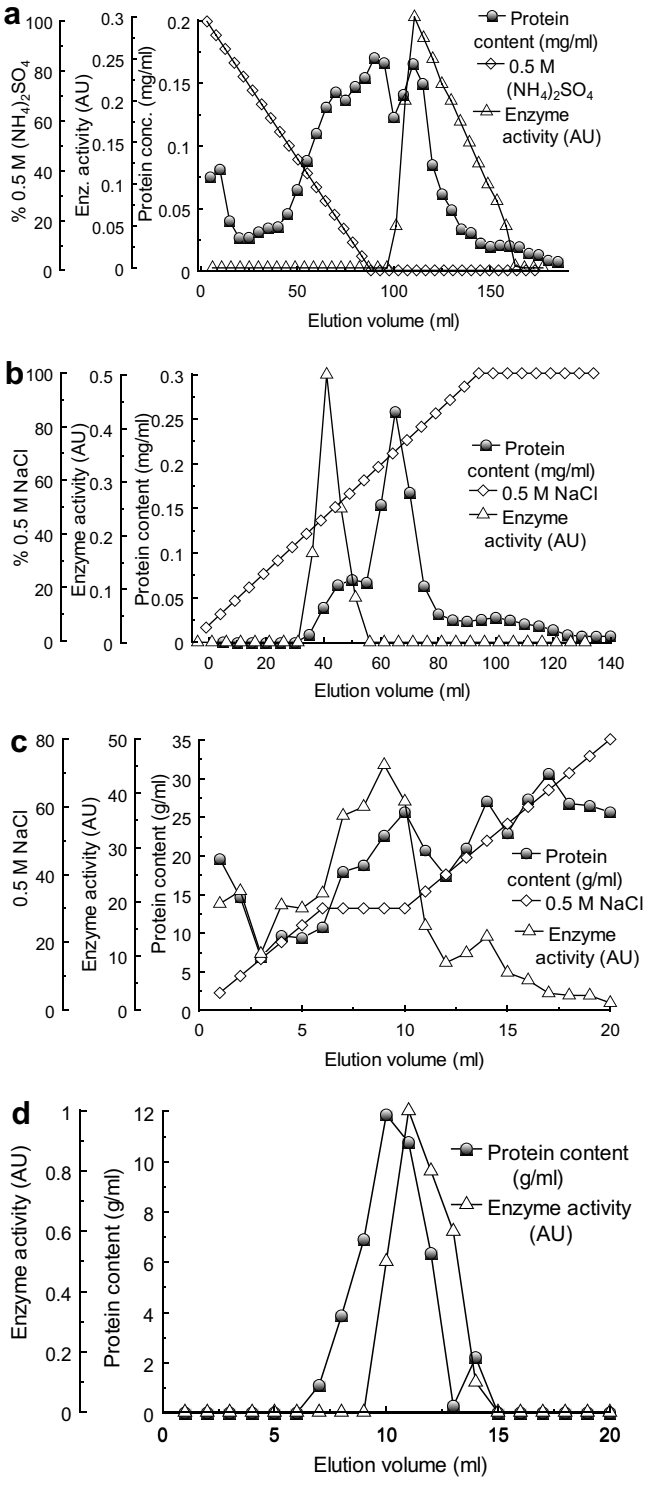


Fig. 1. Purification steps of the *Dp*21MaT. (a) Hydrophobic interaction chromatography on Phenylsepharose 6 FF; (b) anion exchange chromatography on Source 30 Q, repeat; (c) affinity chromatography on Cibacron Blue F3G-A; (d) gel filtration on Superdex 75.

Table 2
Purification of *Dp*21MaT from fresh leaves of *D. purpurea* L.

Purification step	Total protein (mg)	Total activity (pkat)	Specific activity (μkat kg^{-1})	Purification (-fold)	Recovery (%)
Crude extract[a]	776	739	0.95	1	100
$(NH_4)_2SO_4$ precipitation	668	692	1.03	1.1	94
Phenylsepharose 6 FF	105	598	5.7	6	81
Source 30 Q	16	479	30	31	65
Source 30 Q repeat	3.14	108	32	34	15
Cibacron Blue F3G-A	0.12	11	92	96	1.5
Superdex 75	0.025	6	240	252	0.8

[a] After passing through Sephadex G-25.

An ammonium sulphate gradient from 0.5 to 0 M was applied and the enzyme activity eluted with ammonium sulphate-free elution buffer. This step led to a six-fold purification with an 81% recovery of the enzyme activity. The enzyme adsorbed at pH 9.5 to Source 30 Q from which it could be eluted with 0.2 M NaCl. Repeating this step, the enzyme was purified 65-fold with a 31% recovery of its activity (Fig. 1b). Affinity chromatography on Cibacron Blue (Fig. 1c) yielded a purification factor of 100. The final step consisting of gel filtration on Superdex 75 (Fig. 1d) led to a 252-fold purified enzyme with a 0.81% recovery of activity. The specific activity at this level was 240 μkat kg^{-1} protein (Table 2).

Native PAGE and SDS–PAGE were used to identify the band representing the investigated enzyme during purification. After gel filtration, a protein of 27 kDa was seen in all fractions containing *Dp*21MaT activity. Similarly, in native PAGE only the fraction containing the 27 kDa protein showed *Dp*21MaT activity.

This band was eluted from the native PAGE gels and its malonyltransferase activity demonstrated. The purified *Dp*21MaT was a monomer showing a single protein band in native PAGE and a molecular mass of 27 kDa as determined by SDS–PAGE (Fig. 2). The isoelectric point (p*I*) of *Dp*21MaT as determined by isoelectric focusing was 4.66.

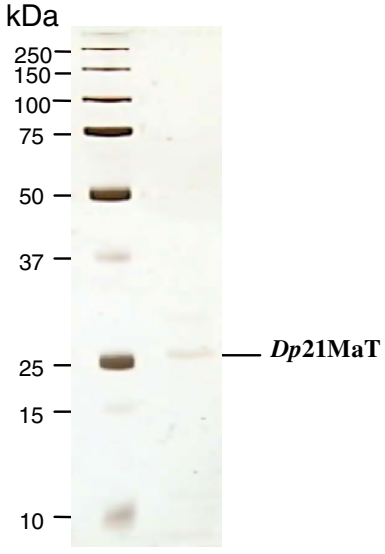


Fig. 2. *Dp*21MaT silver-stained after SDS–PAGE (right lane). Protein standards in the left lane. The *Dp*21MaT fraction analysed here was obtained from a native PAGE gel by gel elution.

2.3. Temperature and pH optima

The highest *Dp*21MaT activity was observed at 42 °C, half-maximal activities at 25 and 60 °C. The Arrhenius plot of the data covering the activation range indicated the energy of activation to be 29.28 kJ mol^{-1}, whereas the energy of inactivation was 48.57 kJ mol^{-1} (Fig. 3a). Q_{10} determined between 30 and 40 °C was 0.88. The influence of the pH on *Dp*21MaT activity was examined at 42 °C. The highest activity was seen at pH 6.5 in 50 mM McIlvaine and 50 mM Na–Pi buffer with half-maximal activities at pH 5.8 and 8.3 (Fig. 3b).

2.4. Acyl-acceptor and donor specificities

The purified enzyme was used to determine acyl-acceptor and donor specificities as well as kinetic constants of

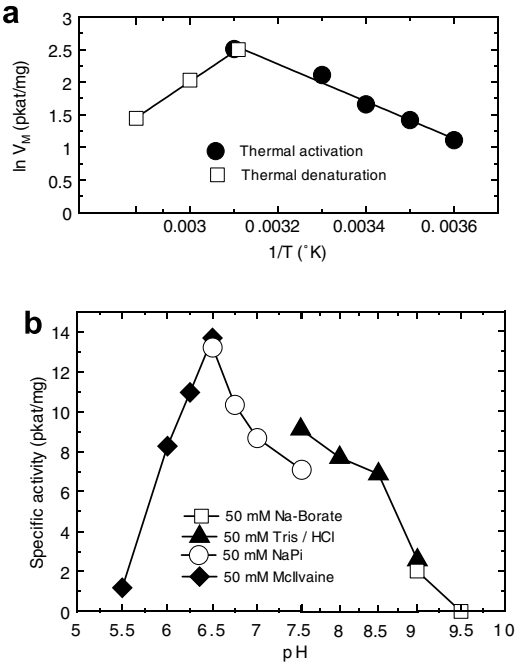


Fig. 3. (a) Thermal inactivation and activation of the *Dp*21MaT. (b) Effect of pH on *Dp*21MaT activity.

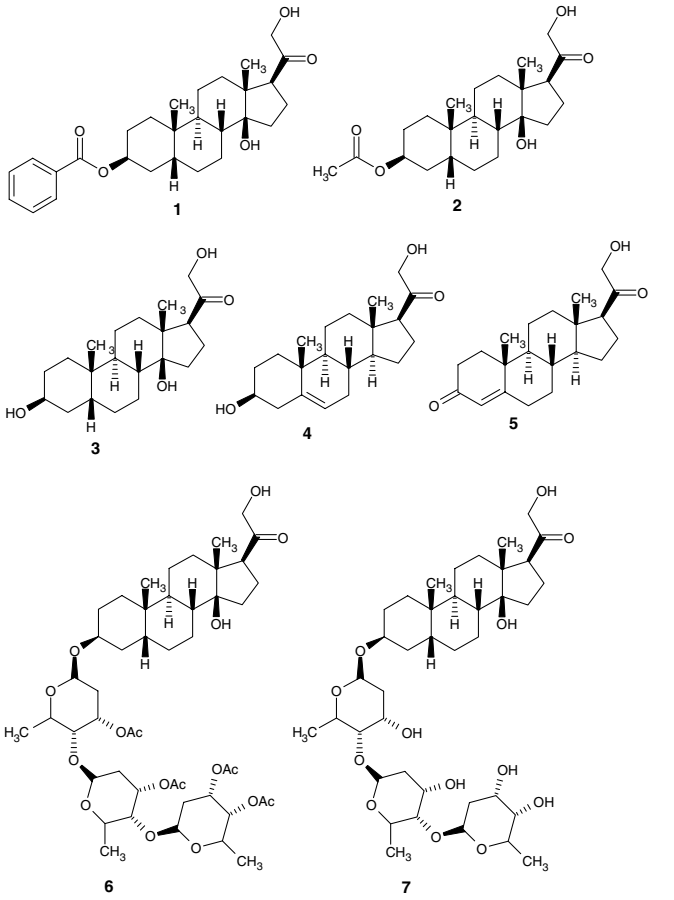


Fig. 4. Structures of 21-hydroxylated steroids that were examined as acyl-acceptors of *Dp*21MaT. (**1**) 3β-Benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one; (**2**) 3β-acetoxy-5β-pregnane-14β,21-dihydroxy-20-one; (**3**) 5β-pregnane-3β,4β,21-trihydroxy-20-one; (**4**) 3β,21-dihydroxypregn-5-en-20-one (21-hydroxypregnenolone); (**5**) 21-hydroxypregn-4-en-3,20-dion (21-hydroxyprogesterone); (**6**) 21-hydroxy ketone from digitoxin tretraacetate; (**7**) 21-hydroxy ketone obtained from digitoxin.

*Dp*21MaT. Apart from the standard acyl-acceptor 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one (**1**) we also examined the specificities of *Dp*21MaT toward several other substrates for their possible acceptance in standard assay conditions, namely 3β-acetoxy-5β-pregnane-14β,21-dihydroxy-20-one (**2**), 5β-pregnane-3β,4β,21-trihydroxy-20-one (**3**), 21-hydroxypregnenolone (**4**), 21-hydroxyprogesterone (**5**), 21-hydroxyketone prepared from digitoxin tretraacetate (**6**) and 21-hydroxyketone prepared from digitoxin (**7**) (Fig. 4). It was found that **5** was not accepted at all, whereas substrates **1**, **2** and **3** were accepted very well. Substrates **4**, **6**, **7** were malonylated albeit to a lesser extent (Table 3).

Apart from malonyl-CoA, other CoA esters like acetyl-CoA, benzoyl-CoA and acetoacetyl-CoA were tested as acyl-donors, but only malonyl-CoA served as an acyl-donor. In another set of experiments all of these compounds, as well as coenzyme A, were tested for their possible inhibitory effects, but only coenzyme A (0.4 mM) was found to impair the malonylation reaction, reducing it by 73%.

Table 3
Substrate specificity of *Dp*21MaT:acyl-acceptor

Substrate[a]	Relative activity[b] (%)
1	100
2	200
3	150
4	20
5	0
6	60
7	50

[a] Chemical structures of acyl-acceptors listed in this table are presented in Fig. 4.

[b] The specific activity seen with 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one and malonyl-CoA are taken to be 100%.

Kinetic studies using the standard substrate **1** together with malonyl-CoA as co-substrate were performed keeping the concentration of one substrate constant while varying the other. Hyperbolic plots indicating Michaelis–Menten kinetics were obtained. Hanes plots $[S]/v = f[S]$ (Fig. 5) of the data indicated K_m values of 99 and 28.44 μM for compound **1** and malonyl-CoA, respectively, and V_{max} values of 47.57 and 39.4 μkat kg^{-1} protein, respectively, for compound **1** and malonyl-CoA. An excess of **1** did not result in substrate inhibition, whereas concentrations of malonyl-CoA greater than 0.3 mM inhibited *Dp*21MaT.

2.5. Effect of divalent metals and ascorbic acid on the activity of *Dp21MaT*

Various divalent cations were tested for their possible inhibitory or stimulatory effect on the *Dp*21MaT activity.

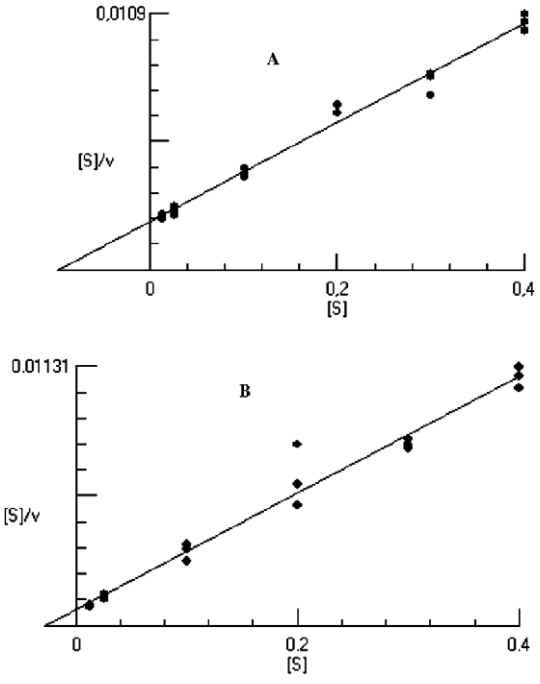


Fig. 5. Hanes plot used to determined K_m and V_{max} values for substrate 3β-benzoyloxy-5β-pregnane-14β,21-dihydroxy-20-one [a] and malonyl-CoA [b].

Ni^{2+}, Fe^{2+}, Cd^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} were added as their dichloride salts ranging in concentration from 0.1 to 5 mM. None of the ions tested influenced the enzyme activity significantly. Ni^{2+}, Ba^{2+} and Mg^{2+} seemed to slightly enhance *Dp*21MaT activity. Ascorbic acid also slightly promoted the activity.

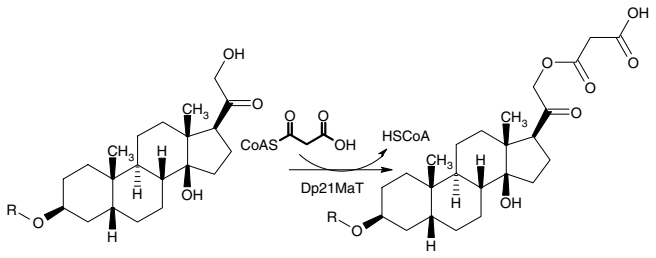
2.6. Stability of the enzyme

The enzyme stability was investigated at different temperatures and with various additives. Samples were stored either with glycerol 5% and β-mercaptoethanol 30 mM or without any additive at room temperature, +4, −20 or at −80 °C. The stability study was carried out in sodium phosphate buffer (50 mM, pH 6.5). Samples stored at −80 °C with glycerol and β-mercaptoethanol conserved considerable enzyme activity for more than 6 months. After 5 weeks only three of the samples still exhibited any enzyme activity, namely the samples stored at −20 and −80 °C without additives, as well as the above mentioned sample stored at −80 °C with glycerol and β-mercaptoethanol. Relative remaining activities were 60%, 80% and 95%, respectively. Samples stored in β-mercaptoethanol only lost their enzyme activity already after 3 days of storage at either −20 or −80 °C, whereas samples at RT and at +4 °C still showed 90% and 100% remaining activity. Except at RT, no significant change occurred between the first and the third week of storage. In summary, *Dp*21MaT can be stored for more than 6 months at −80 °C without loss of activity and it is stable for 3 weeks when stored at 4 °C in the presence of 30 mM β-mercaptoethanol and 5% glycerol.

3. Discussion

Enzyme purification requires careful selection of a suitable source for the enzyme (Ersson et al., 1989). The malonyltransferase *Dp*21MaT is abundant in leaves but not in suspension-cultured cells of *D. lanata* and *D. purpurea*. However, other cell suspensions have been successfully used as the starting material for the purification of enzymes involved in the cardenolide metabolism (Wendroth and Seitz, 1990; Warneck and Seitz, 1990; Finsterbusch et al., 1999). *Dp*21MaT activity in cell suspension might have been obscured by the presence of the highly active malonylesterase during screening of different putative sources of the enzyme. Attempts to separate *Dp*21MaT completely from the esterase have only recently been successful (Kuate, Hahn, Kreis, unpublished data). Young leaves of *D. purpurea* proved to be the best starting material for enzyme purification taking into account the abundance of *Dp*21MaT combined with a low malonylesterase activity.

The *Dp*21MaT catalyses the transfer of a malonyl moiety from malonyl-CoA to a 21-hydroxypregnane. This is supposed to initiate the butenolide ring formation, which



Scheme 1. Reaction catalysed by the malonyl-coenzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT).

is a specific reaction in all cardenolides (Scheme 1). Preliminary studies demonstrated that ammonium sulphate was highly detrimental to *Dp*21MaT activity (data not shown). The combination of dithiothreitol (DTT) and ascorbic acid used as antioxidants during the purification, especially in hydrophobic interaction chromatography, proved to be disadvantageous. Using only one of these agents at a time was less damaging to the enzyme activity which suggests the formation of an inhibitory complex between both compounds. Preliminary assays showed that most of the chlorophyll still present in the crude extract was pelleted between 0% and 20% saturation using $(NH_4)_2SO_4$ precipitation, whereas most of the *Dp*21MaT activity was found in the 20–60% ammonium $(NH_4)_2SO_4$ fraction. The purification procedure described here omits cation exchange chromatography. At pH 5.5 the enzyme was only partly adsorbed to a SP Sepharose matrix and at lower pH values an irreversible loss of activity was observed. The isoelectric point of *Dp*21MaT was 4.66, which corroborates the above observation that the enzyme could not be fully attached to the ion exchange resin at pH 5.5. Changes in the ionization state of some amino acid side chains in the enzyme do not contribute to the expression of the *Dp*21MaT activity. Amino acids, like histidine, glutamic acid and to some extent aspartic acid, where the side chain pK_a is 6.0, 4.25 and 3.65, respectively (Voet et al., 2006) may play an essential role in the catalytic activity of the enzyme (Suzuki et al., 2003).

The *Dp*21MaT is insensitive to salt addition up to 5 mM. Even Cu^{2+}, known to impair the activity of many transferases at low concentrations (Suzuki et al., 2001, 2002; Akermoun et al., 2002) did not affect *Dp*21MaT activity.

Acylation is a common and biochemically significant modification of plant secondary metabolites. BAHD acyltransferases constitute a large family of acyl CoA-utilizing enzymes involved in the formation of many plant secondary metabolites (St-Pierre and De Luca, 2000). To date, more than 45 acyltransferases of the BAHD family including 10 malonyltransferases have been genetically and biochemically characterized. They were all found to be monomeric enzymes with a molecular mass ranging from 48 to 55 kDa (D'Auria, 2006). Only the tomato fruit variety showed a lower molecular mass of 38 kDa (Martin and Saftner, 1995). Malonyltransferases have also been purified

from bacteria yielding molecular masses of around 35 kDa. *Dp*21MaT measuring only 27 kDa appears to be one of the smallest malonyltransferases described so far. At a glance it seems that many authors have investigated plant malonyltransferases. However, taking a closer look, it appears that the majority of them only concentrated on the biosynthesis of anthocyanins and flavonoids (Callebaut et al., 1996; Suzuki et al., 2001, 2002, 2003, 2004). Only few have looked at this type of enzyme in others metabolic pathways (Martin and Saftner, 1995; Revill et al., 1995; Liu et al., 2006).

4. Experimental

4.1. Chemicals

3β-Benzoyloxy-5β-pregnane-14β,21-diol-20-one (**1**), the substrate for malonyltransferase assay is not commercially available and was thus synthesized according to the methods of Euw and Reichstein (1964), Rabitzsch (1971) and Kuate et al. (unpublished). 3β-Acetoxy-5β-pregnane-14β,21-dihydroxy-20-one (**2**), 5β-pregnane-3β,4β,21-trihydroxy-20-one (**3**), 21-hydroxy ketone from digitoxin tretraacetate (**6**) and 21-hydroxy ketone obtained from digitoxin (**7**) were obtained in a similar way. Malonyl-coenzyme A as well as the compounds 3β,21-dihydroxypregn-5-en-20-one (21-hydroxypregnenolone) (**4**) and 21-hydroxypregn-4-en-3,20-dione (21-hydroxyprogesterone) (**5**) were purchased from Sigma (Deisenhofen, Germany) and dimethylsulfoxide from Serva (Heidelberg, Germany). Chromatography columns and media used throughout the purification were from Amersham Biosciences (Freiburg, Germany).

4.2. Plant material

D. lanata Ehrh., *D. purpurea* L., *I. canariensis* (L) Lindl. ex G. Don, *I. sceptrum* L., *G. glauca* Cav. were grown in the institute's greenhouse. Cell suspension cultures of *D. lanata* (strains K3OHD and W.1.4) and *D. purpurea* were provided by our tissue culture laboratory.

4.3. Standard enzyme assay for Dp21MaT

*Dp*21MaT activity was assayed routinely using the method described by Stuhlemmer and Kreis (1996). The assay contained 0.2 mM substrate in 96% ethanol, 0.2 mM malonyl-coenzyme A in DMSO and 240 μl of protein solution in a final volume of 250 μl. Samples denatured for 10 min at 100 °C served as controls. The reaction was run in 2 ml-tubes for 1 h at 42 °C under agitation (650 rpm) in a thermomixer (Eppendorf, Hamburg, Germany). At the end of the enzyme reaction 15 μl of testosterone (10 mM) were introduced as internal standard. Subsequently, the medium was acidified with an equal volume of citrate buffer (50 mM, pH 3.5) and immediately extracted with 1 ml of dichloromethane. The Eppendorf tubes were shaken thoroughly for 5 s and phase separation was facilitated by centrifugation (2 min at 13,000 × *g*) (Centriguge Biofuge 13; Heraeus, Nuremberg, Germany). The organic phase was dried under a stream of air in a dry block (Dry-Block DB 2A; Techne, Cambridge, England) and the residue was taken up in 60 μl methanol. This solution was either analysed by thin layer chromatography or by high performance liquid chromatography. For the former, the solution was spotted on silica gel 60 TLC F_{254} plates which were subsequently developed with dichloromethane:methanol:water (80:18:2). Pregnane spots were dyed with anisaldehyde reagent (3 min, 100 °C) (York, 1990), whereas the method recently described (Kuate et al., unpublished) was employed for HPLC analysis. The separation of substrate and product was achieved on Reprosil-Pur 120-C_{18}, particle size 5 μM, 4.6 × 250 mm). UV absorbance was measured at 195 and 228 nm. A gradient (acetonitrile: H_2O, adjusted to pH 2.5 with H_3PO_4) was used for the separation.

4.4. Purification of Dp21MaT

Every purification step was performed in a cold lab (+3 to 5 °C) unless otherwise stated. Äkta *prime* (Amersham Biosciences, Freiburg, Germany) equipment was employed for all column chromatography steps. Buffers used throughout the enzyme purification contained 30 mM β-mercaptoethanol. For the concentration of protein solutions Amicon© tubes 10 kDa (Millipore, Billerica, USA) were used. The amount of protein as well as enzyme activity were assessed after each purification step.

4.4.1. Preparation of the crude extract

Fresh leaves of *D. purpurea* (150 g), collected from the greenhouse, were washed thoroughly with tap water and dried with tissue paper. The central leaf veins were excised and leaves cut into small pieces and finally ground in a Waring blender (Waring, Torrington, USA) in the presence of 600 ml of Na–Pi buffer (50 mM, pH 7.0) and polyvinylpolypyrrolidone (Aug. Hedinger, Stuttgart, Germany) (20%, w/w). The slurry preparation was homogenized for 30 min and squeezed through four layers of cotton gauze Mullro® (Hartmann AG, Heidenheim, Germany). The filtrate was centrifuged at 20,000 × *g* for 20 min. The crude extract was finally filtered through two layers of Miracloth® (Calbiochem, La Jolla, USA). Protein concentration was determined with the method of Bradford (1976) using bovine serum albumin (BSA) as the protein standard.

4.4.2. Enzyme purification

$(NH_4)_2SO_4$ was added as a fine powder to the crude extract while stirring for 30 min in order to achieve 20% salt saturation. After centrifugation (20,000 × *g*, 20 min) the pellet was discarded, the concentration of $(NH_4)_2SO_4$ in the supernatant increased to 60% saturation and the solution then treated as described above. The resulting supernatant was discarded and the pellet obtained was dis-

solved in the 50 mM Na–Pi pH 7.0 containing 0.5 M $(NH_4)_2SO_4$ (buffer A) and centrifuged again. The supernatant was applied to a Phenylsepharose 6 FF column previously equilibrated with buffer A and washed with the same buffer. Proteins were eluted with 150 ml of decreasing $(NH_4)_2SO_4$ (0.5–0.0 M) in 0.02 M Na–Pi pH 7.0 (buffer B) followed by 225 ml of buffer B without $(NH_4)_2SO_4$. Fractions of 5 ml each were collected at a flow rate of 0.5 ml min^{-1}. Fractions were analysed by TLC and active fractions were pooled and concentrated.

The protein was exchanged into buffer C (20 mM Tris/HCl, pH 9.5). The solution was loaded onto column Source 30 Q equilibrated with buffer D (20 mM Tris/HCl, pH 9.5, 0.5 M NaCl) prior to equilibration with buffer C. The bound proteins were eluted with a linear gradient of buffer D at a flow rate of 0.7 ml min^{-1}. Fractions of 2.5 ml each were collected and checked for *Dp*21MaT activity. Pooled active fractions were concentrated and loaded onto the same column again, employing the same experimental conditions as described above.

The most active fractions were exchanged into buffer E (20 mM Na–Pi, pH 7.0) and applied to Cibacron Blue F3G-A FF column (5 ml) previously equilibrated with 20 mM Na–Pi, pH 7.0 containing 0.5 M NaCl (buffer F). Unbound proteins were washed with buffer E until the protein content approached zero. *Dp*21MaT was eluted with a gradient formed from buffers E and F: 0–6 ml (30% buffer F), isocratic until 10 ml, 10–20 ml (80% buffer F). Fractions of 1.0 ml each were collected and enzyme activity was checked.

The most active fractions were pooled, concentrated and loaded onto Superdex 75 (Amersham Biosciences, Freiburg, Germany), already equilibrated with Na–Pi buffer (20 mM, pH 7.0) containing 0.15 M NaCl. The enzyme was eluted using the same buffer. Fractions of 1 ml each were collected at a flow rate of 0.2 ml min^{-1}.

4.5. Enzyme characterization

Enzyme characterization was carried out using a 34-fold purified enzyme obtained from the second anion exchange chromatography on Source 30 Q. The isoelectric point (p*I*) of *Dp*21MaT was determined by isoelectric focusing on a Criterion™ IEF gel (pH 3–10) using the Criteron cell system. Gels 13.3 cm × 8.7 cm × 1.0 mm ($W \times L \times$ thickness) as well as the IEF Markers were from BioRad (Hercules, CA, USA). Buffers employed to determine the optimal pH were 50 mM McIlvaine for pH 5.5–6.5, 50 mM Na–Pi for pH 6.5–7.5, 50 mM Tris/HCl for pH 7.5–9.0 and 50 mM Na–Borate for pH 9–9.5. Buffer exchange was carried out with PD 10 columns (Amersham Biosciences, Freiburg, Germany). The concentrations of divalent cations tested were 0.1, 0.5, 1 and 5 mM. Assays without salt addition served as controls. Hyper32, a hyperbolic regression programme for the analysis of enzyme kinetic data (http://homepage.ntlworld.com/john.easterby/software.html) was used to determine kinetic constants.

4.6. SDS–polyacrylamide gel electrophoresis and isoelectric focusing

SDS–polyacrylamide gel electrophoresis (PAGE) was carried out using the BioRad mini PROTEAN 3 slab gel apparatus (BioRad, Richmond, CA, USA) and the Laemmli buffer system (Laemmli, 1970). Polyacrylamide gels of 0.75 mm thickness consisting of 10% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) were cast and samples of 17 μl (0.5–0.6 μg) of protein were loaded. The proteins were denatured by heating them to 100 °C in the presence of 2-mercaptoethanol for 3 min. Molecular weight markers (BioRad) ranging in size from 10 to 250 kDa were used as molecular weight standards. Gels were silver-stained according to Heukeshoven and Dernick, 1985. Native PAGE was treated similarly, only denaturing agents and conditions were avoided.

The isoelectric point (p*I*) of *Dp*21MaT was determined by isoelectric focusing on a Criterion™ IEF gel (pH 3–10). Gels 13.3 cm × 8.7 cm × 1.0 mm ($W \times L \times$ thickness), as well as the IEF Markers were from BioRad (Hercules, CA, USA). Isoelectric focusing was performed using the Protean IEF Cell and Criterion Precast System (BioRad) according to the instruction manual provided by the manufacturer. Using an electrophoresis power supply (ISCO Model 494, Lincoln, Nebraska, USA). The experiment was run at room temperature with a constant voltage step change of 100 V for 1 h, followed by 250 V for 1 h and finally focusing at 500 V for 30 min. The voltage was supplied by an electrophoresis power supply (ISCO Model 494, Lincoln, Nebraska, USA). The gel was silver-stained as described earlier.

Acknowledgements

S.P.K. and R.M.P. acknowledge the financial support from the Deutscher Akademischer Austauschdienst (DAAD). We thank Barbara White for linguistic advice.

References

Akermoun, M., Testet, E., Cassagne, C., Bessoule, J.J., 2002. Inhibition of the plastidial phosphatidylcholine synthesis by silver, copper, lead and mercury induced by formation of mercaptides with the lyso-PC acyltransferase. Biochim. Biophys. Acta 1581, 21–28.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Callebaut, A., Terahara, N., Decleire, M., 1996. Anthocyanin acyltransferases in cell cultures of *Ajuga reptans*. Plant Sci. 118, 109–118.

D'Auria, C.J., 2006. Acyltransferases in plants: a good time to be BAHD. Curr. Opin. Plant Biol. 9, 331–340.

Dec, G.W., 2003. Digoxin remains useful in the management of chronic heart failure. Med. Clin. N. Am. 87, 317–337.

Ersson, B., Rydén, L., Janson, J.-C., 1989. Introduction to protein purification. In: Janson, J.-C., Rydén, L. (Eds.), Protein Purification: Principles, High Resolution Methods and Applications. VCH Publishers, New York, pp. 3–32.

Euw, J.V., Reichstein, T., 1964. Die Biosynthese des Digitoxigenins, Herkunft des C-20. Helv. Chim. Acta 74, 711–724.

Finsterbusch, A., Lindemann, P., Grimm, R., Eckerskorn, C., Luckner, M., 1999. Δ^5-3β-Hydroxysteroid dehydrogenase/Δ^5-Δ^4-ketosteroid isomerase from *Digitalis lanata*. A multifunctional enzyme in steroid metabolism. Planta 209, 478–486.

Heukeshoven, J., Dernick, R., 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6, 103–112.

Jessup, M., Brozena, S., 2003. Hearth failure. N. Engl. J. Med. 348, 2007–2018.

Kreis, W., Hensel, A., Stuhlemmer, U., 1998. Cardenolide biosynthesis in foxglove. Planta Med. 64, 491–499.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 225, 680–685.

Liu, W., Han, C., Hu, L., Chen, K., Shen, X., Jiang, H., 2006. Characterization and inhibitor discovery of one novel malonyl-CoA:Acyl carrier protein transacylase (MCAT) from *Helicobacter pylori*. FEBS Lett. 580, 697–702.

Luber, E., 2002. Reinigung der Malonyl-coenzym A: 21-Hydroxypregnan 21-malonyltransferase und Versuche zur Isolierung einer Steroid-21-hydroxylase aus *Digitalis lanata* Ehrh. Doctorate Thesis, Universität Erlangen-Nürnberg.

Luckner, M., Wichtl, M., 2000. *Digitalis*: Geschichte, Biologie, Chemie, Physiologie, Molekularbiologie, Pharmakologie, Medizinische Anwendung. Wissenschaftliche Verlags-Gesellschaft, Stuttgart.

Martin, N.M., Saftner, R.A., 1995. Purification and characterization of 1-aminocyclopropane-1-carboxylic acid *N*-malonyltransferase from tomato fruit. Plant Physiol. 108, 1241–1249.

Pervaiz, M.H., Dickinson, M.G., Yamani, M., 2006. Is digoxin a drug of the past?. Cleveland Clin. J. Med. 73 821–824.

Rabitzsch, G., 1971. Ozon/pyridin-abbau der cardenolidtridigitoxoside digitoxin und digoxin zu isopregnanolontridigitoxosiden. Pharmazie 26, 592–597.

Revill, P.W., Bibb, M.J., Hopwood, D.A., 1995. Purification of a malonyltransferase from *Streptomyces coelicolor* A3(2) and analysis of its genetic determinant. J. Bacteriol. 177, 3946–3952.

Rising, S.E., Welch, J.M., Koo, J.M., 2006. Association of mortality risk with high serum digoxin concentrations. Hosp. Pharm. 41, 342–347.

St-Pierre, B., De Luca, V., 2000. Evolution of acyltransferase genes: origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism. In: John, R.I., Romeo, T., Varin, L., De Luca, V. (Eds.), Recent Advances in Phytochemistry. Evolution of Metabolic Pathways, vol. 34. Elsevier Science Publishing, Oxford, pp. 285–315.

Stuhlemmer, U., Kreis, W., 1996. Does malonyl-coenzyme A: 21-Hydroxypregnane 21-hydroxymalonyltransferase catalyse the first step in the butenolide ring of cardenolides? Tetrahedron Lett. 37, 2221–2224.

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Nakao, M., Tanaka, Y., Yamaguchi, M., Kumusi, T., Nishino, T., 2001. Malonyl-CoA:anthocyanin 5-*O*-glucoside-6′-*O*-malonyltransferase from scarlet sage (*Salvia splendens*) flowers. J. Biol. Chem. 272, 49013–49019.

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Yamaguchi, M., Tanaka, Y., Kumusi, T., Nishino, T., 2002. cDNA cloning, heterologous expressions and functional characterization of malonyl-coenzyme A:anthocyanidin 3-*O*-glucoside-6′-*O*-malonyltransferase from dahlia flowers. Plant. Physiol. 130, 2142–2151.

Suzuki, H., Nakayama, T., Nishino, T., 2003. Proposed mechanism and functional amino acid residues of malonyl-coA:anthocyanin 5-*O*-glucoside-6′-*O*-malonyltransferase from flowers of *Salvia splendens*, a member of the versatile plant acyltransferase family. Biochemistry 42, 1764–1771.

Suzuki, H., Nakayama, T., Nagae, S., Yamaguchi, M., Iwashita, T., Fukui, Y., Nishino, T., 2004. cDNA cloning and functional characterization of flavonol 3-*O*-glucoside-6′-*O*-malonyltransferase from flowers of *Verbena hybrida* and *Lamium purpureum*. J. Mol. Catal. B: Enzym. 28, 87–93.

Voet, D., Voet, J.G., Pratt, C.W., 2006. Fundamentals of Biochemistry, 2nd ed. John Wiley & Sons, Hoboken, USA.

Warneck, H.M., Seitz, H.U., 1990. 3β-Hydroxysteroid oxidoreductase in suspension cultures of *Digitalis lanata* Ehrh.. Z. Naturforsch. 45c, 963–972.

Wendroth, S., Seitz, H.U., 1990. Characterization and localization of progesterone 5α-reductase from cell cultures of foxglove (*Digitalis lanata* Ehrh.). Biochem. J. 266, 41–46.

Withering, W., 1785. An Account of the Floxglove and Some of its Medical Uses with Practical Remarks on Dropsy and Other Diseases. M. Swinney, Birmingham, UK.

York, H., 1990. Thin layer chromatography: reagents and detection methods. In: Jork, H., Funk, W., Fischer, W., Wimmer, H. (Eds.), Thin-Layer-Chromatography, vol. 1. VCH Publishers, New York, pp. 195–198.