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Discovery and Characterization of a Novel Lachrymatory Factor Synthase (LFS) in *Petiveria alliacea* and its Influence on Alliinase-mediated Formation of Biologically Active Organosulfur Compounds.

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Abstract

A novel lachrymatory factor synthase (LFS) was isolated and purified from the roots of the Amazonian medicinal plant *Petiveria alliacea*. The enzyme is a heterotetrameric glycoprotein comprised of two $\alpha$ subunits (68.8 kD each), and $\gamma$ (22.5 kD) and $\delta$ (11.9 kD) subunits. The two $\alpha$ subunits are glycosylated and connected by a disulfide bridge. The $\alpha$, $\gamma$, and $\delta$ subunits are shared in common with the $\alpha$, $\gamma$, and $\delta$ subunits of *P. alliacea* alliinase. The LFS has an isoelectric point of 5.2. It catalyzes the formation of a sulfine lachrymator, (Z)-thiobenzaldehyde S-oxide, only in the presence of *P. alliacea* alliinase and its natural substrate, S-benzyl-L-cysteine sulfoxide (petiveriin). Depending on its concentration relative to that of *P. alliacea* alliinase, the LFS sequesters, to varying degrees, the sulfenic acid intermediate formed by alliinase-mediated breakdown of petiveriin. At an LFS to alliinase ratio of 5:1, LFS sequesters all of the sulfenic acid formed by alliinase action on petiveriin, and converts it entirely to thiobenzaldehyde S-oxide. However, starting at an LFS to alliinase ratio of 5:2, the LFS is unable to sequester all of the sulfenic acid produced by the alliinase, with the result that sulfenic acid that escapes the action of the LFS, condenses with loss of water to form S-benzyl phenylmethanethiosulfinate (petivericin). The results show that the LFS and alliinase function in tandem, with the alliinase furnishing the sulfenic acid substrate on which the LFS acts. The results also show that the LFS modulates the formation of biologically active thiosulfinates that are downstream of the alliinase, in a manner dependent upon the relative concentrations of the LFS and the alliinase. These observations suggest that manipulation of LFS to alliinase ratios in plants displaying this system, may provide a means by which to rationally modify organosulfur small molecule profiles to obtain desired flavor and/or odor signatures, or increase the presence of desirable biologically active small molecules.
Introduction

Lachrymatory factor synthase (LFS) is the term coined to refer to the recently discovered enzyme shown to catalyze the formation of the sulfine responsible for the lachrymatory effect of onion, thiopropanal S-oxide (TPSO) (Imai et al., 2002). Until the discovery of the onion LFS, the formation of the onion lachrymatory factor (LF) was thought to be mediated by only a single enzyme, onion alliinase. Alliinases, which are pyridoxal 5'-phosphated dependent cysteine sulfoxide lyases most often found in members of the Allium genus, catalyze the breakdown of cysteine sulfoxide derivatives to yield fleeting sulfenic acid intermediates, and α-aminoacrylic acid (Scheme 1) (Block, 1992; Shimon et al., 2007). Once formed, the sulfenic acids are most often observed to spontaneously condense, with loss of water, to form thiosulfinates, whereas the α-aminoacrylic acid is further hydrolyzed, with loss of ammonia, to form pyruvate. The S-substituted cysteine sulfoxides that are acted upon by alliinases differ from one another by the identity of the sulfur-bound R group. In Allium plants, the R groups are alk(en)yl, with R = methyl, and 2-propenyl appearing in large quantities in garlic, and R = methyl, and (E)-1-propenyl preponderating in onion (Scheme 1). The cysteine sulfoxide that serves as the precursor to the onion lachrymator is (E)-S-(1-propenyl)-L-cysteine sulfoxide (isoalliin). It is structurally distinct from other naturally occurring S-substituted cysteine sulfoxides so far reported, in that it is α,β-unsaturated. This structural feature affords its corresponding sulfenic acid the possibility of undergoing a [1,4]-sigmatropic rearrangement that, in principle, would furnish the onion lachrymator, TPSO. Indeed, the formation of the onion lachrymator was proposed to occur by such a mechanism (Scheme 2) (Block, 1992). Thus, it was surmised that were the α,β-unsaturation to be absent in the precursor S-substituted cysteine sulfoxide, the [1,4]-sigmatropic rearrangement that would lead to sulfine formation could not occur. Consequently, it was not surprising that other S-substituted cysteine sulfoxides constitutively present in garlic, onion, and other alliinase containing plants, but devoid of this α,β-unsaturation in the sulfur-bound R group, did not themselves yield lachrymators on plant tissue wounding. It has since been discovered, however, that formation of the onion
lachrymator is not catalyzed by onion alliinase, but instead by a novel class of enzyme—lachrymatory factor synthase (LFS). Imai et al. (2002) observed that although a crude preparation of onion alliinase yielded both the LF and the corresponding thiosulfinate, the protein fraction with lachrymator forming ability could be completely separated from that with alliinase activity, by passing the crude onion protein preparation through a hydroxyapatite column. The LFS was subsequently purified and shown to be highly substrate specific, producing the LF from only isoalliin, which is (E)-S-(1-propenyl)-L-cysteine sulfoxide that occurs constitutively in onion. Interestingly, the LF was detected only when three components, namely, purified onion alliinase, isoalliin, and the onion LFS, were present in the reaction mixture simultaneously (Imai et al., 2002). Omission of the LFS from the reaction mixture resulted in an increased yield of thiosulfinates, but no LF. Although the complete cDNA sequence of the onion LFS has been determined (Imai et al., 2002), to our knowledge, full biochemical characterization of the enzyme has yet to be reported.

In the course of our studies on organosulfur chemistry in non-Allium plants, we isolated and characterized the S-benzyl-L-cysteine sulfoxides (petiveriins) and S-(2-hydroxyethyl)-L-cysteine sulfoxides (2-hydroxyethiins) from the Amazonian medicinal plant Petiveria alliacea (Figure 1) (Kubec and Musah, 2001; Kubec et al., 2002). These compounds are S-substituted cysteine sulfoxide derivatives with R = benzyl and 2-hydroxyethyl respectively, that had never before been isolated from plants. We showed that, as has been observed in garlic and onion, symmetrical and mixed thiosulfinate derivatives of the corresponding petiveriin and 2-hydroxyethiin precursors could be extracted with ether solvent (Figure 1) (Kubec et al., 2002) upon root tissue disruption. We have also shown that an alliinase that mediates the transformation of the petiveriins and 2-hydroxyethiins to their corresponding thiosulfinates is present in P. alliacea (He et al., 2009). Interestingly, while working with P. alliacea root extracts, we noted the presence of a potent lachrymator which we subsequently determined to be a sulfine—thiobenzaldehyde S-oxide (TBSO) (Figure 2) (Kubec et al., 2003). However, the biochemical precursor of TBSO and the pathway(s) leading to its formation upon disruption of P. alliacea tissue, remain to be
determined. Given that the onion LF (TPSO), whose formation is mediated by LFS, is also a sulfine, we were prompted to investigate the possibility of the presence of a LFS in *P. alliacea*. In this report, we describe our confirmation of the existence of a LFS in *P. alliacea*, and detail, for the first time, biochemical characterization of this novel class of enzyme.

**RESULTS**

**Confirmation of the Existence of a LFS in *P. alliacea***

Since TBSO production was only observed when LFS was exposed to both petiverin and *P. alliacea* alliinase, the presence of the LFS in protein fractions was determined by tracking which protein fractions, when combined with a solution of petiverin and *P. alliacea* alliinase in buffer, reliably produced TBSO, as monitored by reversed phase C-18 HPLC (Figure 3). Over the course of these experiments, a chromatographic protocol was developed that resulted in the isolation of purified LFS whose activity in the production of LFS could be completely separated from the activity of the *P. alliacea* alliinase.

**Isolation and Purification of *P. alliacea* LFS**

The LFS enzyme was purified from homogenized *P. alliacea* roots through the sequential use of ammonium sulfate precipitation, and anion exchange, hydroxyapatite (twice) and gel filtration chromatographies. The results of a typical purification of the *P. alliacea* LFS are summarized in Table I. A crude protein sample derived from 60% ammonium sulfate precipitation of a 150 g *P. alliacea* root macerate in phosphate buffer, was subjected to anion exchange chromatography. The protein eluted between 65 mM and 120 mM NaCl. The eluent was subjected to hydroxyapatite chromatography (second column) where it eluted between 160 mM and 220 mM phosphate. Further purification of the eluent obtained from the first hydroxyapatite column by a second hydroxyapatite column yielded the LFS, which eluted between 88 mM and 134 mM phosphate. The LFS eluent from the second hydroxyapatite chromatographic separation was subjected to further purification by
gel filtration chromatography (fourth column), which furnished purified LFS.

Analysis of the protein by native PAGE using Coomassie Blue G-250 stain showed a single band (Figure 4, Panel B), indicating the success of the purification. The diffuse appearance of the band even at low protein concentrations suggested that the protein might be glycosylated, which was confirmed through the “in-gel” detection of carbohydrate by oxidation of the protein-bound sugars within the gel to aldehydes, followed by reaction of the aldehyde with a hydrazide, which produced an easily detectable fluorescent conjugate (Figure 4, Panel C). The molecular mass of the protein was determined by Ferguson Plot analysis to be 217.7 kD (Figure 5), and its isoelectric point was observed by chromatofocusing to be 5.2 (Figure 6). SDS PAGE analysis in the absence of β-mercaptoethanol (BME) (Figure 7, Panel B) showed three bands termed: α' (136.0 kD), γ (22.5 kD), and δ (11.9 kD). In the presence of BME, SDS PAGE analysis revealed that although the γ and δ bands were retained, the α' band had collapsed to a new band, termed α, of molecular mass 68.8 kD (Figure 7, Panel D). Thus, in the native protein, two α subunits are linked together by a disulfide bond to form the subunit α'. In-gel fluorescent glycoprotein detection indicated that the source of the glycosylation observed by native PAGE were the α' subunits (Figure 7, Panels C and E).

Characterization of *P. alliacea* LFS Activity

The conditions under which LFS mediates the formation of TBSO, were determined through monitoring the formation of TBSO under a variety of conditions by HPLC. Specifically, TBSO formation as a function of (a) ratio of LFS to alliinase; and (b) as a function of LFS in the presence of petiveriin, thiosulfinate (i.e. petivericin) and/or *P. alliacea* alliinase, was determined. The results of these experiments are shown in Figure 8. In the absence of LFS, alliinase, when exposed to petiveriin, produced only S-benzyl phenylmethanethiosulfinate (petivericin) (Figure 8, Panel A). LFS, in the presence of petiveriin or petivericin, but in the absence of alliinase, produced no product (Figure 8, Panels B and C). When petiveriin was exposed to LFS and alliinase in a ratio of 5:1, only TBSO, along with its hydrolysis product,
benzaldehyde, were formed (Figure 8, Panel D). When the ratio of LFS to alliinase was changed to 5:2, and in the presence of petiverin, the amount of TBSO and benzaldehyde increased, compared with the case when the LFS to alliinase ratio was 5:1, and trace amounts of petivericin were observed (Figure 8, Panel E). With a change in the LFS to alliinase ratio to 5:6, substantial amounts of both petivericin and TBSO were formed (Figure 8, Panel F).

DISCUSSION

In this work, we report the successful isolation and purification of an enzyme exhibiting activity similar to that observed for the lachrymatory factor synthase in onion (Figure 3). The protein, purified to homogeneity, exhibits a single diffuse band by native PAGE (Figure 4, Panel B). The protein was purified from pulverized root extracts by a sequence of steps comprised of ion exchange, hydroxyapatite and gel filtration chromatographies (Table 1).

SDS PAGE analysis of the protein showed it to be comprised of a total of four subunits: two α subunits connected via a disulfide bond, and two additional subunits, termed γ and δ (Figure 4). The molecular mass estimates based on SDS PAGE in the absence of BME, from largest to smallest, are ~138 kD (from two α subunits of ~69 kD each), 22.5 kD, and 11.9 kD for the α', γ and δ subunits respectively. This gives a molecular mass estimate for the whole protein of ~172 kD. However, the molecular mass of the protein observed by Ferguson plot analysis was 217.7 kD (Figure 5), ~46 kD more than was estimated by SDS PAGE. This discrepancy was not unexpected. It is well documented that glycoproteins as well as proteins with rigid disulfide linkages often exhibit anomalous migration profiles in SDS PAGE (Segrest et al., 1971; Marciani et al., 1978). Additionally, we observed similar discrepancies with molecular weight determination for P. alliacea alliinase, which shares with the P. alliacea LFS, four of its five protein subunits (He et al., 2009). In-gel carbohydrate detection experiments showed that the α-subunit is glycosylated (Figure 4, Panel C). The isoelectric point of the LFS was determined by chromatofocusing to be 5.2 (Figure 6).

After comparison of the SDS PAGE results obtained for the LFS with those
determined for the *P. alliacea* alliinase reported in the accompanying paper, we were surprised to observe that the LFS appears to be identical to the alliinase, with the exception of the absence in the LFS of the β subunit that is present in the alliinase (Figure 7, Panel F)! The native PAGE of both proteins shows an α’ subunit (130~140 kD) which, in the presence of BME, collapses into a single band of molecular mass 68~69 kD, and both proteins exhibit two additional subunits of similar molecular mass (~24 kD and ~13 kD for the γ and δ subunits respectively). It is possible that these subunits have very slight differences that are a consequence of alternative gene splicing. This would result in small differences in protein sequences that would yield unique protein isoforms. However, the low level of resolution of the SDS PAGE method used here does not permit us to state this with certainty. Additionally, the limited availability of protein did not allow us to explore this hypothesis further. Our results may imply though, that each of these units is encoded by a single gene. Indeed, a single gene product can be a member of multiple complexes, such that the same protein or peptide can be utilized in different ways, depending upon the other peptides or proteins with which it is complexed. Surprisingly, even though the *P. alliacea* alliinase contains all the subunits of which the LFS is comprised, it is totally devoid of LFS activity. Likewise, the presence in the LFS of four of the five subunits contained in the *P. alliacea* alliinase does not render the LFS able to catalyze the breakdown of *S*-substituted cysteine sulfoxides! These results are in sharp contrast to what has been shown in onion. Unlike the *P. alliacea* LFS, that of onion is a monomeric protein of ~18.6 kD, whose primary structure is totally unlike that of the onion alliinase, which functions as a monomer, or homomultimer (Clark *et al.*, 1998). Since detailed biochemical characterization of the onion LFS has heretofore not been reported, we are unable to further compare and contrast the two LFS enzymes.

The isoelectric point of the *P. alliacea* LFS was determined to be 5.2 by chromatofocusing (Figure 6), higher than the 4.78 value observed for the *P. alliacea* alliinase. This fact facilitated separation of the two proteins during the purification process. When the protein precipitate obtained by treatment of macerated *P. alliacea* roots with ammonium sulfate was subjected to anion exchange chromatography, the
pH used was 7.6. Thus, the LFS with its higher pI would be expected to have less affinity for the ion exchange column than the alliinase, with the consequence that it should elute at a lower salt concentration than the alliinase. This was observed to be true, with the LFS eluting at 65 to 120 mM NaCl, and the alliinase eluting at 220 to 265 mM NaCl.

When petiveriin was exposed to *P. alliacea* alliinase with no LFS present, no LF (i.e., TBSO) was formed, and only the expected thiosulfinate petivericin was observed (Figure 8, Panel A). This is analogous to what has been reported for onion (Imai et al., 2002). When the LFS was incubated with petiveriin, as expected, no products were formed (Figure 8, Panel B). Exposure of petivericin to the LFS in the absence of alliinase also did not result in the formation of any product (Figure 8, Panel C). Although we expected that exposure of petiveriin to a combination of LFS and alliinase would yield the LF, we were surprised by the observation that the relative amounts of petivericin and TBSO shifted as a function of LFS to alliinase ratios. Thus, when the ratio of LFS to alliinase was 5:1, only TBSO and its decomposition product, benzaldehyde, were observed (Figure 8, Panel D). When the LFS to alliinase ratio was reduced to 5:2, increased amounts of TBSO and benzaldehyde were observed, along with a trace amount of petivericin (Figure 8, Panel E). When the LFS to alliinase ratio was further reduced to 5:6, substantial amounts of both TBSO and petivericin were formed (Figure 8, Panel F).

Given that, consistent with what has been observed in onion, the LFS does not act directly on a cysteine sulfoxide precursor, and only forms TBSO when in the presence of alliinase, we conclude that the substrate of the LFS is the sulfinic acid generated by alliinase mediated breakdown of petiveriin (Figure 9). In the absence of LFS, the sulfinic acid produced by alliinase action on petiveriin spontaneously condenses with loss of water to form petivericin (Figure 9A). However, the presence of LFS in the milieu provides the opportunity for a second fate for the sulfinic acid (Figure 9B). When the amount of LFS far exceeds that of the alliinase, all of the sulfinic acid produced by the alliinase is sequestered by the LFS, which catalyzes its conversion to TBSO. Such a scenario is represented in Figure 8, Panel D, in which
the only product produced is TBSO. As the amount of LFS is decreased relative to that of the alliinase (Figure 8, Panels E and F), the LFS is unable to sequester all of the sulfenic acid, and that which escapes condenses to form substantial amounts of petivericin. The effective entrapment of the sulfenic acid by the LFS to form TBSO exclusively, implies that the LFS and alliinase function not only in tandem, but are in close proximity, and many in fact be conjoined in the intact plant tissue.

It is worth noting that our analyses of *P. alliacea* plants from both Florida and the Caribbean have consistently shown the amount of LFS to exceed that of the alliinase, with the actual ratios of the two enzymes varying, depending upon the location and time of the year when the plants are harvested. Indeed, prior to our appreciation of the extent to which TBSO and petivericin formation were influenced by the ratio of LFS to alliinase, we assumed, in every case in which root tissue disruption did not result in formation of significant quantities of petivericin, that the alliinase that mediates its formation had been destroyed or inactivated by some unknown mechanism. Our studies have now revealed that this notion was erroneous, and that the presence of a large excess of LFS relative to the alliinase, can effectively eclipse thiosulfinate formation. In such cases, it is the formation of the sulfine lachrymator (TBSO), rather than that of thiosulfinate, that serves as evidence of the presence of the alliinase, since the alliinase is what furnishes the sulfenic acid substrate that is acted upon by the LFS to produce the sulfine.

It is worth mentioning that although both onion and *P. alliacea* lachrymatory factor synthases mediate formation of sulfines (TPSO and TBSO respectively), there is a significant difference in their action. The onion LFS catalyzes only a rearrangement of propenesulfenic acid into the isomeric sulfine (TPSO). On the other hand, the *P. alliacea* LFS mediates the dehydrogenation of benzylsulfenic acid to yield the sulfine (TBSO, Figure 10).

Our observations may have important implications for onion, the plant in which the presence of a LFS was first reported. The ability to rationally modify the organosulfur small molecule profile of agriculturally and/or medicinally important plants is a desired goal, permitting as it would, the capability of directing the cascade
of enzyme-mediated reactions, to produce a range of predicted secondary compounds with unique and desirable sensory, odor, and flavor notes, as well as health promoting attributes. Although, to our knowledge, biochemical characterization of the onion LFS has not been reported, the connection between the discovery of the LFS, and the possibility of creating an onion whose pungency can be reduced and thiosulfinate profile modified, through down-regulation of the onion LFS gene, has not been lost on researchers. For example, Eady et al. (2008) have shown that RNAi silencing of the onion LFS gene yields an onion with substantially reduced pungency, this attribute being replaced by a sweeter aroma reminiscent of cooked onions. Because of the dramatically reduced level of the LFS in the genetically modified onion, much of the isoalliin-derived sulfenic acid remained unsequestered by the LFS. As predicted, this resulted in the formation of much higher concentrations of isoalliin-derived thiosulfimates, which ultimately reacted further to form zwiebelanes, which were present in high concentrations, relative to what is observed in regular onion. Zwiebelanes have been shown to possess antiplatelet aggregation activity (Block, 1992; Block et al., 1996; Keusgen, 2002). In fact, the bulk of the plethora of biological activities reported for Allium crops such as garlic and onion, and their extracts, have been ascribed to the organosulfur small molecule derivatives of precursor S-substituted cysteine sulfoxides, most notably, the thiosulfimates, and complex compounds that are formed from them that appear further downstream, such as di-, tri- and polysulfides, cepaenes, and the aforementioned zwiebelanes. These molecules are reported to exhibit cardiovascular effects, antimicrobial activity, and anticancer activity (Bayer et al., 1989; Dorsch et al., 1990; Block, 1992; Iranshahi et al., 2008). We have shown that petivericin, and other thiosulfimates produced from P. alliacea S-substituted cysteine sulfoxide precursors, also exhibit significant antibacterial and antifungal activity (Kim et al., 2006). As increased information becomes available on the unique structural features that render many of these organosulfur derivatives biologically active, it will become increasingly desirable to have ways in which molecules with the desired features can be acquired. Our results suggest that in addition to RNAi suppression of LFS gene expression, another way in
which to modify the pungency and thiosulfinate profile (and by extension the formation and identity of other organosulfur molecules further downstream) in plants containing LFS/alliinase-type systems, may be through manipulation of LFS to alliinase ratios. Studies on the possibility of influencing the thiosulfinate/LF profile in *P. alliacea* and onion, and the effects that such manipulations may have on organosulfur small molecule signatures, are currently underway.

**Materials and Methods**

**Plant and Materials.**
Unless otherwise noted, all chemicals were obtained from 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 Barbados. They were collected in Vero Beach, Indian River County, 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.

**Reference Compounds**
*S*-Substituted-*L*-cysteines and the corresponding *S*-substituted-*L*-cysteine sulfoxides, as well as petivericin, were synthesized according to the method of Kubec and Musah (2001) and Kubec *et al.* (2002).

**Analytical Methods**
HPLC separations were performed on a Dynamax SD-200 binary pump system, employing a Varian PDA 330 detector (Varian, Palo Alto, CA, USA).

**Native PAGE Analysis**
Native PAGE was performed according to the method of Davis (1964) with 10% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA). Prestained SDS-PAGE Broad Range Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as molecular mass markers. One part protein sample was mixed with two parts sample buffer. The sample buffer was comprised of 62.5 mM Tris, 40% (v/v) glycerol, and 0.01% (w/v) bromophenol blue at pH 6.8. The running buffer was comprised of 0.1 M Tris and 0.1 M Tricine at pH 8.3. The gel was run at a constant voltage of 120 V, with a starting current of 63 mA, to a final current of 32 mA. The total runtime was 45 min.

**SDS PAGE Analysis**

SDS-PAGE was carried out by the method of Laemmli (1970) using 10% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA). Prestained SDS PAGE Broad Range Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as molecular mass markers. One part protein sample was mixed with two parts sample buffer, and the resulting solution was heated at 100 °C for 15 min. The sample buffer was made from 980 μL Tricine Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) and 20 μL BME. The running buffer was comprised of 0.1 M Tris, 0.1 M Tricine, and 0.1% (w/v) SDS at pH 8.3. The gel was run at a constant voltage of 120 V, with a starting current of 63 mA, to a final current of 32 mA. The total runtime was 45 min.

**In-gel Staining of Proteins**

To detect the proteins, Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories, Hercules, CA, USA) was used after native PAGE and SDS PAGE.

**Molecular Mass Determination**

Ferguson plot analysis (Ferguson, 1964) was used to determine the molecular mass of *P. alliacea* LFS: Bio-Rad’s Precision Plus Protein All Blue Standards (50 kD to 150 kD range) were used as the molecular mass markers. Native PAGE, according to the method described above, was run at three different gel concentrations (5%, 10%,...
The molecular mass of the protein was estimated from plotting the logarithm of the protein’s mobility as a function of the gel concentration. The slope of the resulting line, i.e. the retardation coefficient ($K_r$), is proportional to the protein’s molecular mass. The subunit molecular masses were estimated by SDS-PAGE as already described.

**Isoelectric Point Measurement**

The isoelectric point was determined using a chromatofocusing column (5.0 i.d. × 200 mm Mono P™ 5/200 GL, Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s specifications. The pH interval was 5.7 to 3.5. The flow rate was 0.5 mL min⁻¹. The UV monitor wavelength was set at 280 nm.

**Carbohydrate Detection**

Protein glycosylation was detected using a high sensitivity fluorescent glycoprotein detection kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s specifications. Following PAGE, the proteins were fixed in the gel with a acetic acid:methanol:water (3:50:47, v/v/v) solution. The carbohydrates on the proteins are oxidized to aldehydes with periodic acid. A hydrazide dye was reacted with the aldehydes, forming a stable fluorescent conjugate which was viewed using a standard fluorescent UV-transilluminator with emission at 312 nm.

**Purification of LFS from *P. alliacea***

*P. alliacea* LFS was purified following a procedure modeled after published protocols for the isolation of alliinase from shiitake mushrooms (Kumagai et al., 2002), and LFS from onion (Imai et al., 2002). All steps were carried out at 4 °C. Fresh *P. alliacea* roots (150 g) were carefully cleaned in water, and homogenized with a blender in 400 mL of 20 mM phosphate buffer at pH 7.0, containing 20 μM PALP, 10% (v/v) glycerol, 5% (w/v) NaCl, 5% (w/v) polyvinylpolypyrrolidone, 5 mM EDTA and 0.05% (v/v) BME. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 15,000 × g for 30 min. The 250 mL of supernatant obtained was
brought to 60% (w/v) saturation with ammonium sulfate. The precipitate obtained after centrifuging at 15,000 × g for 30 min was resuspended in 15 mL of 20 mM Tris-HCl at pH 7.6, containing 20 μM PALP and 10% (v/v) glycerol, and dialyzed overnight against the same buffer. The dialysate obtained (20 mL) was centrifuged at 10,000 × g for 10 min, and then applied by HPLC in 5.0 mL aliquots to a HiPrep™ 16/10 DEAE FF column (16 i.d. × 100 mm, Amersham Biosciences, Piscataway, NJ, USA) that had been pre-equilibrated with 20 mM Tris-HCl buffer at pH 7.6 containing 10% (v/v) glycerol. The protein sample was washed first with 65 mL 20 mM Tris-HCl buffer at pH 7.6, containing 10% (v/v) glycerol. Then the column was washed again using a linear gradient, over 100 min as follows: solvent A: 20 mM Tris-HCl buffer at pH 7.6 containing 20 μM PALP, 10% (v/v) glycerol; solvent B: 20 mM Tris-HCl buffer at pH 7.6 containing 20 μM PALP, 10% (v/v) glycerol, and 280 mM NaCl. Finally, the column was washed with 30 mL of 20 mM Tris-HCl buffer at pH 7.6 containing 20 μM PALP, 10% (v/v) glycerol and 280 mM NaCl. The flow rate for this column was 2.0 mL min⁻¹. Active fractions (see Enzyme activity detection) were pooled and concentrated to 4.0 mL using a Millipore centrifugal filter (molecular mass cutoff of 30 kD), and then dialyzed overnight against 20 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol. The dialysate obtained was applied in 2 mL aliquots to a hydroxyapatite column (15 i.d. × 113 mm, Bio-scale CHT20-I, Bio-Rad Laboratories, Hercules, CA, USA) that had been pre-equilibrated with 20 mM phosphate buffer at pH 7.0, containing 20 μM PALP and 10% (v/v) glycerol. The column was washed first with 60 mL 20 mM phosphate buffer at pH 7.0, containing 20 μM PALP and 10% (v/v) glycerol, then with the following solvent combination over a linear gradient lasting 100 min: solvent A: 20 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol; solvent B: 400 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol, and finally with 30 mL 400 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol. The flow rate for this column was 1.0 mL min⁻¹. Active fractions were pooled and concentrated to 4.0 mL using a Millipore centrifugal filter (molecular mass cutoff of 30 kD), then dialyzed overnight against a 20 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol. The
dialysate obtained was applied to the same hydroxyapatite column that had been equilibrated with 20 mM phosphate buffer pH 7.0, containing 20 μM PALP and 10% (v/v) glycerol. The column was washed first with 60 mL of 20 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol, then with the following solvent combination over a linear gradient lasting 100 min: solvent A: 20 mM phosphate buffer at pH 7.0 containing 20 μM PALP, 10% (v/v) glycerol; solvent B: 200 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol, and finally with 30 mL of 200 mM phosphate buffer at pH 7.0 containing 20 μM PALP and 10% (v/v) glycerol. The flow rate for this column was 1.0 mL min⁻¹. Active fractions were pooled and concentrated to 2.6 mL. The resulting sample was applied in 200 µL aliquots to a gel filtration column (7.8 i.d. × 300 mm, Bio-Sil SEC-250, Bio-Rad Laboratories, Hercules, CA, USA) that had been pre-equilibrated with 20 mM phosphate buffer at pH 6.8 containing 0.15 M NaCl and 10% (v/v) glycerol. The loaded protein was washed with the same phosphate buffer at a flow rate of 1.0 mL min⁻¹. Active eluents were pooled and concentrated to 2.6 mL using a Millipore centrifugal filter (molecular mass cutoff of 30 kD). Protein concentrations were estimated based on the sample’s absorbance at 260 nm (A₂₆₀) and 280 nm (A₂₈₀) at a 1.0 cm path length, using the following equation: protein concentration (mg mL⁻¹) = 1.55 × A₂₈₀ - 0.75 × A₂₆₀ (Simpson, 2004).

**Enzyme activity detection**

LFS activity detection was carried out in vitro in solution with the substrate petiveriin. The reaction mixture in 10 mM phosphate buffer, pH 8.0 (in a total volume of 1.0 mL), was comprised of 1.5 mM petiveriin, 25 μM pyridoxal 5’-phosphate (PALP), 0.1 mM NAD⁺, 1.0 μg purified alliinase (~6.9 nM) and 20 to 60 μL of LFS extract in the protein concentration range of 0.4~7.6 mg mL⁻¹, depending on the stage of purification and activity of the sample (see Purification of LFS from *P. alliacea*). The mixtures were incubated for 10 min at room temperature, and then 10 to 20 μL of the reaction solution was analyzed by HPLC using an analytical RP C-18 column (Microsorb-MV 100Å, 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. Under these conditions, benzaldehyde, the TBSO hydrolysis product elutes at 3.75 min, whereas TBSO elutes at 3.94 min. Both are observed if the fraction being analyzed exhibits LFS activity. The concentration of TBSO and benzaldehyde were calculated from their molar extinction coefficients (4,387 L mol$^{-1}$ cm$^{-1}$ for TBSO and 11,040 L mol$^{-1}$ cm$^{-1}$ for benzaldehyde) at 210 nm. The identity of eluted TBSO and benzaldehyde peaks were verified by ESI-HRMS and by comparison with authentic compounds.

**Enzyme substrate determination.** The enzyme substrate was determined by a method similar to that described above (see Enzyme activity detection) except that: 1) the amount of purified LFS used was 5.7 µg (~34 nM), and several different LFS to *P. alliacea* alliinase molar ratios (5/0, 5/1, 5/2, 5/6, and 0/6) were used; and 2) the reaction mixture was incubated for 10 min with and without petiveriin and petivericin (0.25 mM).

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Figure Legends

**Scheme 1.** Alliinase mediated formation of thiosulfimates from cysteine sulfoxide precursors (Block, 1992; Shimon et al., 2007). Alliin: S-allyl-L-cysteine sulfoxide; PRENCSO: (E)-S-(1-propenyl)-L-cysteine sulfoxide; MCSO: S-methyl-L-cysteine sulfoxide; PCSO: S-propyl-L-cysteine sulfoxide.

**Scheme 2.** Mechanism advanced by Block (1992) to account for formation of the onion lachrymator, thiopropanal S-oxide (TPSO). Alliinase bound pyridoxal 5'-phosphate forms a Schiff base with bound isoalliin. General base catalysis at the active site yields an α,β-unsaturated sulfenic acid that can undergo a [1,4]-sigmatropic rearrangement to furnish the sulfine.

**Figure 1.** Cysteine sulfoxides and their corresponding thiosulfinate derivatives isolated from the Amazonian medicinal plant *P. alliacea*. The breakdown of the cysteine sulfoxides is mediated by *P. alliacea* alliinase.

**Figure 2.** Lachrymatory sulfine isolated from *P. alliacea*.

**Figure 3.** Confirmation of the production of TBSO and benzaldehyde by *P. alliacea* LFS action on the benzyl sulfenic acid generated through *P. alliacea* alliinase catalyzed breakdown of petiveriin, by RP C-18 HPLC. A 10 µL aliquot of a reaction mixture comprised of 1.0 mL of 10 mM phosphate buffer at pH 8.0, 25 µM pyridoxal 5'-phosphate, 0.1 mM NAD⁺, 1.5 mM petiveriin, and a *P. alliacea*-derived protein was, after incubation for 10 minutes at rt, analyzed by HPLC (flow rate: 1.0 mL min⁻¹; mobile phase: water:acetonitrile (30:70, v/v); detection wavelength: 210 nm).
**Figure 4.** Native PAGE characterization of *P. alliacea* LFS. Panel A: native PAGE molecular weight marker. Panel B: native PAGE with Coomassie blue staining showing the LFS enzyme purified to homogeneity. Panel C: native PAGE of the LFS after oxidation of carbohydrates bound to the protein, followed by treatment with a hydrazide dye to yield a highly fluorescent conjugate (i.e. a positive test for the presence of sugars).

**Figure 5.** Molecular mass determination of *P. alliacea* LFS by Ferguson plot analysis. The retardation coefficient (*Kr*) was derived from the calibration curve constructed by plotting the mobility of the following standards against the gel concentration: A. 50 kD protein; B. 75 kD protein; C. 100 kD protein; D. 150 kD protein; E. *P. alliacea* LFS; F. *P. alliacea* LFS molecular mass estimation from the calibration curve of the molecular masses of standards against the retardation coefficient, *Kr*.

**Figure 6.** *P. alliacea* LFS isoelectric point determination by chromatofocusing. The pH interval was 5.7-3.5. The protein was monitored at 280 nm.

**Figure 7.** SDS PAGE analysis of *P. alliacea* LFS and *P. alliacea* alliinase. Panel A: molecular weight marker; Panel B: SDS PAGE of *P. alliacea* LFS in the absence of BME, with Coomassie blue staining showing the three bands representing the subunits of which *P. alliacea* LFS is comprised; Panel C: SDS PAGE of *P. alliacea* LFS in the absence of BME after oxidation of carbohydrates bound to the protein, followed by treatment with a hydrazide dye to yield a highly fluorescent conjugate. The results show that the α' subunit is glycosylated; Panel D: SDS PAGE of *P. alliacea* LFS in the presence of BME showing that the α' band in Panel B collapses into the α band seen in Panel D; Panel E: SDS PAGE of *P. alliacea* LFS in the presence of BME after oxidation of carbohydrates bound to the protein, followed by treatment with a hydrazide dye to yield a highly fluorescent conjugate. The results show that the α subunit is glycosylated; Panel F: SDS PAGE of *P. alliacea* alliinase in the absence of BME showing the bands representing the subunits of which *P. alliacea* alliinase is
comprised; Panel G: SDS PAGE of *P. alliacea* alliinase in the presence of BME showing that the $\alpha'$ band in Panel F collapses into the $\alpha$ band. Comparison of Panel's D and G reveal that the *P. alliacea* LFS shares with the *P. alliacea* alliinase subunits $\alpha$, $\gamma$ and $\delta$, but not its $\beta$ subunit; Panel H: SDS PAGE of *P. alliacea* alliinase in the presence of BME after oxidation of carbohydrates bound to the protein, followed by treatment with a hydrazide dye to yield a highly fluorescent conjugate. The results show that it's $\alpha$ and $\beta$ subunits are glycosylated.

**Figure 8.** Substrate determination for *P. alliacea* LFS using RP C-18 HPLC. A 20 $\mu$L of a reaction mixture comprised of 1.0 mL of 10 mM phosphate buffer, pH 8.0, 25 $\mu$M PALP, 0.4 mM NAD$^+$, 1.5 mM petiveriin (or 0.25 mM petivericin), and 5.7 $\mu$g of purified LFS (~100 nM) at several LFS to alliinase molar ratios was incubated for 10 minutes at room temperature and then analyzed by HPLC to reveal what products, if any, were formed.

**Figure 9.** Fate of the sulfenic acid intermediate produced by alliinase catalyzed breakdown of petiveriin. **A.** When no LFS is present, no TBSO is formed. **B.** When LFS is present, and depending on its concentration relative to that of the alliinase, the benzyl sulfenic acid that is formed can be intercepted by the LFS, and converted to TBSO.

**Figure 10.** The difference in the mode of action of the onion LFS and *P. alliacea* LFS.

**Table I.** Purification of the LFS from 150 g of *P. alliacea* roots.
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![Scheme 1 Diagram](image-url)
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Figure 10. The difference in the mode of action of the onion LFS and *P. alliacea* LFS.
| Fraction               | Volume (ml) | Total Protein (mg) | Total activity (nkat) | Specific activity (nkat mg⁻¹) | Recovery (%) | Purification (fold) |
|------------------------|-------------|--------------------|----------------------|-------------------------------|--------------|---------------------|
| Homogenate             | 230         | 289                | —                    | —                             | —            | —                   |
| 60% (NH₄)₂SO₄          | 20          | 152                | —                    | —                             | —            | —                   |
| Anion exchange         | 4.0         | 26                 | 140                  | 5.5                           | 100          | 1.0                 |
| Hydroxyapatite (1ˢᵗ)   | 4.0         | 9.2                | 58                   | 6.3                           | 40           | 1.1                 |
| Hydroxyapatite (2ⁿᵈ)   | 2.6         | 2.9                | 23                   | 7.9                           | 16           | 1.4                 |
| Gel filtration         | 2.6         | 1.0                | 12                   | 12                            | 8            | 2.2                 |

¹ 1 nkat = 1 nmol TBSO s⁻¹