Phytochemistry 72 (2011) 1939–1946

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

First insights into the mode of action of a "lachrymatory factor synthase" – Implications for the mechanism of lachrymator formation in *Petiveria alliacea*, *Allium cepa* and *Nectaroscordum* species

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ARTICLE INFO

Article history:
Received 16 December 2010
Received in revised form 11 July 2011
Available online 15 August 2011

Keywords:
Petiveria alliacea
Phytolaccaceae
Lachrymatory factor synthase
Sulfenic acid
Sulfenic acid dehydrogenase
Phenylmethanesulfenic acid dehydrogenase
Sulfenic acid isomerase
1-Propenesulfenic acid isomerase
1-Butenesulfenic acid isomerase
Onion
Petiveriin
Isoalliin
Homoisoalliin
Sulfine

ABSTRACT

A study of an enzyme that reacts with the sulfenic acid produced by the alliinase in *Petiveria alliacea* L. (Phytolaccaceae) to yield the *P. alliacea* lachrymator (phenylmethanethial *S*-oxide) showed the protein to be a dehydrogenase. It functions by abstracting hydride from sulfenic acids of appropriate structure to form their corresponding sulfines. Successful hydride abstraction is dependent upon the presence of a benzyl group on the sulfur to stabilize the intermediate formed on abstraction of hydride. This dehydrogenase activity contrasts with that of the lachrymatory factor synthase (LFS) found in onion, which catalyzes the rearrangement of 1-propenesulfenic acid to (*Z*)-propanethial *S*-oxide, the onion lachrymator. Based on the type of reaction it catalyzes, the onion LFS should be classified as an isomerase and would be called a "sulfenic acid isomerase", whereas the *P. alliacea* LFS would be termed a "sulfenic acid dehydrogenase".

1. Introduction

The familiar lachrymatory effect elicited when onions are cut is caused by a volatile small molecule sulfine, (*Z*)-propanethial *S*-oxide [PTSO, (**1**), Fig. 1] (Brodnitz and Pascale, 1971). The formation of this molecule has been shown to be catalyzed by a novel enzyme, termed lachrymatory factor synthase (LFS, Fig. 1) (Imai et al., 2002). The PTSO precursor is (*E*)-*S*-(1-propenyl)-L-cysteine *S*-oxide [isoalliin, (**2**)], a cysteine sulfoxide derivative that is constitutively present in the cytoplasm of onion cells. When onion tissue is disrupted, alliinase, which is a C–S lyase present in onion cell vacuoles (Lancaster and Collin, 1981; Pickering et al., 2009), comes into contact with isoalliin, and catalyzes its breakdown into two cleavage products. These are the highly reactive 1-propenesulfenic acid [PSA, (**3**) Fig. 1], and α-aminoacrylic acid (not shown), which undergoes rapid hydrolysis to yield ammonia and pyruvate. PSA (**3**) is then acted upon by the LFS, and the highly volatile and lachrymatory PTSO (**1**) is formed.

Prior to the discovery of the LFS, it was believed that PTSO was formed *via* a [1,4]-sigmatropic rearrangement of PSA (**3**) at the onion alliinase active site, by analogy to non-enzymatic conversion of PSA (**3**) to PTSO (**1**) at elevated temperatures in the gas phase (Block et al., 1996). It has since been shown however, that in the absence of the onion LFS, onion alliinase is incapable of catalyzing the formation of PTSO (**1**). Thus, when the alliinase alone interacts with isoalliin (**2**), the PSA (**3**) product undergoes a variety of reactions that yield a plethora of downstream organosulfur compounds (Block, 1992, 2010), but no PTSO (**1**) is formed (Imai et al., 2002; Eady et al., 2008). Although the onion LFS has been cDNA cloned, little is known of its catalytic mechanism.

In previous work conducted on the Amazonian medicinal plant *Petiveria alliacea* L. (Phytolaccaceae), it was shown that like onion,

Abbreviations: BAD, benzyl alcohol dehydrogenase; BSA, 1-butenesulfenic acid; BTSO, butanethial *S*-oxide; LFS, lachrymatory factor synthase; PMSA, phenylmethanesulfenic acid; PMTSO, phenylmethanethial *S*-oxide; PSA, 1-propenesulfenic acid; PTSO, propanethial *S*-oxide; SAD, sulfenic acid dehydrogenase; TASO, thioacrolein *S*-oxide.

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doi:10.1016/j.phytochem.2011.07.013

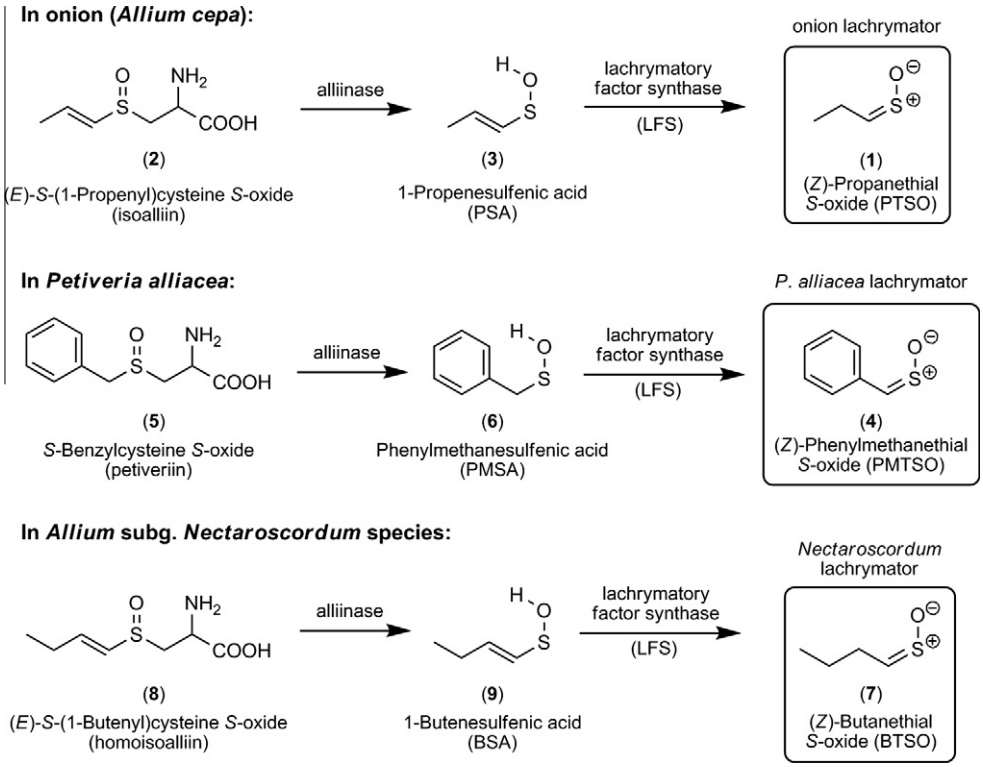


Fig. 1. Lachrymator formation in onion (*A. cepa*) and *P. alliacea*. In onion, upon tissue disruption, the onion LFS acts on PSA, which is produced by the catalytic action of the onion alliinase on isoalliin, to afford the lachrymator PTSO. In *P. alliacea*, upon tissue disruption, the *P. alliacea* LFS acts on PMSA, which is furnished by the catalytic action of the *P. alliacea* alliinase on petiveriin, to yield the lachrymator PMTSO. In *Allium* subg. *Nectaroscordum* species, the sulfenic acid BSA, which is furnished by the action of alliinase on homoisoalliin, is acted upon by an LFS to give the lachrymator BTSO.

P. alliacea contains not only an alliinase, but also an LFS-type enzyme that catalyzes the formation of a sulfine lachrymator, (*Z*)-phenylmethanethial *S*-oxide [PMTSO (**4**), Fig. 1] (Kubec et al., 2003; Musah et al., 2009a,b). Similar to onion, the *P. alliacea* alliinase acts on a cysteine sulfoxide derivative, in this case, *S*-benzyl-L-cysteine *S*-oxide [petiveriin (**5**)], to yield a sulfenic acid [phenylmethanesulfenic acid – PMSA (**6**)] that is acted upon by an LFS to furnish the sulfine, PMTSO (**4**) (Fig. 1).

Recently, another lachrymatory sulfine, (*Z*)-butanethial *S*-oxide [BTSO (**7**)], was identified in two *Allium* subg. *Nectaroscordum* species (*A. siculum* and *A. tripedale*). It was shown that this higher homologue of the onion lachrymator is formed from (*E*)-*S*-(1-butenyl)cysteine *S*-oxide [homoisoalliin (**8**)] *via* 1-butenesulfenic acid [BSA (**9**)] [(Kubec et al., 2010; Block et al., 2010), Fig. 1]. It is reasonable to assume that the conversion of BSA (**9**) into BTSO (**7**) is also mediated by an LFS-type enzyme.

A cursory glance at the LFS-catalyzed reactions that yield the sulfines in onion, subgenus *Nectaroscordum* species and *P. alliacea* implies that they may employ similar mechanistic tactics in their reactions with isoalliin (**2**), homoisoalliin (**8**) and petiveriin (**5**), respectively (Fig. 1). However, a fundamental difference in the structures of the sulfenic acid precursors to the sulfines [i.e., PSA (**3**), PMSA (**6**) and BSA (**9**), Fig. 1] suggests otherwise. Whereas PSA (**3**) and BSA (**9**) possess α,β-unsaturation, a vestige of the isoalliin/homoisoalliin from which they are derived, PMSA (**6**) is devoid of this feature. A consequence of this is that the sp^2-hybridized carbon that is attached to sulfur in the sulfenic acids from which PTSO (**1**) and BTSO (**7**) originate [i.e., PSA (**3**) and BSA (**9**) respectively] is more highly oxidized than that [i.e., PMSA (**6**)] from which PMTSO (**4**) is derived. Therefore, it is clear that despite the fact that the LF synthases from onion, *Nectaroscordum* species and *P. alliacea* act on sulfenic acids to yield sulfine lachrymators, they perform these reactions by different mechanisms. Mechanistic studies involving determination of the kinetics of LF synthases are complicated by the following unique challenges: (a) their substrates are reactive intermediates (sulfenic acid) which are created *in situ* by the enzyme alliinase; (b) quantification of the substrate (which again, is a sulfenic acid intermediate) is hampered by the fact that under the reaction conditions, two molecules of sulfenic acid condense with loss of water to form a thiosulfinate product; (c) the product of the action of the LFS (i.e., a sulfine) is itself labile and is degraded under the reaction conditions to an aldehyde; (d) because the action of the LFS requires the presence of an alliinase (in order to produce the sulfenic acid substrate), it is challenging to ascribe any particular kinetic effect that one might observe, to a particular enzyme in the system; and (e) the results of solvent isotope effect studies would be ambiguous, since this represents a two-enzyme system, and convincingly attributing the observed effects to one enzyme and not the other is difficult. As such, very little is known about the mechanisms of this class of enzymes, despite their importance in plant defense. Thus, we sought to glean information about the mode of action of this novel class of enzymes by alternative methods, beginning first with the *P. alliacea* LFS. Herein, we report the results of our studies. Our observations suggest that the *P. alliacea* LFS is actually a dehydrogenase that acts on the sulfenic acid produced by the *P. alliacea* alliinase, to form PMTSO (**4**) *via* a hydride transfer mechanism.

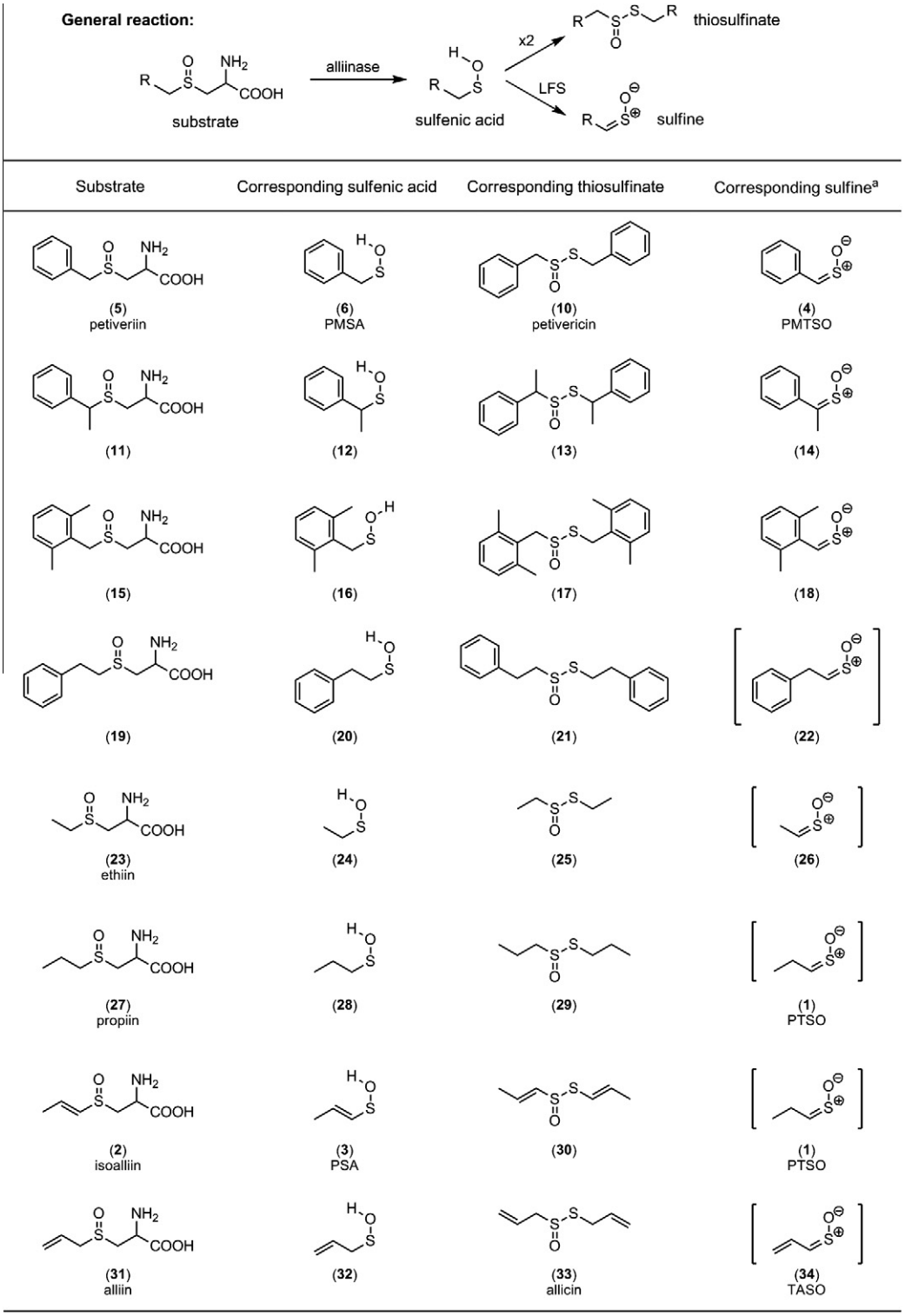
2. Results and discussion

2.1. Substrate specificity of the P. alliacea lachrymatory factor synthase

The substrates for LF synthases have been shown to be the sulfenic acid intermediates furnished by the reaction of an alliinase with a suitable *S*-substituted cysteine *S*-oxide precursor (Imai

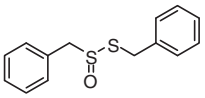
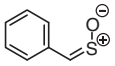
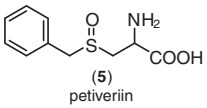
et al., 2002; Musah et al., 2009a,b). When an alliinase catalyzes the cleavage of *S*-substituted cysteine *S*-oxides in the absence of an LFS, the resulting sulfenic acids condense spontaneously to form thiosulfinates (Table 1). Therefore, thiosulfinate products serve as direct evidence of the formation of sulfenic acids by an alliinase. Like the onion LFS, the *P. alliacea* LFS produces a sulfine only in the presence of both an *S*-substituted cysteine *S*-oxide derivative and an alliinase. In the case of *P. alliacea*, it has also been shown that when suitable *S*-substituted cysteine *S*-oxides are exposed to its LFS/alliinase complex, the relative amounts of thiosulfinate and sulfine formed are dependent upon the ratio of LFS to alliinase (Musah et al., 2009a). When the LFS to alliinase molar ratio is 5:1,

Table 1
The products formed when various substrates were exposed to alliinase in the presence of the *P. alliacea* LFS enzyme. The analysis of the scope of reactivity of the LFS was conducted by reacting various *S*-substituted cysteine sulfoxides with an LFS/alliinase complex, in which the two proteins were present in a 5:3 M ratio respectively.



[a] Structures in square brackets are sulfines that were not observed.

Table 2
The products formed when petiveriin was exposed to the *P. alliacea* alliinase under various conditions.

Entry no.	substrate	added enzyme and (or) cofactor	reaction products[1,2]	
			 thiosulfinate (petivericin, **10**)	 sulfine (PMTSO, **4**)
1	 (**5**) petiveriin	alliinase →	+	–
2	petiveriin	alliinase + LFS →	+	1.0
3	petiveriin	alliinase + NAD^+ →	+	0.08
4	petiveriin	alliinase + $NADP^+$ →	+	0.08
5	petiveriin	alliinase + FAD →	+	–
6	petiveriin	alliinase + FMN →	+	–
7	petiveriin	alliinase + NAD^+ + LFS →	+	2.4
8	petiveriin	alliinase + $NADP^+$ + LFS →	+	2.0
9	petiveriin	alliinase + FAD + LFS →	+	1.0
10	petiveriin	alliinase + FMN + LFS →	+	1.0

[a] The "+" and "–" signs indicate whether the product was formed ("+") or not formed ("–").
[b] The values represent the ratios of sulfine formed relative to the amount formed in the system consisting of LFS, petiveriin and alliinase (entry no. 2).

all of the sulfenic acid produced by the alliinase is sequestered, so that only the sulfine product is observed. On the other hand, when the LFS to alliinase molar ratio is $\leqslant$5:2, the LFS is unable to sequester all of the sulfenic acid formed by the alliinase, with the result that some of the sulfenic acid self-condenses to form thiosulfinate (Musah et al., 2009a). Therefore, our analysis of the scope of reactivity of the LFS was conducted by reacting various *S*-substituted cysteine *S*-oxides with a *P. alliacea*-derived LFS/alliinase complex, in which the two proteins were present in a 5:3 M ratio respectively. It was anticipated that the use of this LFS/alliinase ratio would reveal whether sulfenic acids were available to the LFS (through observance of thiosulfinates) in those cases where no sulfine was formed by the LFS. The group of compounds tested is listed in Table 1, and is comprised of petiveriin and its derivatives (compounds **5**, **11** and **15**, a chain elongated petiveriin homologue (compound **19**), and various *S*-alk(en)yl-L-cysteine *S*-oxides (compounds **2**, **23**, **27** and **31**). The substrate specificity of the *P. alliacea* LFS was determined by tracking the formation of sulfine products by HPLC, UV–Vis, and electrospray ionization mass spectrometry (ESI-MS).

Examination of the results of these experiments revealed clear structural distinctions between those sulfenic acids with which the LFS reacted to form sulfines, and those with which it did not. When the LFS/alliinase complex was reacted with its natural substrate petiveriin (**5**), as expected, PMTSO (**4**) was formed (Table 1). Similarly, sulfines were also formed when the protein complex was reacted with α- and ring-substituted derivatives of petiveriin (**5**) (Table 1, compounds **11** and **15**). However, with the presence of an additional methylene group between the benzene ring and the α-carbon adjacent to the sulfur, no sulfine formation was observed (Table 1, compound **19**). Additionally, no sulfines were formed from precursor molecules **2**, **23**, **27** and **31**, including the precursor to the onion lachrymator [isoalliin (**2**)]. On the other hand, formation of the corresponding thiosulfinates was observed for all of the other compounds tested.

2.2. P. alliacea LFS catalyzes hydride transfer from a sulfenic acid intermediate

To further define the conditions under which sulfines are formed, petiveriin (**5**) was exposed to the *P. alliacea* alliinase in the presence of selected enzyme cofactors. The results of these experiments are shown in Table 2. Although the combination of petiveriin (**5**) and the alliinase in the absence of the *P. alliacea* LFS produced only the corresponding thiosulfinate (**10**) (Table 2, reaction 1), the addition of either of the naturally occurring cofactors NAD^+ or $NADP^+$ to the reaction system resulted in the formation of PMTSO (**4**), the *P. alliacea* lachrymatory sulfine, albeit at concentrations 13 times lower than were observed in the presence of LFS (Table 2, reactions 3 and 4). The use of the redox cofactors FAD or FMN in a similar experiment yielded only the thiosulfinate, and no sulfine (Table 2, reactions 5 and 6). When petiveriin (**5**), alliinase and LFS were reacted together in the presence of added NAD^+ or $NADP^+$, PMTSO was produced at relative concentrations of 2.4 and 2.0 times higher, respectively, than observed with a combination of petiveriin (**5**), alliinase and LFS (Table 2, reactions 7 and 8).

2.3. P. alliacea LFS substrate specificity studies

Although onion, *Nectaroscordum* species and *P. alliacea* contain several constitutively present *S*-substituted cysteine *S*-oxides, only a single compound in each plant serves as a precursor for the lachrymator that is formed on tissue disruption. However, despite the limited number of sulfine precursors that are available in *P. alliacea*, its LFS has an active site that can accommodate not only its natural substrate [i.e., PMSA (**6**)], but other non-natural sulfenic acids (Table 1, compounds **11** and **15**). Thus, the LFS is capable of transforming PMSA (**6**) analogues that bear substituents on the

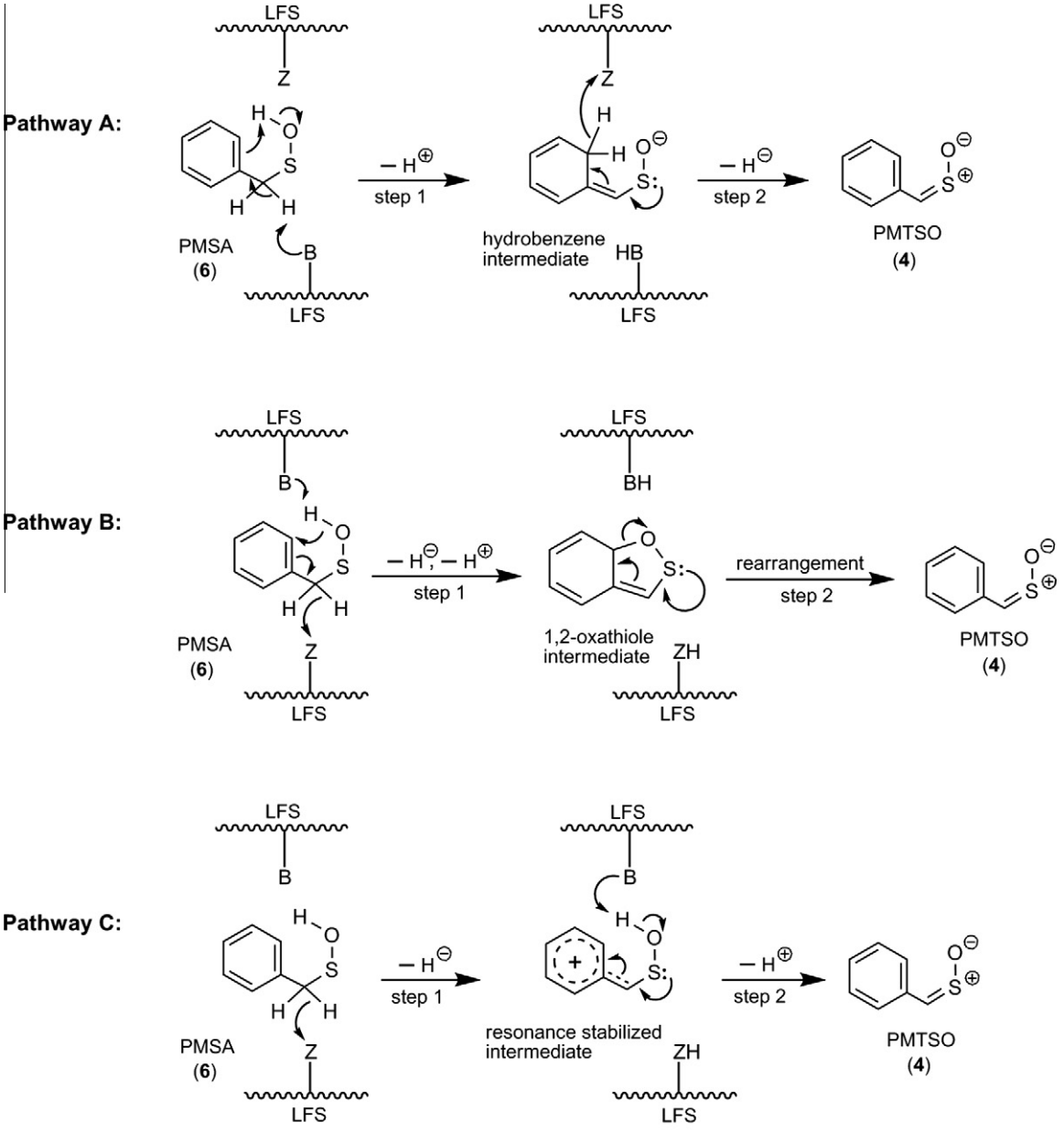


Fig. 2. Proposed mechanisms for formation of the *P. alliacea* lachrymator PMTSO *via* a hydride transfer from PMSA to an electron acceptor.

carbon α to the benzene ring (Table 1, compound **11**), and on the benzene ring (Table 1, compound **15**). However, this ability does not extend to the chain-elongated derivative (Table 1, compound **19**), nor to the sulfenic acids derived from propiin and isoalliin (Table 1, compounds **2** and **27**). Ethiin (Table 1, compound **23**) also did not react to form its corresponding sulfine. That sulfenic acid substrates were available to the LFS for all of the compounds tested was demonstrated by the observation of thiosulfinates, the condensation products of the sulfenic acids formed by the alliinase, in all cases. It is clear from the results that the sulfenic acids corresponding to compounds **5**, **11** and **15** are distinguished from those corresponding to **2**, **19**, **23** and **27** in that abstraction of an α-hydrogen (either as H^+ or H^-) would yield a resonance stabilized benzylic ion.

It was surprising though, that the observation of sulfine formation did not require the presence of the LFS, and that the cofactors NAD^+ or $NADP^+$ (but not FAD or FMN), could serve in its stead, albeit much less efficiently (Table 2, entries 3–6). The inclusion of $NAD(P)^+$ with petiveriin, alliinase and the LFS resulted in increased product formation, when compared to identical reactions in which $NAD(P)^+$ were not present. However, FAD and FMN did not elicit this effect.

A characteristic function of $NAD(P)^+$ in biological systems is the transfer of electrons from one redox center to another. In these reactions, a proton and two electrons, in the form of hydride, are transferred from a donor to $NAD(P)^+$ to form NAD(P)H, with concomitant release of H^+. Therefore, the observation that $NAD(P)^+$ in the presence of petiveriin (**5**) and alliinase furnished PMTSO (**4**), suggested not only that the cofactor served the role of abstracting an α-hydride from the sulfenic acid to afford the sulfine product, but also that the LFS functions similarly.

Fig. 2 outlines three possible mechanisms that involve hydride transfer from PMSA (**6**) to an electron acceptor to form PMTSO (**4**). In Pathway A, a base at the active site abstracts a proton from the benzylic carbon, and the *ortho* carbon of the benzene ring abstracts the proton from the oxygen atom of the PMSA (**6**). The resulting intermediate then looses hydride to an electron acceptor, which yields the product sulfine. In Pathway B, an active site base abstracts the proton that is attached to the PMSA (**6**) oxygen. Single bond formation occurs between oxygen and the *ortho* carbon of the benzene ring, and double bond formation occurs between the ring and the α-carbon, with loss of hydride from the α-carbon to an electron pair acceptor. The resulting 1,2-oxathiole intermediate then rearranges *via* a six electron exocyclic ring opening to form the product. Pathway C is similar to Pathway B in that hydride is lost from the benzylic carbon. However, Pathway C differs from B in that a bicyclic intermediate is not formed, and there is considerable positive charge buildup in the intermediate.

The observations made thus far favor Pathway C. For starters, Pathways A and B appear rather unlikely due to the lack of an obvious driving force to destroy the aromatic ring system. Second, Pathway A involves the transfer of a proton from the PMSA (**6**) oxygen to the *ortho* carbon of the benzene ring. Sulfenic acid protons are acidic and have been shown to readily exchange in D_2O (Penn et al., 1978; Davis and Billmers, 1984; Ishii et al., 1996; Goto et al., 1997). Therefore, if the solvent system is D_2O, then this exchange would result in deuteration of the ring, regardless of whether the ring deuteration step is rate-limiting. This would result in formation of at least some deuterium-labeled PMTSO (**4**). However, when petiveriin (**5**), alliinase and the LFS were reacted in D_2O and the HPLC-purified product was analyzed by ESI-TOF, only unlabeled PMTSO (**4**) was detected (data not shown). Third, Pathways A and B both involve attack at the *ortho* carbons of the benzene ring. It might be expected that blocking these positions with substituents would curtail formation of the corresponding sulfine for steric reasons. However, we observed that the corresponding sulfine was readily formed even when the hydrogens at both *ortho* positions were replaced with methyl groups (Table 1, compound **15**). This issue is circumvented in Pathway C, because the Pathway C mechanism does not involve attack at the *ortho* carbons of the benzene ring.

The nature of the discrimination shown by the *P. alliacea* LFS for different substrates, coupled with the finding that $NAD(P)^+$, in the presence of the alliinase and petiveriin, furnishes PMTSO (**4**), provided additional clues regarding the mode of action of LFS reactions with sulfenic acids. Our results show that an essential structural requirement for the LFS-sulfenic acid substrate is that it possess a free hydrogen at a fully saturated α-carbon that is adjacent to an unsaturated center capable of providing significant resonance stabilization. Petiveriin (**5**), as well as compounds **11** and **15**, meet this requirement. Although compound **19** possesses an α-hydrogen at a fully saturated carbon, the α-carbon to which it is bonded is not adjacent to an unsaturated center. The situation is similar for compounds **23** and **27**, neither of which is converted to a sulfine, even though both are converted to sulfenic acids by the alliinase. That sulfenic acids are actually formed in these cases is evidenced by the formation of thiosulfinates under the reaction conditions. From the pattern that emerged, it was predictable that the sulfenic acid PSA (**3**) derived from isoalliin (**2**), the precursor of the onion lachrymator, would not react with the *P. alliacea* LFS, as was observed (Table 1).

The reaction of alliin (Table 1, compound **31**) with the *P. alliacea* LFS/alliinase complex is an interesting case. Like the other substrates that were oxidized to sulfines, it possesses an α-hydrogen attached to the carbon that is adjacent to an unsaturated center. Therefore, loss of hydride from alliin (**31**) might be expected to occur in a fashion similar to that observed for the benzyl systems, which would yield (*Z*)-thioacrolein *S*-oxide [TASO (**34**)] (Pelloux-Léon et al., 1997). Analogous to what has been observed for the PTSO (**1**) that is formed from isoalliin (**2**), which decomposes in an aqueous environment to form propanal (Brodnitz and Pascale, 1971), TASO (**34**) could undergo a similar reaction to form acrolein (2-propenal). Therefore, the observation of acrolein would serve as evidence for the formation of TASO (**34**). Such a finding would imply that the *P. alliacea* LFS may be similar to bacterial benzyl alcohol dehydrogenases (BADs), in terms of their ability to catalyze the oxidation of both benzylic and allylic substrates. BADs have been shown to catalyze the oxidation to aldehydes of both benzylic and allylic alcohols (but not their non-allylic or non-benzylic counterparts) through an intermediate that is partially positively charged (Curtis et al., 1999).

However, under the reaction conditions employed, neither formation of TASO (**34**), acrolein, nor any other product besides the thiosulfinate allicin (**33**) was observed, and it is concluded that the sulfenic acid derived from alliin (**31**) is not a suitable substrate for the *P. alliacea* LFS. It is proposed that the reason 2-propenesulfenic acid does not serve as a suitable substrate for the LFS, despite having an abstractable α-hydrogen on the carbon that is adjacent to an unsaturated center, is that the allyl substituent is not as well accommodated by the enzyme active site when compared to the benzyl substituent, and therefore, its corresponding intermediate is not as well stabilized. This could indicate that pi-pi stacking interactions between the benzene ring of the substrate and active site residues are important determinants for substrate binding. In support of this, it has been observed that the K_m for the interaction of alliin (**31**) with the *P. alliacea* alliinase was 4.32 mM, whereas that for petiveriin (**5**) was 0.39 mM (Musah et al., 2009b). This implies that the binding of alliin (**31**) to the alliinase active site is ~11 times less tight than that for petiveriin (**5**). Additionally, the catalytic efficiencies of the alliinase for alliin (**31**) and petiveriin (**5**) were found to be 42,800 and 100,000 $s^{-1}\,M^{-1}$ respectively, indicating that the breakdown of petiveriin (**5**) by

the alliinase is much more efficient than for alliin (**31**) (Musah et al., 2009b). Although it is not customary to compare the catalytic efficiencies of distinct enzymes that catalyze different reactions, it has been found that the *P. alliacea* LFS shares with the *P. alliacea* alliinase four of the alliinases five protein subunits (Musah et al., 2009a,b). Thus, it may be possible that as a consequence of the structural similarity between the *P. alliacea* LFS and alliinase, the discrimination shown by the alliinase for different *S*-substituted cysteine *S*-oxides based upon differing R group substituents, is shared by the LFS. However, the possibility cannot be ruled out that TASO (**34**) is formed, but is quickly transformed into derivatives that we did not detect under the experimental conditions used.

The loss of hydride from the sulfenic acid, a conclusion implied by the ability of $NAD(P)^+$ to furnish PMTSO (**4**) under appropriate conditions, coupled with the aforementioned substrate structural requirements, implicates the involvement of an intermediate possessed of significant positive charge buildup. The Pathway C mechanism shown in Fig. 2 is in alignment with this conclusion. Once a sulfenic acid is formed by the alliinase, it has two possible fates. It can condense with another sulfenic acid molecule to form a thiosulfinate, or be sequestered by the LFS and undergo further transformation. When the sulfenic acid possesses an α-hydrogen adjacent to a benzene ring, this hydrogen is abstracted by the LFS to yield an intermediate in which significant positive charge buildup can be stabilized by the adjacent aromatic ring. Either concomitantly, or as a second step, a bonding interaction is established between a basic residue at the active site, and the proton that is attached to the oxygen of the sulfenic acid. This proton is ultimately abstracted by the active site base, and subsequently released to the surroundings. Although the timing of abstraction of the proton from the sulfenic acid is unclear, its loss, along with the loss of hydride from the α-carbon of the sulfenic acid, would yield the sulfine. It is also possible that the divalent sulfur contributes significantly to stabilization of the cation. In that case, a protonated sulfine would be formed which could be expected to release its proton faster than occurs in the sulfenic acid.

The Pathway C mechanism proposed in Fig. 2 provides a rationale for why the *P. alliacea* LFS does not catalyze sulfine formation from isoalliin (**2**), the precursor of the onion lachrymator. As a consequence of its α,β-unsaturation, PSA (**3**) is an isomer of its corresponding sulfine, PTSO (**1**) (i.e., the onion lachrymator). Thus, it need simply undergo a rearrangement in order to furnish the sulfine. Apparently, it is this rearrangement that is catalyzed by the onion LFS. The absence of α,β-unsaturation in petiveriin (**5**) means that the α-carbon in PMSA (**6**) is less oxidized than that in PSA. Thus, sulfine formation from the PMSA (**6**) intermediate requires loss of two electrons and two protons, which is the reaction catalyzed by the *P. alliacea* LFS. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), the naming and classification of enzymes is based on the reactions they catalyze. Given that PMTSO (**4**) formation involves oxidation of PMSA (**6**) *via* loss of an electron pair and two protons to an acceptor, it would be more appropriate to refer to the *P. alliacea* enzyme that mediates this process by the common name "sulfenic acid dehydrogenase" (SAD), and more specifically as "phenylmethanesulfenic acid dehydrogenase". According to this categorization, the enzyme would belong to the enzyme classification 1 (EC 1) group of oxidoreductases. Regardless of mechanism, enzymes that catalyze the structural rearrangement of isomers are termed isomerases, and thus the onion LFS can be classified as such, even though the mechanism by which the isomerization occurs is unknown. Thus, by the current convention, the onion LFS would be categorized as a "sulfenic acid isomerase", or more specifically as 1-propenesulfenic acid isomerase. This would place it in the EC 5 group of enzymes. Similarly, the LFS presumably present in subgenus *Nectaroscordum* species should be termed 1-butenesulfenic acid isomerase.

The fact that various redox cofactors are capable of promoting the formation of sulfines from sulfenic acids in the absence of SAD implies that SAD itself utilizes a cofactor as part of its catalytic mechanism. Detailed exploration of this hypothesis is the subject of ongoing studies.

3. Conclusions

These studies of the enzyme isolated from *P. alliacea* that exhibits LFS activity show it to be a dehydrogenase that functions by abstracting hydride from sulfenic acids of suitable structure. A benzene ring adjacent to the carbon α to sulfur is an important determinant for successful hydride abstraction because it stabilizes the positively charged intermediate formed upon abstraction of hydride. The ability of various redox cofactors to promote formation of sulfines from sulfenic acids in the presence of this enzyme implies that the enzyme itself might utilize a co-factor as part of its catalytic mechanism. Although onion (*Allium cepa*), *Allium* subg. *Nectaroscordum* species and *P. alliacea* contain LFSs that mediate formation of sulfines, the mechanisms by which they function are fundamentally different. Sulfine formation in onion and *Nectaroscordum* species from the precursor sulfenic acids is formally a rearrangement reaction, whereas sulfine formation from the precursor sulfenic acid in *P. alliacea* is formally an oxidation. Thus, it may be more appropriate to term the onion and *Nectaroscordum* LFSs "sulfenic acid isomerases" and the *P. alliacea* LFS a "sulfenic acid dehydrogenase".

4. Experimental

4.1. Plants and materials

Unless otherwise noted, all chemicals were obtained from the Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Whole fresh plants of *P. alliacea* were obtained from Native Habitat Landscaping (Vero Beach, FL, USA), and stored at −30 °C until analysis. A voucher specimen is deposited at the herbarium PIHG at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL, USA, under accession number 7801.

4.2. ESI-TOF

An Agilent dual ESI source ESI-MSD-TOF mass spectrometer at the Scripps Research Institute (La Jolla, CA) was used for accurate mass determination of compounds. A mixture of standards (Agilent ESI-TOF TUNE mix) was used to spray two lock masses at 121 and 922 from the second sprayer for internal calibration of each mass spectrum to get the highest mass accuracy. The samples were introduced by flow injection analysis at 4000 V using an 8 μL sample injection at 100 μL/min using an Agilent 1100 HPLC system with a solvent consisting of 50% ethanol.

4.3. Reference compounds

S-Substituted-L-cysteines and the corresponding *S*-substituted-L-cysteine *S*-oxides, as well as petivericin, were synthesized according to the methods of Kubec and Musah (2001) and Kubec et al. (2002). Isoalliin was isolated from white onion bulbs obtained at a local market according to the method of Shen and Parkin (2000). 2,6-Dimethylbenzyl bromide was synthesized according to the method of Soloshonok et al. (2001).

4.4. Purification of alliinase and LFS from P. alliacea

The alliinase and LFS enzymes in *P. alliacea* were purified according to the protocols of Musah et al. (2009a,b).

4.5. Determination of LFS substrate specificity

Sulfenic acid substrates with which the LFS could react were generated *in situ* through the action of a *P. alliacea* alliinase/LFS complex on cysteine sulfoxide derivatives as reported in the thesis of He (2010). The reaction mixtures in 10 mM phosphate buffer, pH 8.0 (in a total volume of 1.0 mL), were minimally comprised of 1.5 mM substrate, 25 μM pyridoxal 5′-phosphate (PLP), 3.0 μg of purified alliinase (~21 nM) and 5.7 μg of purified LFS (~34 nM). In those cases where the effects of cofactors were determined, 0.32 mM $NAD(P)^+$, FAD or FMN was also present. The mixtures were incubated for 20 min at room temperature, and then 10–20 μL of the reaction solution was analyzed by HPLC using an analytical RP C-18 column (Microsorb-MV 100 Å, 250 × 4.6 mm, 5 μm, Varian, Palo Alto, CA, USA) under the following conditions: flow rate: 1.0 mL min^{-1}; mobile phase: water:acetonitrile (30:70, v/v); detection wavelength: 210 nm. Eluted products were analyzed by UV–Vis and ESI-TOF. The reaction between the alliinase/LFS complex and petiveriin was also conducted in 10 mM phosphate buffer prepared with D_2O, and the HPLC eluted products were analyzed by ESI-TOF.

Acknowledgements

The authors thank Distinguished Professor Dr. Eric Block for helpful suggestions and critical reading of the manuscript. The financial support of the National Science Foundation (Grant #0239755 to R.A.M.) and the Research Foundation of SUNY are gratefully acknowledged.

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