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Requirement of Catalytic-Triad and Related Amino Acids for the Acyltransferase Activity of *Tanacetum cinerariifolium* GDSL Lipase/Esterase TcGLIP for Ester-Bond Formation in Pyrethrin Biosynthesis

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We have recently discovered that a GDSL lipase/esterase (TcGLIP) in *Tanacetum cinerariifolium* catalyzed acyltransferase activity to form an ester bond in the natural insecticide, pyrethrin. TcGLIP contained Ser40 in Block I, Gly64 in Block II, Asn168 in Block III and Asp318 and His321 in Block V, suggesting underlying hydrolase activity, although little is known about their role in acyltransferase activity. We expressed TcGLIP here in *Escherichia coli* as a fusion with maltose-binding protein (MBP), part of the fusion being cleaved with a protease to obtain MBP-free TcGLIP. A kinetic analysis revealed that the MBP moiety scarcely influenced the kinetic parameters. The effects on acyltransferase activity of mutations of Gly64, Asn168, Asp318 and His321 were investigated by using MBP-fused TcGLIP. Mutations of these amino acids markedly reduced the acyltransferase activity, suggesting their critical role in the production of pyrethrins.

Key words: acyltransferase activity; GDSL lipase; pyrethrin; *Tanacetum cinerariifolium*

Pyrethrins are natural insecticides that are biosynthesized by *Tanacetum cinerariifolium* in the Asteraceae family.[1)] Pyrethrins consist of six esters: pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I and jasmolin II, resulting from the esterification of two acid moieties (chrysanthemic acid and pyrethric acid) with three alcohol moieties (pyrethrolone, jasmololone and cinerolone). Pyrethrins act by keeping voltage-sensitive sodium channels open, thereby inducing hyperexcitation, conduction blocking, or both, in the nervous system.[2)] In respect of their selective neurotoxicity, pyrethrins are used for household and pet pest control, and also for agricultural pest control. However, since pyrethrins are readily degraded in the field and their production is climate-sensitive, synthetic analogs (pyrethroids) with longer persistency and higher potency have been developed. Pesticide research has consequently been redirected from pyrethrins to pyrethroids, and the biochemistry of pyrethrins remains elusive. We therefore examined the biosynthetic pathway to pyrethrins by using [1-^{13}C] D-glucose as the precursor. We found that the acid moiety was biosynthesized by the non-mevalonate pathway, whereas the alcohol moiety was biosynthesized by the oxylipin pathway, sharing part of the pathway with the plant hormone, jasmonic acid.[3)]

The acid and alcohol moieties in pyrethrin biosynthesis are biosynthesized by different pathways which join to yield pyrethrins in the final step. Since an acyltransferase was postulated to catalyze this esterification step (Fig. 1A), chrysanthemoyl CoA and (*S*)-pyrethrolone were added to a crude enzyme from the flower bud, resulting in the production of pyrethrin I. Purifying this enzyme and cloning the gene unexpectedly led to the discovery that the isolated enzyme showed similarities to GDSL lipases/esterases which have been named after the Gly-Asp-Ser-Leu motif.[4)] This family consists of many members with such diverse functions as ceramidase,[5)] sinapine esterase,[6)] transesterification,[7)] cutin synthesis[8)] and cutinase activities[9)] in plants.[10)] We confirmed that the recombinant enzyme expressed in *Escherichia coli* as a fusion with maltose-binding protein (MBP) showed acyltransferase activity for pyrethrin synthesis. The enzyme displayed high substrate specificity for the acid and alcohol moieties, recognizing the absolute configuration of the three asymmetric carbons in the substrates. Gene expression was also highly correlated with the pyrethrin content in *T. cinerariifolium*, suggesting that the acyltransferase, referred to as TcGLIP, underlaid the ester-bond formation in pyrethrin biosynthesis.[4)]

GDSL lipases/esterases generally contain serine in Block I, glycine in Block II and asparagine in Block III, as well as aspartate and histidine in Block V as the amino acids involved in hydrolase activity.[10,11)] Histidine in Block V deprotonates serine in Block I with the assistance of aspartate in Block V, and activated serine attacks the carbonyl carbon of substrates in the "cata-

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Abbreviations: TcGLIP, *Tanacetum cinerariifolium* GDSL lipase/esterase; MBP, maltose-binding protein; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

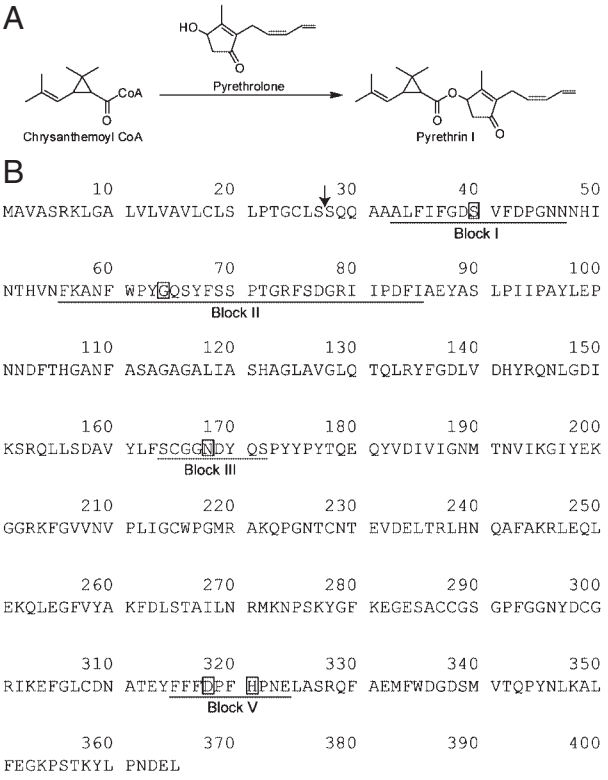

Fig. 1. Acyltransferase Activity for Pyrethrin I Synthesis (A) and Primary Structure of TcGLIP (B).

Ser40 in Block I, Gly64 in Block IIA, Gly68 in Block IIB, Asn168 in Block III, and Asp318 and His321 in Block V are enclosed by squares (amino acid numbers are counted from Met1). The signal peptide cleavage site is indicated by an arrow.

lytic triad." Backbone NH of serine in Block I and that of glycine in Block II, as well as the amide side chain of asparagine in Block III serve as oxyanion acceptors.[11] All these structural features are preserved as Ser40, Gly64, Asn168, Asp318 and His321 in TcGLIP (Fig. 1B). A GDSL lipase-like acyltransferase has been found in tomato to exhibit glucarate caffeoyltransferase activity for phenylpropanoid synthesis. In respect of this enzyme, mutations of the catalytic-triad serine in Block I, as well as of the aspartate and histidine in Block V had only a small impact on the activity.[7] However, this may not be the case for the pyrethrin-forming acyltransferase activity of TcGLIP, since we have observed that the S40A mutation eliminated the activity.[4]

We prepared in this present study MBP-fused and MBP-free TcGLIPs to examine the effects of MBP on the acyltransferase activity. Having observed no significant impact of the MBP moiety on the activity, we investigated the effects of mutations on the acyltransferase activity of several amino acids that are presumably involved in hydrolase activity.

Materials and Methods

Chemicals. The substrates, (1*R*,3*R*)-chrysanthemoyl CoA, (1*R*,3*R*)-pyrethroyl CoA and (*S*)-pyrethrolone, were synthesized and their chemical structures were confirmed as described in the supplementary information of Ref. 3.

Site-directed mutagenesis and enzyme preparation. The cDNA of wild-type and mutant TcGLIPs (GenBank accession no. JN418994) cloned in the pMAL-c4E vector (New England Biolabs, Ipswich, MA, USA) was expressed in *E. coli* as previously described.[4] When preparing the cDNA vector, the enterokinase cleavage site (DDDDK) was replaced by the PreScission Protease (GE Healthcare Life Sciences, Uppsala, Sweden) cleavage site (LEVLFQGP) to facilitate removal of the MBP and protease. The mutant cDNAs were prepared from wild-type cDNA by using a QuikChange kit (Agilent Technologies, Santa Clara, CA, USA) with the primers listed in Table 1, according to the manufacturer's protocol. The entire DNA sequences of the mutant cDNAs were determined by automated sequencing with a 3100 genetic analyzer (Life Technologies, Carlsbad, CA, USA).

Table 1. DNA Primers for Generating Mutant cDNAs of TcGLIP

Mutation	Primer	Sequence (5′-3′)
G64A	Forward	CTTTTGGCCATATGCTCAATCCTACTTCAG
	Reverse	CTGAAGTAGGATTGAGCATATGGCCAAAAG
G78A	Forward	GATTCTCTGATGCCCGTATCATCCCTG
	Reverse	CAGGGATGATACGGGCATCAGAGAATC
N168A	Forward	TAGCTGTGGAGGTGCCGACTACCAAAGCCC
	Reverse	CTTTGGTAGTCGGCACCTCCACAGCTAAAC
D318A	Forward	GTATTTTTTCTTTGCCCCTTTTCATCCTAATG
	Reverse	GGATGAAAAGGGGCAAAGAAAAAATAC
H321A	Forward	CTTTGACCCTTTTGCTCCTAATGAATTG
	Reverse	CCAATTCATTAGGAGCAAAAGGGTCAAAG

The wild-type and mutant cDNAs were expressed in *E. coli* BL21 (DE3) pLysS and purified with Amylose Resin (New England Biolabs) to obtain the recombinant TcGLIP as an MBP-fused protein. We confirmed that the wild-type MBP-fused TcGLIP purified with Amylose Resin always showed acyltransferase activity. The wild-type and N168A mutant were further purified by using an ÄKTAexplorer 10S with a Mono Q 5/50 GL column (GE Healthcare Life Sciences) to determine their specific activity, whereas the other mutants were not purified with the Mono Q column because they had no acyltransferase activity, as described later. The purity was checked by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the protein bands were stained by the Coomassie Brilliant Blue reagent, using a SimplyBlueSafeStain kit (Life Technologies).

Some of the Mono-Q-purified MBP-fused TcGLIP was treated at 4 °C with PreScission Protease (GE Healthcare Life Sciences) in a 50 mM Tris–HCl buffer (pH 7.5) to cleave the MBP–TcGLIP linkage. The digest was purified with a mixture of Amylose Resin and Glutathione Sepharose 4B (GE Healthcare Life Sciences) at a ratio of 2:1 to remove the MBP and the glutathione *S*-transferase-tagged protease, and to obtain MBP-free TcGLIP as an unabsorbed protein in the 50 mM Tris–HCl buffer (pH 7.5).

Enzyme assay. The enzyme assay for pyrethrin synthesis was conducted in a 100-μL solution consisting of (1*R*,3*R*)-chrysanthemoyl CoA, (*S*)-pyrethrolone and TcGLIP as indicated in the Results section in 50 mM Tris–HCl (pH 7.5). Kinetic analyses were conducted by fixing the concentration of one substrate, while that of the other substrate was increased. The reaction mixture was incubated for 10 min at 25 °C and stopped by adding 10 μL of acetic acid. The reaction mixture was extracted with 100 μL of hexane, and 10 μL of the extract was subjected to an HPLC analysis of pyrethrin I, using a Class VP system (Shimadzu, Kyoto, Japan) at 230 nm with a Cadenza CD-C18 column (4.6 × 100 mm; Imtakt, Kyoto, Japan) and an acetonitrile–water mixture (4:1) at a flow rate of 1 mL min^{-1}.

Results

Effects of MBP on the kinetics of pyrethrin synthesis

Wild-type TcGLIP was expressed in *E. coli* as an MBP fusion protein and purified in a Mono Q column to yield a single band by 10% SDS–PAGE (Fig. 2). We measured the specific activity of MBP-fused wild-type TcGLIP in the presence of 1 mM (*S*)-pyrethrolone with various concentrations of (1*R*,3*R*)-chrysanthemoyl CoA to determine the kinetic parameters for the CoA

Table 2. Kinetic Parameters of the Acyltransferase Activity of TcGLIP Determined in the Presence and Absence of the MBP Moiety

Substrates (TcGLIP status)	K_m (μM)	V_{max} [nkat (mg protein)$^{-1}$]	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Chrysanthemoyl CoA (MBP-fused)	751 ± 101	1.53 ± 0.08	0.125 ± 0.007	167
Pyrethrolone (MBP-fused)	29.5 ± 2.0	1.17 ± 0.03	0.095 ± 0.002	3230
Chrysanthemoyl CoA (MBP-free)	832 ± 105	3.24 ± 0.17	0.123 ± 0.007	147
Pyrethrolone (MBP-free)	33.7 ± 3.5	2.63 ± 0.09	0.100 ± 0.004	2960

Data were determined by 250 ng of protein at 25 °C and pH 7.5, and are indicated as the mean ± standard error of the mean (n = 3).

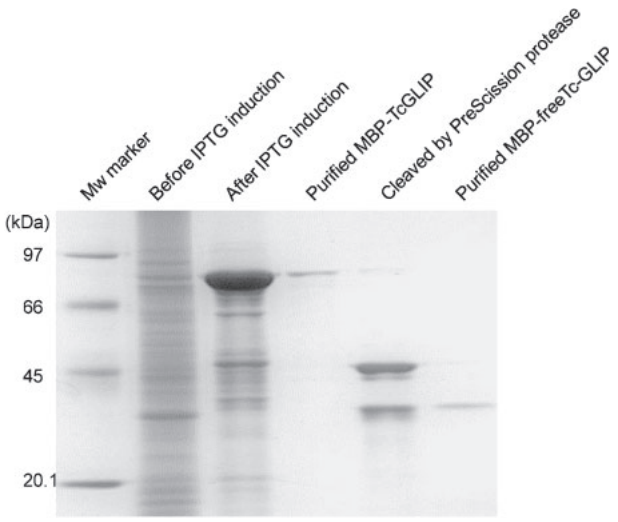

Fig. 2. 10% SDS–PAGE Images for Recombinant TcGLIP Before and After Cleavage by PreScission Protease.
Each sample is indicated above the gel picture.

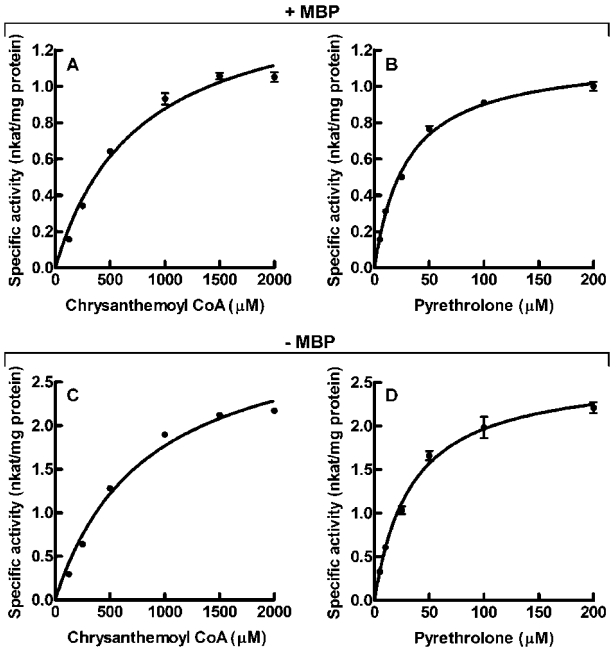

Fig. 3. Specific Acyltransferase Activity for Pyrethrin I Synthesis of Recombinant MBP-Fused and MBP-Free TcGLIP Saturated with an Increase in Substrates at a Constant Temperature of 25 °C.
(A) Specific activity of MBP-TcGLIP saturated with an increase of (1*R*,3*R*)-chrysanthemoyl CoA in the presence of 1 mM (*S*)-pyrethrolone. (B) Specific activity of MBP-TcGLIP saturated with an increase of (*S*)-pyrethrolone in the presence of 2 mM (1*R*,3*R*)-chrysanthemoyl CoA. (C) Specific activity of MBP-free TcGLIP saturated with an increase of (1*R*,3*R*)-chrysanthemoyl CoA in the presence of 1 mM (*S*)-pyrethrolone. (D) Specific activity of MBP-free TcGLIP saturated with an increase of (*S*)-pyrethrolone in the presence of 2 mM (1*R*,3*R*)-chrysanthemoyl CoA. Data are plotted as the mean ± standard error of the mean (n = 3).

substrate (Fig. 3A). A non-linear regression analysis of the isotherm resulted in respective K_m and V_{max} values of 751 ± 101 μM and 1.53 ± 0.08 nkat (mg of protein)$^{-1}$ (n = 3, mean ± standard error of the mean) (Table 2). These values and the molecular weight (81,689 Da) of MBP-fused TcGLIP were used to calculate the respective k_{cat} and k_{cat}/K_m values for (1*R*,3*R*)-chrysanthemoyl CoA of 0.125 ± 0.007 s^{-1} and 167 s^{-1} M^{-1} (Table 2). Similarly, the K_m, V_{max}, k_{cat} and k_{cat}/K_m values for (*S*)-pyrethrolone were also respectively determined in the presence of 2 mM (1*R*,3*R*)-chrysanthemoyl CoA by fitting the isotherm (Fig. 3B) to be 29.5 ± 2.0 μM, 1.17 ± 0.03 nkat (mg of protein)$^{-1}$, 0.095 ± 0.002 s^{-1} and 3230 s^{-1} M^{-1} (Table 2).

We cleaved MBP-fused TcGLIP by PreScission Protease and purified the digest with an Amylose Resin and Glutathione Sepharose 4B resin mixture, resulting in a single band of MBP-free TcGLIP with a molecular weight of about 40 kDa by SDS–PAGE (Fig. 2). We determined the kinetic parameters for the MBP-free enzyme by fitting the isotherm shown in Fig. 3C and D. The K_m (832 ± 105 μM), k_{cat} (0.123 ± 0.007 s^{-1}) and k_{cat}/K_m values (147 s^{-1} M^{-1}) for (1*R*,3*R*)-chrysanthemoyl CoA resembled those obtained for the MBP fusion. The K_m (33.7 ± 3.5 μM), k_{cat} (0.100 ± 0.004 s^{-1}) and k_{cat}/K_m (2960 s^{-1} M^{-1}) values for (1*S*)-pyrethrolone were also similar to those obtained for MBP-fused TcGLIP (Table 2).

Effects of site-directed mutagenesis on acyltransferase activity for the production of pyrethrin I

Since the MBP moiety had no significant effect on the kinetic parameters of the acyl and alcohol substrates and it was easier to prepare the MBP-fused protein than the MBP-free protein as already described, we employed MBP-fused TcGLIP to examine the effects on acyltransferase activity of the mutations to alanine of Gly64 in Block II, Asn168 in Block III, and Asp318 and His321 in Block V which have been proposed as underlying the hydrolase activity of GDSL-lipases/esterases.[11] When assayed with 2 mM (1*R*,3*R*)-chrysanthemoyl CoA and 1 mM (*S*)-pyrethrolone as substrates, the G64A, D318A and H321A mutants did not show any detectable acyltransferase activity (Fig. 4), as was the case for the S40A mutant.[4] Of those tested, only the N168A mutant showed acyltransferase activity, although its level was 18.8% of that of the wild-type enzyme (Fig. 4).

Discussion

We expressed in this study MBP-fused TcGLIP in *E. coli* and digested part of the MBP-fused enzyme with PreScission Protease to prepare a soluble form of MBP-

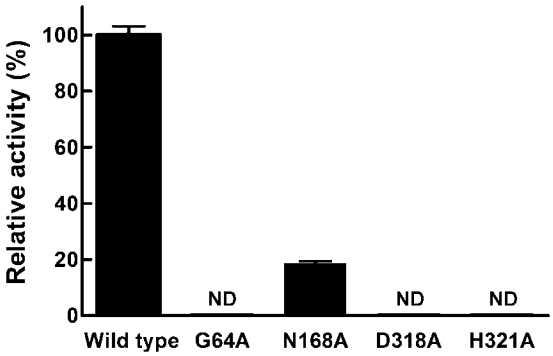

Fig. 4. Effects of Mutations of the Catalytic Triad-Forming Amino Acids on the Acyltransferase Activity of Recombinant TcGLIPs Expressed in *E. coli*.

Bar graphs indicate the mean ± standard error of the mean (n = 3). ND, no detectable activity.

free TcGLIP; we then compared the kinetic parameters for the pyrethrin I-generating acyltransferase activity of the MBP-fused and MBP-free enzymes. (*S*)-Pyrethrolone had 25-fold higher affinity than (1*R*,3*R*)-chrysanthemoyl CoA in terms of the K_m value previously reported, whereas the k_{cat} value of the acyl group donor was about 1.32-fold higher than that of the alcohol (Table 2). The catalytic efficiency (k_{cat}/K_m) for the alcohol moiety eventually became greater than that for the acyl moiety (Table 2), suggesting the rate-limiting substrate to be the acyl donor in the production of pyrethrin I. A comparison of the kinetic parameters for TcGLIP determined in the presence and absence of MBP (Table 2) led to the finding that the MBP moiety had no significant effect on catalytic activity.

The role of "SGNH" amino acids has also been studied for tomato (*Solanum lycopersicum*) glucarate caffeoyltransferase underlying phenylpropanoid biosynthesis. The D328A and H331A mutations, and also even the S27A mutation, only had a small impact on the glucarate caffeoyltransferase activity.[7] In the case of TcGLIP, however, mutations of the corresponding amino acids markedly reduced the pyrethrin-synthesizing acyltransferase activity. The results and our previous observation of the S40A mutation-induced complete loss of hydrolase activity[4] appear to suggest that the acyltransferase and hydrolase activities of TcGLIP shared a common mechanism. Nevertheless, we cannot rule out the possibility that some mutations may change the conformation of the active site, thereby eliminating the activities. Further investigations are therefore needed to better understand the underlying mechanisms involved.

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