

Determination and Isolation of a Thioesterase from Passion Fruit (*Passiflora edulis* Sims) That Hydrolyzes Volatile Thioesters

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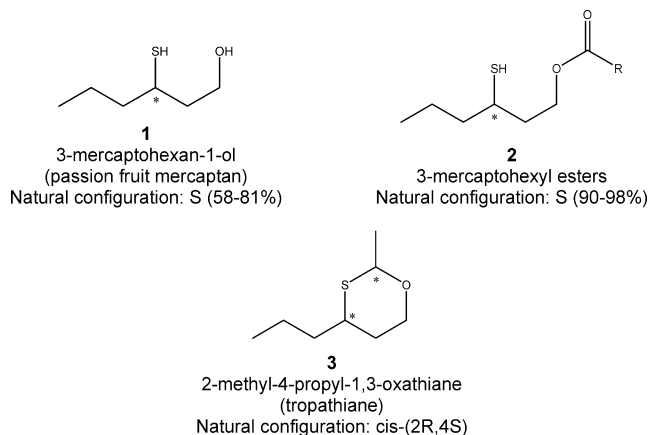
Volatile organosulfur compounds (VOSCs) are high impact aroma chemicals characteristic of tropical fruits which are active as both free thiols and the respective thioesters. Using a simple and sensitive colorimetric enzyme assay, a thioesterase activity toward VOSCs has been identified in ripening purple passion fruit (*Passiflora edulis* Sims). The assay was based on determining the release of free thiols from 2-methyl-3-furanthiol acetate using Ellman's reagent. The major thioesterase in the fruit was found to be a wall-bound protein in the mesocarp. The extracted enzyme activity was purified 150-fold and shown to be associated with a 43 kDa monomeric serine hydrolase which was selectively labeled with a fluorophosphonate suicide probe. MS-MS sequencing identified the thioesterase as a class 13 glycoside hydrolase, most similar to pectin acetyltransferase, an enzyme involved in cell wall modifications in the peel of a number of fruit. Our results suggest that cell wall hydrolases in tropical fruit may have additional useful roles in biotransforming VOSCs.

KEYWORDS: Cell wall hydrolase; Ellman's reagent; microbial lipase; porcine liver esterase; pectin acetyltransferase

INTRODUCTION

Plants can synthesize a diverse range of volatile natural products, which we know as perfumes and flavors, from both floral and vegetative tissues (1). This is particularly well demonstrated by the rich exotic tastes and aromas found in the fruits of tropical plants, with many of the associated high impact aroma chemicals derived from volatile organosulfur compounds (VOSCs) (2). For example, in purple passion fruit (*Passiflora edulis* Sims), 3-mercaptohexan-1-ol (1) and its derivatives (2, 3) (Figure 1) provide the characteristic tropical notes (3, 4). In addition to having unique odor characteristics, VOSCs are extremely active, being detectable at concentrations lower than ten parts per billion (10 μ g/L) in water (5). Thus, while VOSCs are extremely important in determining flavor and fragrance characteristics of tropical fruits, these compounds are often produced in only trace quantities. As such, the biosynthetic pathways leading to their biogenesis have proven difficult to study. Recently the identification of a cysteine conjugate of 3-mercaptohexan-1-ol (1) in passion fruit has suggested a biosynthetic route for the generation of such VOSCs routed through the *S*-glutathionylation of reactive alkenals arising from

fatty acid oxidation (6). The VOSCs are then released from the respective *S*-cysteinyl conjugated catabolites through the action of a C–S β lyase, with an analogous mechanism for the incorporation of sulfur into natural products proposed for the generation of allyl sulfides in *Allium* species (6–8). The VOSCs can then undergo either direct release, or metabolism to more stable derivatives.



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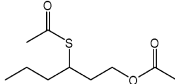
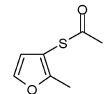
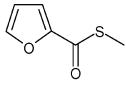
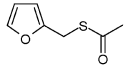
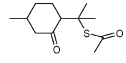

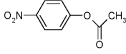
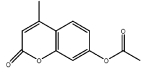
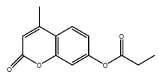
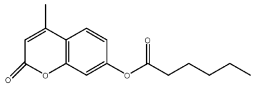
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R: CH₃, C₃H₇, C₅H₁₁

*: Chiral centre

Figure 1. Examples of the passion fruit VOSC 3-mercaptohexanol and its derivatives.

Table 1. Specific Activities (nkat/ mg protein) of the Enzymatic Hydrolysis of Thioester and Carboxylester Substrates by Passion Fruit (Crude Protein and Partially Purified Thioesterase Preparation), Porcine Liver Esterase, and the Lipase from *Candida rugosa*^a

Compound number	Thioester/ ester substrates	Crude passion fruit extract (S.a. nkat/mg)	Pure passion fruit thioesterase (S.a. nkat/mg)	Porcine liver esterase (S.a. nkat/mg)	<i>Candida rugosa</i> lipase (S.a. nkat/mg)
4	 3-(acetylthio) hexylacetate	0.04 +/- 0.002	1.12 +/- 0.02	14.02 +/- 0.72	ND
5	 2-methyl -3-furanthiol acetate	0.97 +/- 0.003	149.38 +/-0.0021	537.88 +/- 24.65	0.35 +/- 0.02
6	 Methyl thiofuroate	ND	ND	100.0 +/- 9.75	ND
7	 Furfuryl thioacetate	0.24 +/- 0.04	10.06 +/-	35.04 +/- 1.98	0.05 +/- 0.01
8	 8-acetylthio-p-menthan-3-one	0.13 +/- 0.02	8.84 +/- 0.08	4.17 +/- 0.02	0.02 +/- 0.01
9	 α -Naphthyl acetate	0.26 +/- 0.9	4.16 +/- 0.72	159.4 +/- 16.14	0.58 +/- 0.01
10	 P-Nitrophenyl acetate	0.67 +/- 0.10	11.39 +/- 1.2	2117.6 +/- 87.5	5.14 +/- 0.23
11	 4-Methylumbelliferyl acetate	0.04 +/- 0.003	0.66 +/- 0.08	426.15 +/- 72.55	4.26 +/- 0.18
12	 4-Methylumbelliferyl propanoate	0.03 +/- 0.008	0.57 +/- 0.09	1589.26 +/- 102	118.36 +/- 21.39
13	 4-Methylumbelliferyl hexanoate	0.0005 +/- 0.0001	0.006 +/- 0.0004	1302.97 +/- 97	36.59 +/- 4.00

^a The 8-acetylthio-p-menthanone was assayed as a mixture of *E/Z* isomers. Activities are means \pm SDs ($n = 3$). ND; no activity detected. Boiled protein samples (95 °C, 5 min) were used for the determination of chemical hydrolysis, which was corrected for in the enzyme specific activities shown.

One important set of VOSCs are the respective thioesters, with the acetyl, butyl and hexyl derivatives of 3-mercaptohexyl acetate all associated with the flavor and fragrance of tropical fruits (5). Based on their ability to act in a controlled stereospecific manner, there has been interest in identifying hydrolases that catalyze the cleavage of thioesters to release optically active VOSCs (9, 10). These studies have tended to use microbial lipases and esterases and as such, little attention has been focused on the endogenous thioesterases that might catalyze these reactions in tropical fruits. With an interest in studying

enzymes involved in biotransforming tropical fruit VOSCs we now report on the purification and identification of an enzyme from purple passion fruit that selectively hydrolyzes thioesters using a simple and robust colorimetric assay.

MATERIALS AND METHODS

Chemicals and Plant Material. Thioester substrates (Tables 1 and 2; compounds 4–8) and the respective free thiols were obtained from Oxford Chemicals Limited (Hartlepool, U.K.).

Table 2. Sequencing and Identification of the Candidate 43 kDa Enriched Thioesterase^a

peptide fragment	database hit (score/E value)	identification	predicted molecular mass (kDa)	accession
LPLTLVQSAVAR (12 amino acids)	LTLVQSAVAR LTL+QSA A+ LTLIQSAAAK <i>Arabidopsis thaliana</i> (23.5/47)	carboxylic ester hydrolase (member of pectin acylesterase family)	43.0	NP 176072.3
AVLDPMLEK (9 amino acids)	AVLDPMLE AV++PMLE AVVEPMLE <i>Brassica juncea</i> (21.8/129)	methyl transferase	43.9	AAV52268.1
LAPSAVD (7 amino acids)	LAPSAVD LAP+AVD LAPTAVD <i>Arabidopsis thaliana</i> (21.0/216)	pectinacylesterase, putative (member of pectin acylesterase family)	42.0	NP 567585.1
NVGLGNPSR (10 amino acids)	GNSPSR GNSPSR GNSPSR <i>Lactuca sativa</i> (21.8/436)	pectinacylesterase (member of pectin acylesterase family)	43.7	AAP72959.1
TFGFAWLGGK (10 amino acids)	FAWLGGK FAWLK FAWLGTK <i>Arabidopsis thaliana</i> (21.0/234)	alliinase C-terminal domain-containing protein	44.7	NP 177213.1

^a Database hits obtained from MS-MS sequencing of tryptic digests of the purified PeWH. For each peptide, the top BLAST hits are presented for known or putative proteins. Both score and expect values are shown to highlight the significance of the match.

Carboxylesters (**Table 1**; compounds **9–13**), porcine liver esterase and the lipase from *Candida rugosa* were obtained from Sigma-Aldrich. A trifunctional probe bearing a fluorophosphonate suicide inhibitor of serine hydrolases linked to a rhodamine dye and biotin was synthesized as described previously (11). Purple passion fruit (*Passiflora edulis* Sims) were obtained from commercial sources (H2H wholesalers, Newcastle-Upon-Tyne, U.K.) and allowed to ripen in a 20 °C constant temperature room to a water content of 70% (w/w). The water content served as a means of determining fruit maturity and was calculated from representatives ($n = 20$) of each batch of fruit analyzed by drying the fruit at 37 °C to a constant dry weight.

Enzyme Extraction and Purification. The peel and flesh of the fruit was separated, weighed and the peel homogenized in a blender on ice in 5 v/w 0.1 M potassium phosphate buffer pH 7.2 containing 0.25 M NaCl, 2 mM EDTA, 1 mM dithiothreitol, and 2% (w/v) polyvinylpyrrolidone. The slurry was stirred at 4 °C for 2 h, prior to filtration through Miracloth (Calbiochem Novabiochem, Nottingham, U.K.). The filtrate was centrifuged (10,000 g, 15 min), ammonium sulfate added and the fraction precipitating between 40–80% saturation recovered by recentrifugation. Following overnight dialysis against 20 mM potassium phosphate buffer pH 7.2, the 40–80% fraction was applied onto a column (41 mL) of DEAE-Sepharose (GE Healthcare) at a flow rate of 4 mL/min. After washing off unbound material, proteins were eluted with a linearly increasing gradient of NaCl (0–0.5 M; total vol = 200 mL), with the UV absorbance monitored at 280 nm and 8 mL fractions assayed for thioesterase activity. Retained active fractions were pooled and ammonium sulfate added to a final concentration of 1M, prior to loading onto a column (10 mL) of octyl-Sepharose (GE Healthcare) at a flow rate of 1 mL/min. Protein was eluted with a linearly decreasing concentration of ammonium sulfate (1.0–0 M; total vol 50 mL) with 2 mL fractions collected and assayed for thioesterase activity. Active fractions were pooled and

ammonium sulfate added (to 1 M), prior to loading onto a 0.69 mL high performance phenyl-Superose column (GE Healthcare) at 0.5 mL/min. Protein was eluted with a linearly decreasing gradient of ammonium sulfate 1.0–0 M; total vol = 20 mL) with 1 mL fractions collected. The polypeptide content of the active fractions was monitored by SDS-PAGE and active serine hydrolases present labeled with the biotinylated fluorophosphonate suicide probe (2 μ M) as described previously (11). The molecular mass of the native enzyme was determined by gel filtration chromatography using a precalibrated column (12).

Enzyme Assay. Protein samples in 0.1 M potassium phosphate buffer pH 7.2 were incubated with 1 mM thioester/ ester substrates at 30 °C. Carboxylesterase activity was determined using the colorimetric and fluorescent assays described previously (11, 12). Thioesterase activity was determined by adding an equal volume of 4 mg/mL Ellman's reagent (dithio-bis(2-nitrobenzoic acid)) dissolved in 0.1 M potassium phosphate buffer pH 7.2 to the assay at timed intervals. After a 1 min incubation, the absorbance was determined at 412 nm ($\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). All assays were run in triplicate, with boiled protein samples used to correct for nonenzymic hydrolysis. To confirm the formation of hydrolyzed thiols, thioesterase assays were run in parallel without the addition of dithio-bis(2-nitrobenzoic acid) and reaction products partitioned into dichloromethane and dried over sodium sulfate prior to injection onto a Varian capillary GC column, CP SIL coated (length: 30 m, diameter, 0.25 mm, film thickness 0.25 μ m) using splitless injection. The injector temperature: was 200 °C, initial oven temperature: 80 °C with a ramp rate 5 °C/min to maximum temperature of 200 °C. The carrier gas was nitrogen at 30 kPa column head pressure, with compounds being detected by flame ionization detection. The identity of the VOSCs was confirmed and quantified using authentic standards. Enzyme activity is quoted as nmol product formed per second = nkat.

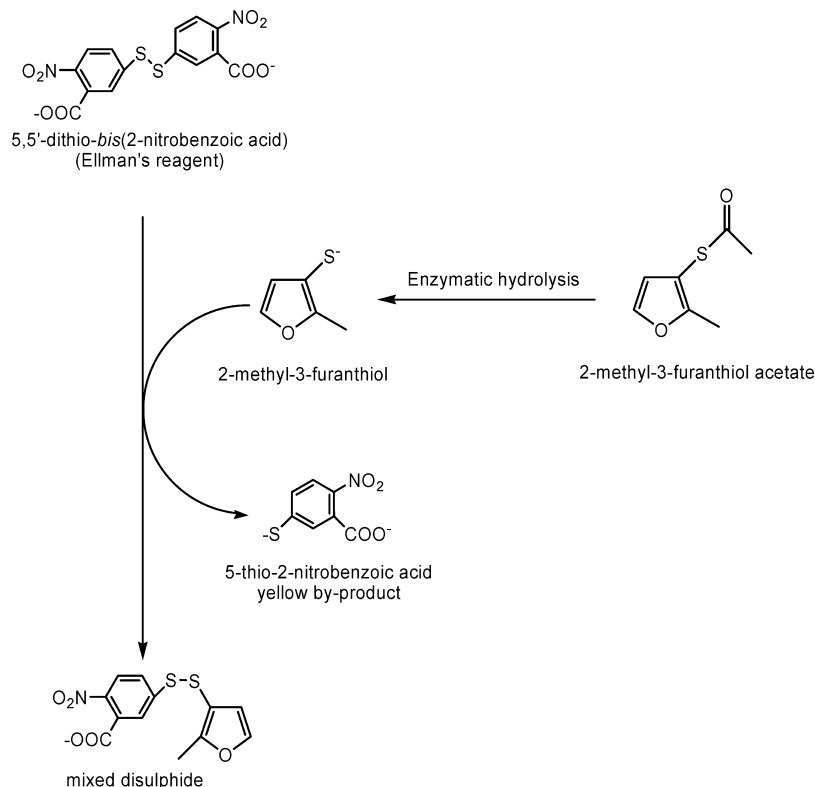


Figure 2. Determination of thioesterase activity with 2-methyl-3-furanthiol acetate as substrate using Ellman's reagent.

Protein Identification. Proteins were visualized in SDS-PAGE gels using Sypro Ruby stain (Invitrogen) prior to excision from the gel. Protein was destained and digested with trypsin as described previously, prior to tandem MS sequencing of the resulting peptides (12). Peptide sequences were used to interrogate the plant genome databases using short nearly exact match BLAST to identify protein hits.

RESULTS AND DISCUSSION

Thioesterase Assay Development and Associated Enzyme Activity in Passion Fruit. The conventional method for determining the release of VOSCs in enzyme assays requires the partitioning of products into organic solvent and their quantification by gas chromatography (10). While such assays are sensitive, they are prone to loss of VOSCs during the processing of the organic phase, are time-consuming and require access to capillary GC equipment. To simplify the assay of thioesterases, a method was developed which measured the release of free thiols using Ellman's reagent (dithiobis(2-nitrobenzoic acid)), which is more commonly used to measure cysteine and its derivatives in cell extracts (13). Several VOSCs were selected as potential thioesterase substrates (Table 1). These included 3-(thioacetyl)hexylacetate (4), which is a VOSC reminiscent of passion fruit, 8-acetylthio-*p*-menthan-3-one (8), which has been reported in buchu plant extracts (14), and 2-methyl-3-furanthiol acetate (5), a roasted note identified in cooked foods (15). In a preliminary screen, each thioester was incubated with a commercially available lipase from *Candida rugosa*, or porcine liver esterase, with both enzyme preparations having been reported to show thioesterase activity (9). Using a GC-based assay, 2-methyl-3-furanthiol acetate, was found to be the optimal thioester substrate (Table 1) and was used for subsequent assay validation. When 2-methyl-3-furanthiol acetate was incubated with either porcine liver esterase or the lipase and Ellman's reagent added, an enzyme-dependent release of

the yellow 5-thio-2-nitrobenzoic acid was determined due to the 2-methylfuran-3-thiol taking part in a thiol exchange reaction with dithiobis(2-nitrobenzoic acid) (Figure 2). Porcine liver esterase was found to be far more active as a thioesterase than the lipase, with the associated increase in colored product determined at 412 nm being strictly dependent on protein content and incubation time (Figure 3A,B). To confirm that the Ellman's assay was quantitative, the reaction products from the assay were analyzed by GC and the amounts of 2-methyl-3-furanthiol produced determined after calibrating the column with the authentic thiol (Figure 3C). An excellent correlation of activities as determined using the two methods was established for both porcine liver esterase and the lipase; the two sources of enzyme varying 500-fold in their specific activity (Figure 3D).

Having validated a sensitive colorimetric assay for thioesterase activity, crude protein extracts from the peel and flesh of ripened purple passion fruit were independently assayed using 2-methyl-3-furanthiol acetate as substrate. Passion fruits were first extracted with 0.1 M potassium phosphate buffers to extract soluble proteins. Thioesterase activity could be determined in the peel in extracts from the exocarp and thin purple endocarp tissues (0.404 nkat mg⁻¹ protein). No activity was determined in the flesh of the fruit. Sequential extraction of the peel with buffers containing 0.25 M saline to extract proteins which were ionically bound to wall components, resulted in a 2.4-fold increase in the recovery of thioesterase activity (0.976 nkat mg⁻¹ protein). To standardize the source of the thioesterase activity, the fruit were put into store and the peel sampled over a 24-day period. During the early time points when the fruit was unripe, a great deal of nonproteinaceous coextractives were obtained during processing, which made subsequent purification of the proteins difficult. Over the first 10 days of storage, the extractable activity remained relatively constant (total activity 73–78 nkat per fruit), while after this time, and as the fruit

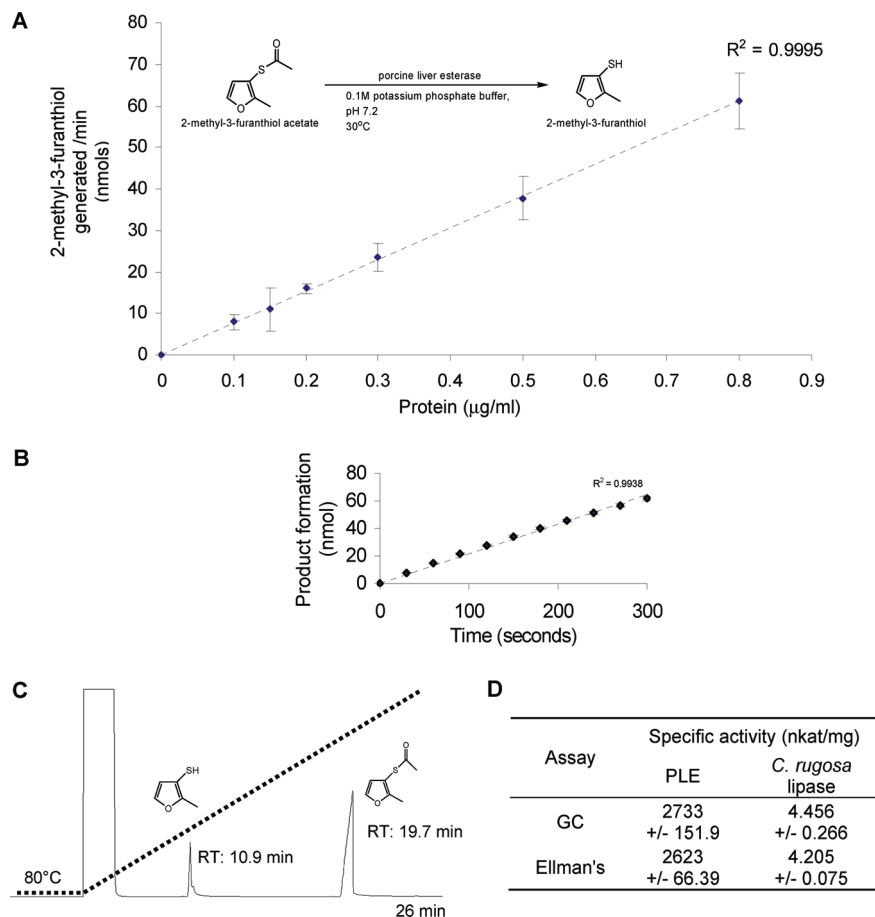


Figure 3. Validation of determination of thioesterase activity using Ellman's reagent. Protein (A) and time dependence (B) of 2-methyl-3-furanthiol formation following action of porcine liver esterase (0.1 μg/mL) on 1 mM 2-methyl-3-furanthiol acetate as determined using Ellman's assay. The nature of the thiol reaction products were subsequently confirmed by GC (C). Thioesterase activity toward 2-methyl-3-furanthiol acetate as determined by GC and by Ellman's reagent assays was then compared using porcine liver esterase and the lipase from *Candida rugosa* as enzymes (D). Activities are means ± SDs ($n = 3$). Boiled protein samples were used to correct for the rates of chemical hydrolysis.

began to visibly dry out, recovery of the thioesterases declined such that by 24 days less than 40% remained. As a compromise between the difficulties in processing the unripe tissue and losing activity over time, it was determined that fruit with a total water content of 70% by weight was ideal for enzyme extraction.

The crude enzymes from the passion fruit peel were then assayed for hydrolytic activity toward a series of model carboxylesters (Table 1). The peel preparations showed low activities toward *p*-nitrophenol acetate and α -naphthol acetate but were not active toward the esters of methylumbelliferol. The thioesterase activity in the crude preparations from passion fruit was then characterized in more detail prior to its purification. Activity was optimal at pH 8 using potassium phosphate buffer and was enhanced by increasing the assay temperature up to 37 °C. Above 45 °C, the enzyme(s) became rapidly inactivated. The passion fruit enzyme was found to be relatively stable with its activity having a half-life of 16 days when stored at 30 °C at pH 7.2. Treatment of the crude protein extracts with EDTA, mercuric chloride or iodoacetamide (each at 1 mM) gave minimal inhibition of thioesterase activity, indicating that the enzyme(s) neither required divalent metal ions nor free cysteines to be catalytically active. In contrast, treatment of crude protein extracts with 0.1 mM paraoxon (*O,O*-diethyl-*O-p*-nitrophenylphosphoric acid), a potent organophosphate inhibitor of plant serine hydrolases (11, 12), reduced hydrolysis by 87% and indicated a catalytic serine was important for the observed thioesterase activity.

Purification and Sequencing of a Putative Thioesterase from Passion Fruit. Salt extracts from the passion fruit peel were precipitated with ammonium sulfate and the thioesterase activity sequentially purified using DEAE anion exchange chromatography followed by hydrophobic interaction chromatography using first octyl-Sepharose and then phenyl-Sepharose (Figure 4). During the initial DEAE-Sepharose chromatography, thioesterase activity eluted in two distinct pools (Figure 4A), with approximately half of the recovered activity unretained on the column. To test the possibility that its lack of retention was due to overloading of the column, this fraction was reappplied to freshly conditioned DEAE-Sepharose. Again the activity was not bound, suggesting that the respective enzyme was basic in nature. Due to its relative instability, further purification of the unretained enzyme was not pursued. The DEAE-retained thioesterase activity which was recovered with 0.196 M salt, was applied onto an octyl-Sepharose column and eluted with a decreasing concentration of ammonium sulfate. The thioesterase activity eluted at the end of the gradient with the relatively hydrophobic proteins (Figure 4B). The active enzyme was then applied onto a high performance phenyl-Superose column and recovered in 0.545 M salt (Figure 4C). Based on the final specific activity of the thioesterase (149.38 ± 0.002 nkat mg⁻¹ protein), the enzyme was purified 154-fold in 6% yield.

When each stage of the purification was analyzed by SDS-PAGE, it could be seen that the final preparation was far from homogeneous (Figure 4D). The final thioesterase peak from

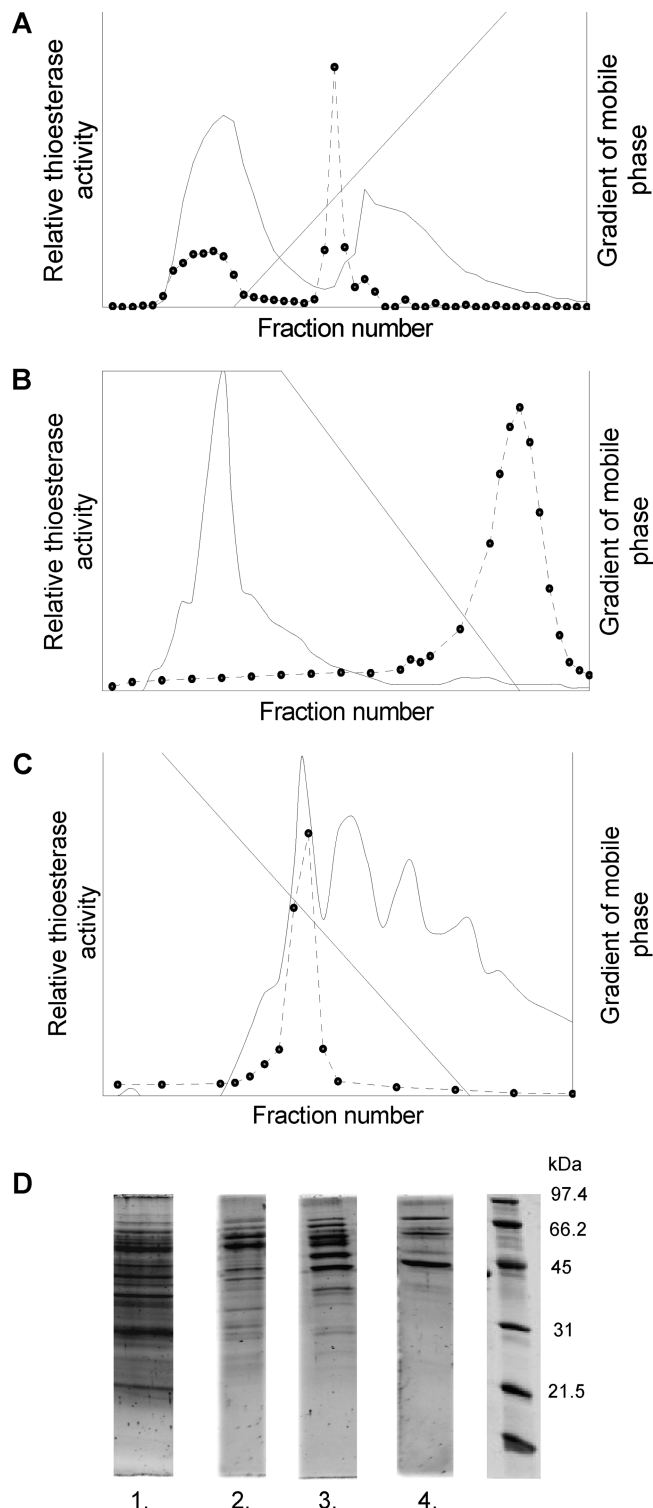


Figure 4. Sequential purification of a thioesterase active toward 2-methyl-3-furanthiol acetate from the peel of passion fruit by chromatography on DEAE-Sepharose (A), octyl-Sepharose (B), and phenyl-Superose (C). The continuous line shows the elution of UV absorbing protein (280 nm) with thioesterase activity in individual fractions determined (· · ·), with the gradient of mobile phases shown. SDS-PAGE analysis of polypeptides from each stage of purification are shown (D) with lanes 1 = crude, 2 = DEAE-Sepharose (pool B), 3 = octyl-Sepharose, 4 = phenyl-Superose. Molecular mass markers are shown for reference.

the phenyl-Superose column was divided into multiple fractions and the elution of enzyme activity compared to changes in the relative abundance of the polypeptides present (Figure 5A). A 43 kDa polypeptide was identified whose relative abundance

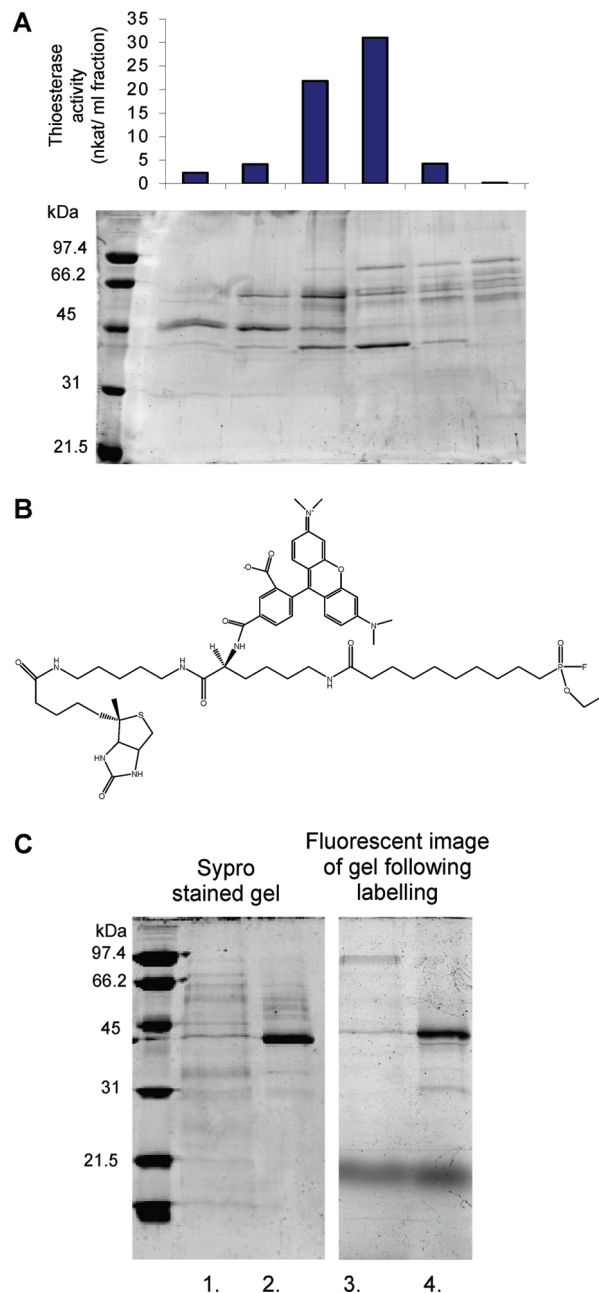


Figure 5. Identification of the semipurified thioesterase purified from passion fruit. The fractions associated with the final enrichment of the thioesterase on phenyl-Superose were analyzed for enzyme activity and polypeptide content (A). The structure of the trifunctional fluorophosphonate probe (B) and its use in labeling thioesterase present in passion fruit (C). Total polypeptide content in the crude (lane 1) and partially purified preparation (lane 2) and the same proteins (lanes 3 and 4, respectively) visualized for fluorescence after treatment with the rhodamine tagged TriFPP.

mirrored eluting thioesterase activity. Similarly, gel filtration analysis of the thioesterase isolated after the first step of the purification indicated that the native enzyme was a 43 kDa protein monomer (data not shown). As our inhibition studies with the crude preparations had suggested that the thioesterase was a serine hydrolase, we could then test whether or not the 43 kDa protein was the enzyme of interest using a chemical probe approach. First, the partially purified preparation was treated with 0.1 mM paraoxon, which resulted in total inhibition of thioesterase activity, supporting the identification of the candidate enzyme as a serine hydrolase. The same preparation

was then treated with a trifunctional fluorophosphonate activity probe (TriFPP). The custom-synthesized TriFPP contained a reactive fluorophosphonate to covalently modify reactive active site serines, and biotin and rhodamine components for recovery and recognition respectively (**Figure 5B**) and had been previously shown to label and selectively inactivate serine hydrolases in *Arabidopsis thaliana* (11). Treatment of the semipurified thioesterase with 10 μ M TriFPP resulted in a $86\% \pm 3\%$ (mean \pm variation in replicates, $n = 3$) inhibition of activity. When the labeled protein was analyzed by SDS-PAGE, the 43 kDa polypeptide could be seen to be labeled with the fluorescent TriFPP confirming its identity as a hydrolase (**Figure 5C**). Based on this, the protein was termed *Passiflora edulis* wall-bound hydrolase (*PeWH*).

The partially purified *PeWH* was then screened for activity against the thioester substrates (**Table 1**). The greatest thioesterase activity was determined with 2-methyl-3-furanthiol acetate (**5**), with appreciable activities also determined with 8-acetylthio-*p*-menthan-3-one (**8**). Reasonable thioesterase activity was also determined with the putative passion fruit substrate 3-(acetylthio)hexyl acetate (**4**). In the case of compound **4**, as determined by GC analysis, *PeWH* was nine times more active in cleaving the thioester bond than the carboxylester in this substrate. Compounds **5–7**, all contained the 5-membered furan ring, with the *PeWH* thioesterase activity being much greater when the acetyl-thioester was substituted at the 3 (**5**) rather than the 2 position (**7**). When the furan moiety became the acyl rather than the alcohol moiety (**6**) all activity was lost. *PeWH* was also a carboxylesterase, showing good activities toward *p*-nitrophenylacetate (compound **10**) and α -naphthyl acetate (**9**) but little turnover of the umbelliferyl esters (**11–13**). As compared with the commercially available hydrolases, *PeWH* showed some interesting selectivity in its thioesterase activity. *PeWH* was much more active toward the thioesters than the microbial lipase and hydrolyzed the thioester 8-acetylthio-*p*-menthan-3-one (**8**) with higher specific activity than porcine liver esterase. It was however much less active as a carboxylesterase. In all the assays it is likely that the true specific activity of *PeWH* was underestimated as the respective protein preparation was not totally pure.

The 43 kDa putative thioesterase was then analyzed for its physical properties. The *PeWH* polypeptide was shown to be glycosylated, based on its reactivity to glycan staining (data not shown). Similarly, when the semipurified protein from phenyl-Superose chromatography was applied onto a concanavalin A–Sepharose column, 100% of the activity was selectively retained, with 91% recovered after elution with 0.1 M methyl- α -D-mannopyranoside. When analyzed by isoelectric focusing gel electrophoresis, the *PeWH* migrated as a basic protein with a pI between pH 9–10. The 43 kDa *PeWH* polypeptide was excised from the SDS-PAGE gels of the final protein preparation (**Figure 4D**) and digested with trypsin, prior to the analysis of individual fractions by tandem MS based sequencing (12). Sequence was obtained from five distinct peptides, which were used to interrogate the plant genome and EST databases. From the five major hits it was apparent the enzyme belonged to a group of proteins labeled as family 13 glycoside hydrolases (16, 17). Of the proteins identified, pectin acetyl esterase (EC 3.1.1.6) was the primary candidate, with three of the five fragments generating significant hits matching this group of enzymes.

Thioesterase Activity and Family 13 Glycoside Hydrolases.

Despite their potential importance in plant cell wall modifications, pectin acetyl esterases have not been well studied at the biochemical level. However, what is known about these proteins

does support the identification of *PeWH* as such an enzyme. First, as determined with *PeWH*, pectin acetyl esterases in a range of Citrus species, including orange, lime, lemon, grapefruit and clementine (18), are found in the exocarp, but not in the flesh of the fruit. The purification of plant pectin acetyl esterases have only been reported from orange and mung bean, with the extraction techniques mirroring the procedures required to isolate *PeWH* (19, 20). For example, high salt concentrations in the extraction buffer (0.2–1 M NaCl) were required to solubilize the ionically bound enzymes from the cell wall (19, 20). Like *PeWH*, the pectin acetyl esterases are highly basic proteins and are of a very similar molecular mass with the enzyme from mung bean also being a 43 kDa protein (19, 21). The endogenous functions of pectin acetyl esterases are not well understood, though their activity in regulating the degree of pectin acetylation, has been ascribed roles in softening fruit tissue (18) and in controlling cell growth and elongation (21). Interestingly, in Citrus fruit peel, pectin acetyl esterases were localized at high concentrations in oil vesicles where functions in wall modifications would seem improbable (18). Based on our observations with *PeWH*, it is also possible that glycoside hydrolases present in fruit peel are able to hydrolyze volatile secondary metabolites. Such a role in natural product metabolism may extend to many fruits. For example, in apples butyl esters are hydrolyzed to release butanol into the surrounding air by undefined esterases found at high concentrations in the cortex and peel (22). In addition a recent study in the tropical snake fruit (*Salacca edulis*) found that pectin methyl esterase activity was linked to the metabolism of volatile methyl esters (23). The diversity of roles adopted by glycoside hydrolases in fruit ripening (24, 25) and their potential to act as bifunctional enzymes contributing to the biogenesis of volatile secondary metabolites is an interesting area for future study. Work will now focus on cloning and expressing the enzyme both as a means of confirming identity and for characterizing the enzyme further.

ABBREVIATIONS USED

PeWH, *Passiflora edulis* wall hydrolase; TriFPP, trifunctional fluorophosphonate activity probe.

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