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Enzyme That Makes You Cry—Crystal Structure of Lachrymatory Factor Synthase from *Allium cepa*

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Abstract

The biochemical pathway that gives onions their savor is part of the chemical warfare against microbes and animals. This defense mechanism involves formation of a volatile lachrymatory factor (LF) ((*Z*)-propanethial *S*-oxide) that causes familiar eye irritation associated with onion chopping. LF is produced in a reaction catalyzed by lachrymatory factor synthase (LFS). The principles by which LFS facilitates conversion of a sulfenic acid substrate into LF have been difficult to experimentally examine owing to the inherent substrate reactivity and lability of LF. To shed light on the mechanism of LF production in the onion, we solved crystal structures of LFS in an apo-form and in complex with a substrate analogue, crotyl alcohol. The enzyme closely resembles the helix-grip fold characteristic for plant representatives of the START (star-related

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M.G. designed the experiments. J.A.S. and M.J.P. expressed, purified, and crystallized LFS. S.B., P.D.K., and M.G. collected X-ray diffraction data. All authors contributed to the data analyses. J.A.S., M.J.P., and M.G. wrote the manuscript with valuable input from S.B. and P.D.K.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.7b00336.

Summary of LFS purification, sequence alignments of LFSs from selected *Allium* species, topology diagrams of onion LFS, structural comparison of LFS and PYL10, and interpretation of electron density maps in the vicinity of the ligand-binding site (PDF)

lipid transfer) domain-containing protein superfamily. By comparing the structures of LFS to that of the abscisic acid receptor, PYL10, a representative of the START protein superfamily, we elucidated structural adaptations underlying the catalytic activity of LFS. We also delineated the architecture of the active site, and based on the orientation of the ligand, we propose a mechanism of catalysis that involves sequential proton transfer accompanied by formation of a carbanion intermediate. These findings reconcile chemical and biochemical information regarding thioaldehyde *S*-oxide formation and close a long-lasting gap in understanding of the mechanism responsible for LF production in the onion.

Graphical Abstract

Plant species that belong to the genus *Allium* include common vegetables such as the onion (*A. cepa*), garlic (*A. sativum*), and leek (*A. porrum*), which are known for their distinct taste and aroma. The origin of this spectrum of flavors is attributed to a conversion of odorless and species-specific precursors, S-alk(en)yl cysteine *S*-oxides to thiosulfonates and other organosulfur compounds.^{1–3} Upon damage to plant tissue, S-alk(en)yl cysteine sulfoxides are enzymatically cleaved by alliinase (E.C. 4.4.1.4) to the corresponding sulfenic acids.^{4–6} These reactive compounds undergo further spontaneous condensation and rearrangement reactions giving rise to a variety of thiosulfonates. In the onion, however, another compound is formed along with thiosulfonates, a volatile small molecule lachrymatory factor (LF) ((*Z*)-propanethial *S*-oxide),⁷ with which we are too familiar for causing inconvenient eye irritation (Figure 1). Thioaldehyde *S*-oxides are very rare in nature. There are only four known natural compounds of this type, all of which are produced by plants: (*Z,Z*)-*d,l*-2,3-dimethyl-1,4-butanedithial *S,S'*-dioxide from *A. cepa*, (*Z*)-butanethial *S*-oxide from *A. siculum*, and (*Z*)-phenylmethanethial *S*-oxide from *Petiveria alliacea*.^{8–11}

Based on the experiments performed in the gas phase, formation of onion's LF was initially suggested to result from a [1,4]-sigmatropic rearrangement of (*E*)-1-propenesulfenic acid.^{12,13} This uncatalyzed intramolecular shift of a hydrogen, which migrates from the oxygen (atom 1) onto carbon (atom 4) is spontaneous; however it is unlikely to occur at RT due to the substantial (33 kcal/mol) energy barrier.¹³ More recent biochemical data clearly indicate that production of LF depends on the activity of a specific enzyme, which was discovered to be lachrymatory factor synthase (LFS).^{14,15} In agreement with the proposed catalytic function of this enzyme, silencing LFS expression resulted in the generation of a “tearless” onion that did not exert eye irritation properties upon disruption of the onion tissue.^{16,17} Importantly, changes in the relative concentration of LFS and alliinase have had a profound effect on the organosulfur metabolite profile in these genetically modified plants.¹⁸

These results directly indicate that alliinase cleaves (*E*)-S-(1-propenyl)cysteine *S*-oxide to provide sulfenic acid substrate for LFS to yield LF (Figure 1).

The enzymatic mechanism by which LFS converts (*E*)-1-propenesulfenic acid into LF is currently unknown. Based on the chemical composition of LF produced in the presence of D₂O, it has been proposed that LFS facilitates an intramolecular proton exchange between the oxygen and the alkene chain.^{12,13,19} However, this model is essentially equivalent to the aforementioned [1,4]-sigmatropic rearrangement and therefore would not require the assistance of a specific enzyme. Notably, a putative catalytic mechanism cannot be inferred from LFS sequence analysis. The enzyme exhibits low sequence similarity ($\approx 18\%$) to the plant pyrabactin resistance-like (PYL) protein family¹⁵ that constitutes a class of abscisic acid receptors and does not contain any catalytically functional domains.²⁰ From an enzymological perspective, the conversion of (*E*)-1-propenesulfenic acid into its corresponding thioaldehyde *S*-oxide signifies a constitutive isomerization reaction that requires shuffling the position of a double bond in the alkene chain without changing the chemical formula of the reactants. This type of enzymatic reaction usually involves protonation/deprotonation steps followed by double bond rearrangement.^{21–23} However, mechanistic studies that could provide unambiguous information regarding LF formation have been hindered by numerous challenges, including inherent reactivity of the substrate (sulfenic acid), volatility and lability of LF, and the necessity for a two enzyme system that involves the presence of alliinase in addition to LFS in the enzymatic assay.²³ To overcome these limitations, we sought an alternative method to determine the mechanism of this reaction. We adopted a structural biology-based approach to elucidate the molecular architecture of LFS and shed light on functional aspects of the unique enzymatic reaction of LF synthesis.

Herein, we provide a mechanistic framework for the production of LF based on high-resolution crystallographic structures of LFS from *A. cepa* in apo-form as well as in complex with a substrate analogue, crotyl alcohol ((2*E*)-but-2-en-1-ol). Based on a comparison to the structurally related PYL proteins, we elucidated the molecular adaptations that enable LFS to utilize an α -grip fold for catalysis. Based on the relative position of the side chains involved in catalysis with respect to the ligand, we propose an alternative catalytic mechanism for LF formation. Thus, our findings offer a robust molecular explanation for the role of the protein scaffold in the conversion of sulfenic acid into *S*-oxide as well as provide evidence for functional diversity of the α -grip fold in plants.

RESULTS AND DISCUSSION

Determination of the LFS Structure

To obtain protein suitable for crystallization trials, LFS was expressed in a truncated form, in which the first 23 amino acids were omitted (Supporting Information Figure 1). Sequence alignment of LFS from several *Allium* species indicated high variability of this N-terminal portion of the enzyme (Supporting Information Figure 2a).¹⁵ Moreover, prediction of the secondary structure of the N-terminus of LFS with PSIPRED (Protein Sequence Analysis Workbench)²⁴ revealed a lack of propensity for a defined fold in this region that could hinder protein crystal formation. Importantly, elimination of this region did not have any

adverse effects on LFS enzymatic activity.¹⁵ Purified LFS produced high-quality crystals that diffracted X-rays to 1.40 Å in the apo-form (Protein Data Bank (PDB) accession 5VGL) and 1.90 Å in complex with crotyl alcohol (PDB accession 5VGS). One protein molecule was found in the asymmetric unit, corresponding to a Matthews volume of 2.2 Å³/Da and 43.0% solvent.²⁵ The initial structure model of apo-LFS was obtained by molecular replacement using BALBES with a final search model based on uncharacterized protein Q6HG14 from *Bacillus thuringiensis* (PDB accession 3F08), which shares 18.9% primary sequence identity with LFS. Subsequent refinement of the structure yielded $R_{\text{work}}/R_{\text{free}}$ values of 15.2/18.9 and 16.7/21.5 for apo- and holo-LFS, respectively. Data collection and refinement statistics are shown in Table 1.

Overall Architecture of the Enzyme

The structure of LFS exhibits a compact fold composed of a seven-stranded antiparallel β -sheet (strands $\beta 1$ – $\beta 7$), which enfolds a long C-terminal α -helix ($\alpha 3$; Figure 2a). Two additional short α -helices ($\alpha 1$ and $\alpha 2$) located between $\beta 1$ and $\beta 2$ complete the structure. The regularity of the extended β -sheet is perturbed by β -bulging at Ser43-Val44, Val79-Ala80, and Thr93-Glu94, which results in the curved shape of the entire β -sheet. The overall 1 β -2 α -6 β -1 α topology closely resembles the helix-grip fold characteristic of the plant START (star-related lipid transfer) protein superfamily (Figure 2b and Supporting Information Figure 2b).^{26,27} Comparison of known structures of START proteins with LFS revealed high similarity to a subclass of the START family called pyrabactin resistance1/PYR1-like/regulatory components of ABA receptors (PYR/PYL/RCAR) that function as intracellular receptors for abscisic acid.^{28–30} The main chain root-mean-square deviation (r.m.s.d) calculated for LFS and PYR/PYL/RCAR proteins represented by PYL1, 2, 3, 5, 9, 10, and 13, for which structural coordinates are available,^{30–35} did not exceed 2.3 Å (Supporting Information Figure 3). Similar to other START proteins, a dominant feature of PYL abscisic acid receptors is a large internal hydrophobic cavity of elongated shape. It is formed by the inside surface of a curved β -sheet and three α -helices. Total volume of this pocket exceeds 500 Å³ in the apo-forms of PYL proteins to accommodate relatively large ligands such as abscisic acid (molecular volume of 254.1 Å³).³⁰ The opening to this binding site is flanked by two β -loops ($\beta 3$ – $\beta 4$ and $\beta 5$ – $\beta 6$) and a linker between $\beta 7$ and $\alpha 3$. They form a gate or entrance portal characteristic for lipid-binding proteins. Notably, in comparison to abscisic acid receptors, the size of the intramolecular cavity in the LFS structure is distinctly reduced to nearly 216 Å³ (Figure 2c,d and Supporting Information Figure 3). The key structural factor that contributes to the reduced size of this pocket is the extended $\beta 3$ – $\beta 4$ loop (Figure 2b). Although spatial positions of the secondary elements are comparable throughout the examined structures, part of $\beta 3$ and $\beta 4$ as well as a loop that connects these two β -strands in LFS revealed major conformational differences that have essential functional consequences for LFS. The main chain of this region appears shifted inward toward the core of the protein and $\alpha 3$ as evident by superimposition of PYL10 and LFS molecules (Figure 2b and Supporting Information Figure 3a–c). Consequently, side chains of two large hydrophobic amino acids (Met77 and Phe84), which are part of the $\beta 3$ – $\beta 4$ loop, protrude into the space that corresponds to the abscisic acid binding site in PYL proteins (Supporting Information Figure 3b,c). In addition, two tryptophan residues, Trp133 and Trp155, limit the volume of the internal cavity in LFS. Reduced size of the binding

pocket in LFS is accompanied by substitution of key residues involved in interaction with abscisic acid in PYL proteins. The carboxyl of abscisic acid forms a salt bridge with the ϵ -amino group of lysine and a water-mediated hydrogen bond network with several side chains of polar residues including two glutamic acids, an asparagine, and a serine.³⁶ In LFS, the lysine residue as well as the cluster of polar amino acids is replaced by hydrophobic methionine, phenylalanine, leucine, and tryptophan residues. Thus, the reduced size of the binding pocket, its much higher hydrophobicity, and the absence of key residues involved in hydrogen bonding with the carboxylic acid group of the ligand in PYL proteins exemplify the primary adaptations of LFS for specific interaction with a relatively small 1-propenylsulfenic acid molecule (molecular volume of 82.8 Å³).

Although the majority of START proteins do not appear to possess any enzymatic activity, and as a result are classified as putative lipid/sterol-binding entities, the helix-grip fold has been found in three plant enzymes of well-established catalytic activities: S-norcoclaurine synthase,^{37,38} TcmN aromatase/cyclase,³⁹ and Hyp-1, an enzyme that catalyzes the formation of hypericin in St. John's wort.^{40,41} Therefore, LFS is among the first illustrations of functional diversity of the START domain in eukaryotes. This combination of enzymatic and non-enzymatic functions makes the helix-grip fold a fascinating example for adaptation of the same molecular architecture for binding diverse ligands and performing efficient catalysis. As demonstrated by comparing LFS to PYL proteins (Figure 2 and Supplemental Figure 3), relatively subtle changes in the geometry of the binding pocket, accompanied by the introduction of polar amino acids in the active site, can have dramatic functional consequences. Thus, systematic analysis of START domain-containing proteins of known structures could provide valuable information regarding the principles of protein/small molecule ligand interaction and evolution of catalysis. The helix-grip fold could also serve as an excellent starting platform for *de novo* design and testing the new enzymes with targeted properties.

Structure of LFS in Complex with Crotyl Alcohol

The intramolecular cavity present in LFS constitutes a putative active site of the enzyme. However, the high propensity for self-condensation represents a major obstacle in obtaining experimental insight into the mode of substrate–enzyme interaction. To overcome this problem, we cocrystallized LFS with crotyl alcohol, a substrate analogue, in which the sulfur atom is substituted with a carbon (Figure 3a). The bound crotyl alcohol was identified from the residual electron density evident in the $F_o - F_c$ map and found to be located centrally in the binding cavity (Supporting Information Figure 4a–c). This electron density was not observed in the crystallographic data obtained for LFS in the absence of the ligand. Comparing the structures with and without crotyl alcohol indicated no conformational changes in the protein structure. Both structures superimpose perfectly with RMSDs of 0.11 and 0.22 Å for the main chain and all atoms, respectively.

The cavity within LFS that harbors the ligand is shielded from the environment by enfolded side chains at the entry portal. Two methionine residues (Met77 and Met143) from the β 3– β 4 and β 7– α 3 loops and a phenylalanine residue (Phe108) located at the β 5– β 6 loop form a nonpolar cap at the presumed entrance to the binding site in the crystal structure (Figure 3b).

It is, however, plausible that the relative position of these side chains depends on the spatial orientation of relatively flexible portal loops allowing spontaneous diffusion of the substrate in and out of the active site. The opposite side of the binding cavity is enclosed predominantly by the polar side chains of $\alpha 2$ and $\alpha 3$. Interestingly, they form a narrow tunnel that leads to the active site. In the crystal structure, this tunnel is occupied by highly ordered water molecules (Figure 3c). Thus, a well-defined water channel connects the active site with the exterior of the enzyme.

Because the crotyl alcohol used for experiments was a mixture of (*Z*)- and (*E*)-isomers, both configurations could potentially bind to the enzyme. Indeed, the overall characteristic of the initial electron density for the ligand seemed to be consistent with this assumption. Subsequent refinement of the structure with either (*Z*)- or (*E*)-crotyl alcohol resulted in an extra electron density adjacent to the ligand that was interpreted as possible binding of both isomers by LFS. The occupancies for the ligand isomers were refined to 57% and 43% with the higher value associated with (*Z*)-crotyl alcohol. Although we found weak evidence for partial occupancy of waters and the alcohol ligand in the active site, the limited resolution of the data precluded definitive occupancy refinement for potential water molecules. The data suggested two distinct modes of crotyl alcohol binding that depend on the geometric configuration of the ligand (Figure 3d,e). The position of the (*Z*)-isomer was determined by hydrogen bonding between the hydroxyl group of the ligand and side chains of two tyrosine residues (Tyr102 and Tyr124). The hydrophobic chain of this ligand projected toward the nonpolar portion of the binding site formed by Leu47, Val73, Phe84, and Trp133. The (*E*)-crotyl alcohol adopted an alternative orientation with its hydroxyl group facing the polar side chains of Glu88 and Arg71 while remaining in proximity (3.3 Å) to the β carboxyl group of Glu88. Thus, the position of this isomer seems to be defined by hydrophobic interaction of the alkene chain with the side chains of Phe84 and -148, Tyr102, Trp133 and -155, and Leu152.

Computational Docking of the Substrate

Although crotyl alcohol was instrumental in mapping the active site of LFS, the promiscuity of the interaction between crotyl alcohol and the enzyme as well as the chemical differences between this analogue and (*E*)-1-propenesulfenic acid may contribute to an alternative binding for the substrate. An additional factor that needs consideration is the geometry of the ligand found in the active site. The only bonds that are not restricted by rotational barriers are C2–C3 in crotyl alcohol and S2–C3 in (*E*)-1-propenesulfenic acid (Figures 3a and 4a). Consequently, they can adopt multiple configurations. Shifting of the C3==C4 double bond of the substrate locks the product of the enzymatic reaction exclusively in the *Z* configuration with respect to the C3==S2 bond (Figure 4a). This fact implies that (*E*)-1-propenesulfenic acid binds in the active site in a preferential configuration enabling formation of only (*Z*)-propanethial *S*-oxide. However, the extended orientation of the oxygen atom in crotyl alcohol bound to LFS would promote formation of product in the (*E*) configuration (Figure 3d,e). Thus, to assess potential differences between the interaction of the enzyme with crotyl alcohol and (*E*)-1-propenesulfenic acid, we conducted a substrate-docking experiment using AutoDock Vina.⁴² Although the docking search area included the whole protein, the software positioned the substrate molecule in the binding pocket

previously defined by crotyl alcohol. Interestingly, despite positional similarities, spatial orientation of docked (*E*)-1-propenesulfenic acid differed from that observed in crotyl alcohol. The substrate's preferred orientation placed the oxygen atom of sulfenic acid in proximity to the carbonyl group of the Glu88 side chain, the hydroxyl moiety of Tyr102, and η^2 amino group of Arg71, engaging the substrate in hydrogen bond interactions (Figure 4b). Trp133 and -155 as well as Phe148 and Leu152 engaged in hydrophobic interactions with the alkene chain. Importantly, the docked substrate preferentially adopted a synperiplanar orientation, which is consistent with the *Z* configuration for the final product of the enzymatic reaction. The differences in position of crotyl alcohol and sulfenic acid are most likely a consequence of elemental differences between these ligands. The atomic radii of a sulfur atom is significantly larger than carbon (70 pm vs 100 pm, respectively).⁴³ Consequently, the bond lengths between C–S and S–O (1.81 and 1.66 Å)⁴⁴ in the sulfenic acid are greater than the corresponding C–C and C–O bonds in crotyl alcohol (1.54 and 1.48 Å).

Mechanistic Insight into LF Formation

Analysis of the crystal structure of LFS enables a detailed view into the mechanism of catalysis and reconciliation of previously reported biochemical data regarding the enzyme's action. The active site architecture unambiguously indicates that two solvent-inaccessible polar amino acids, Glu88 and Arg71, are in close proximity to the substrate molecule. Because the γ carboxyl group of Glu88 is located within hydrogen-bonding distance from sulfenic acid, it is reasonable to propose that the carboxylate oxygen of this side chain is required to polarize the substrate by abstracting an acidic proton through a general base mechanism (Figure 5). Under physiological conditions, glutamic acid carboxylate exists in a resonance form with the negative charge shared between the two oxygens. Interaction with N ϵ of the neighboring Arg71 *via* hydrogen bonding stabilizes a negative charge on one of the oxygens, effectively lowering the p*K*_a of Glu88 and preventing it from becoming protonated.⁴⁵ The presence of Arg71 is critical for the catalytic function of Glu88. The side chain of Glu88 is totally inaccessible to the solvent. This nonpolar environment increases the p*K*_a value for the carboxylate to 8.0 as calculated with the PROPKA server.⁴⁶ The side chain hydrogen bond and more importantly Coulombic interactions with Arg71 diminish the influence of the hydrophobic environment by lowering the p*K*_a by 0.7 and 1.7 units, respectively, to the final value of 5.6. The p*K*_a values of sulfenic acids are relatively high and range from 7.5 for sterically hindered 1-anthraquinone⁴⁷ to 10.5 for 2-methyl-2-propenesulfenic acids.⁴⁸ Because of this moderate acidity, deprotonation of the substrate solely depends on the proximity to the carboxylate anion of glutamic acid that acts as a general base, for which the negative charge is stabilized *via* hydrogen bonding with Arg71. An additional factor that might contribute to the efficient deprotonation of the substrate is formation of a hydrogen bond between O1 and N η^2 of Arg71 and hydroxyl group of Tyr102. This interaction increases the acidity of the sulfenic acid proton and thus lowers the energy barrier required for substrate deprotonation.

As a consequence of proton extraction, a transient double bond forms between the O1 and S2 atoms that rapidly rearranges to form *S*-oxide. This reorganization causes a single proton deficiency at C4 that results in a localized carbanion reaction intermediate (Figure 5). In the

secluded environment of the active site, the hydroxyl group of Tyr102 could serve as a proton donor to quench the carbanion, forming the final product of the enzymatic reaction with a newly acquired hydrogen atom bond at C4. Because of the high pK_a value of the tyrosine side chain, reprotonation of Tyr102 could occur through the transfer of a hydrogen from the carboxyl group of Glu88, restoring the protonation state of the active site prior to the enzymatic reaction. The sequence alignment of LFS enzymes suggests that this tyrosine residue is not absolutely required for catalysis and can be replaced by another proton donor, *i.e.*, cysteine, as it is in LFS from *A. chinense* (Supporting Information Figure 2a). The alternative scenario could include donation of a proton abstracted from the substrate by Glu88 back to the catalytic intermediate yielding the final product.

Shuffling of a double bond usually implies a geometric change within a molecule. Upon deprotonation of (*E*)-1-propenesulfenic acid, the linkage between O1, S2, C3, and C4 atoms becomes planar (dihedral angle $\approx 0^\circ$). This change in the geometry may weaken the hydrogen bonding between the oxygen atom of the substrate and the side chains of Arg71 and Tyr102 preventing spontaneous reprotonation. Additionally, the differences in relative position of the atoms in the substrate and the product in conjunction with charge repulsion may play a decisive role in the diffusion of the LF from the active site.

Although independently derived from the crystal structure, this catalytic mechanism is supported by several lines of evidence. Extensive mutagenesis of the enzyme performed by Masamura and colleagues¹⁵ revealed that substitution of Arg71 and Glu88 with leucine and glutamine, respectively, completely abolished the enzymatic reaction (Figure 4b). Additional evidence that supports the above mechanism is derived from experiments carried out in the presence of D₂O.^{12,19} They resulted in enzymatic formation of a single deuterated LF at the C4 position. Although this important observation was originally interpreted in favor of intramolecular rearrangement of (*E*)-1-propenesulfenic acid, deuteration of a carbon chain in the final product is undeniably in line with the proposed catalytic mechanism involving a carbanion reaction intermediate. Interestingly, only one isotopomer of LF was formed in the presence of D₂O.¹² It contained a deuterium located *syn* to the sulfine oxygen. This profound “syn effect” can be explained by the particular architecture of the enzyme’s active site that enforces directional protonation of the carbanion. In fact, the side chain of Tyr102 faces the substrate molecule from the side of the sulfine oxygen. Hence, regardless of the plausible carbanion inversion, the end product can contain a newly acquired proton exclusively *syn* to the sulfine oxygen.

It is important to mention that the above mechanism does not apply to LF found in other plant species. Formation of (*Z*)-phenylmethanethial *S*-oxide, a LF found in Amazonian medical plant *P. alliacea* is catalyzed by an LFS-like enzyme functionally distinct from the onion’s LFS.^{11,23} LF formation in the onion is formally a rearrangement reaction, whereas production of sulfine from the precursor sulfenic acid in *P. alliacea* involves the loss of two protons. Thus, (*Z*)-phenylmethanethial *S*-oxide results from oxidation of the substrate. Moreover, this reaction depends on redox cofactors such as NAD(P)⁺, indicating that LFS from *P. alliacea* utilizes a cofactor as part of the catalytic mechanism.

Conclusion

The nature and origin for the unique flavors of the species in the genus *Allium* have inspired scientists for over 150 years. The initial isolation and characterization of the principal organosulfur compounds as well as their rather complex chemistry (summarized in refs 1, 2, and 49) were followed by the discovery of the enzymes directly assisting in flavor formation, alliinase⁵⁰ and LFS.¹⁴ However, unlike alliinase, whose molecular architecture and principles of the catalytic mechanism have been explicated in detail,^{6,51} the structural and functional aspects of LFS catalysis have remained largely unexplained. The results of this work allow us for the first time to look at the abundance of chemical and biochemical data regarding LF through the prism of the high-resolution structure of LFS. Consequently, we synthesized numerous pieces of information into a coherent mechanistic model of catalysis by the enzyme. In doing so, the remarkable, eye-irritating property of the onion can be understood at its most fundamental, atomic level. Thus, we have bridged a long-lasting gap in understanding the mechanism responsible for onion's LF formation.

METHODS

Expression and Purification of LFS

Synthetic cDNA of lachrymatory factor synthase (LFS) from *A. cepa* encoding a sequence identical to GenBank entry BAC21275.1 was purchased from DNA2.0. To generate glutathione S-transferase-LFS (GST-LFS) fused constructs, cDNA representing protein fragment ²⁴GKV---CSA¹⁶⁹ was amplified using the following primers: forward, GCAGATGGATCCCCCTGGTATAAGTGGAGGTGGAGGTGGCAAAGTCCATGCTTTGCTTCC and reverse – CGTCTAGAATTCTCAAGCACTGCAAACCTCTTCG. To facilitate efficient thrombin digestion of the fusion protein, the forward primer was designed to incorporate an eight amino acid sequence (PGISGGGG) at the N-terminus of LFS. The PCR product was incorporated into the pGex-2T vector (GE Healthcare Life Sciences) through ligation using restriction enzymes BamH1-Hf and EcoR1-Hf (New England BioLabs). GST-LFS fusion protein was expressed in the *E. coli* BL21 (DE3) strain and cultured in LB broth (USB Affymetrix) at 37 °C in the presence of 50 mg mL⁻¹ of ampicillin (Sigma-Aldrich). When the optical density reached 0.4–0.6, the temperature was lowered to 25 °C. The concentration of the antibiotic was increased to 100 mg mL⁻¹, and the protein expression was induced with 0.5 mM isopropyl β-D-1 thiogalactopyranoside (Roche Applied Science). After 5 h, bacteria were harvested by centrifugation at 6000g for 15 min at 4 °C. The cell pellet was resuspended in water and ruptured by osmotic shock. The lysate was then sonicated and centrifuged at 36 000g for 30 min at 4 °C. Once the supernatant was collected, its buffer composition was adjusted to 10 mM PO₄³⁻, 137 mM NaCl, and 2.7 mM KCl at a pH of 7.2 (PBS) and incubated with glutathione-sepharose resin (GE Healthcare) for 2 h at 4 °C. Resin was then deposited into a chromatography column and washed with 10 volumes of PBS. The fusion protein was eluted with 10 mM reduced glutathione in PBS. Fractions of protein were pooled together and concentrated in 10 kDa cutoff Centricon (Amicon) to a final volume of 5 mL. The concentrate was loaded onto a Superdex 200 (GE Healthcare) size exclusion chromatography column equilibrated with 10 mM Tris/HCl buffer at a pH of 8.0. Fractions containing GST-LFS were collected, combined, and incubated with thrombin (USB Affymetrix) in the presence of 10% glycerol (v/v) for 3 h at 25 °C. The digested

sample was then incubated with glutathione–sepharose resin for 4 h at 4 °C. The resin was pelleted by centrifugation at 5000g for 5 min at 4 °C and washed twice with 10 mM Tris/HCl buffer at a pH of 8.0. The supernatants were collected, combined, and loaded onto a HiTrap Q HP ion exchange column (5 mL; GE Healthcare Life Science) then equilibrated with 10 mM Tris/HCl buffer at a pH of 8.0. LFS was eluted in a linear gradient of NaCl (up to 0.5 M) in 10 mM Tris/HCl buffer at a pH of 8.0. Fractions containing purified protein were collected and concentrated to 10 mg mL⁻¹, aliquoted, snap frozen in liquid nitrogen, and stored at -80 °C.

Crystallization Conditions

LFS crystals were grown using the sitting drop vapor diffusion method. LFS at concentrations between 6 and 10 mg mL⁻¹ was mixed in a 1:1 ratio with 0.1 M sodium acetate at a pH of 4.5 containing PEG 3350 at concentrations between 25% and 32% (w/v). Rod-like crystals with dimensions of 20 μm × 500 μm formed within 1 day at 25 °C. Crystals were cryo-protected in 0.1 M sodium acetate at a pH of 4.5 and 30% PEG 3350 (w/v) plus 10% PEG 2000 and 10% glycerol (v/v) prior to snap freezing in liquid nitrogen for X-ray diffraction data collection. For cocrystallization of LFS with crotyl alcohol (Sigma-Aldrich), the protein was preincubated with 1 mM of the ligand for 30 min on ice prior to setting up crystal drops. The crystals were grown and cryo-protected in the same manner as stated above.

Diffraction Data Collection and Structure Solution

X-ray diffraction data were collected at the Advance Photon Source (APS), The Northeastern Collaborative Access Team (NE-CAT) 24-ID-C and 24-ID-E beamlines at 0.9791 or 1.8785 Å wavelengths. Data from single crystals were indexed, integrated, and scaled with the XDS package⁵² and XDSAPP 2.0.⁵³ The structure of apo-LFS was solved by molecular replacement with an automated molecular replacement pipeline, BALBES CCP4.⁵⁴ The initial molecular replacement phases were improved in PHASER-EP⁵⁵ by using single-wavelength anomalous diffraction phasing based on the anomalous scattering signal from endogenous sulfur atoms present in the protein structure. The resulting model was refined using Python-base Hierarchical Environment for Integrated Xtallography (PHENIX)⁵⁶ software following several cycles of manual adjustment of the structure carried out in Crystallographic Object-Oriented Toolkit (COOT).⁵⁷ The structure of holo-LFS was solved by molecular replacement based on a refined model of the apoprotein. Atomic coordinates and geometric restraints for crotyl alcohol were generated using eLBOW and verified in the Restrain Editor Especially Ligands (REEL) available in the PHENIX software package. Geometry of the refined models was verified with the MolProbity server.⁵⁸ The data collection and refinement statistics are summarized in Table 1. Visualization of the macromolecules and figure preparation was performed in the CHIMERA software package version 1.10.1.⁵⁹

Substrate Docking

Docking calculations for (*E*)-1-propenesulfenic acid were carried out with AutoDock Vina⁴² using a Web service provided by National Biomedical Computation Resources available *via* the CHIMERA software. The ligand coordinates were created using PRODRG server,⁶⁰ and

the stereochemical restraints were generated in eLBOW and validated in REEL. The structure of holo-LFS was prepared for docking by running the DockPrep tool.⁶¹ The initial docking calculations were conducted using the whole LFS molecule as a search area. In this condition, (*E*)-1-propenesulfenic acid was reliably placed in the binding cavity occupied by crotyl alcohol in the holo-LFS structure. Thus, for the final calculations, the search space excluded the surface of the protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GST	glutathione S-transferase
LF	lachrymatory factor ((<i>Z</i>)-propanethial <i>S</i> -oxide)
LFS	lachrymatory factor synthase
PYL	pyrabactin resistance-like
RMSD	root-mean-square deviation
PYR/PYL/RCAR	pyrabactin resistance1/PYR1-like/regulatory components of ABA receptors
START	start-related lipid transfer

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Figure 1.

Formation of lachrymatory factor following tissue destruction in the onion. A precursor molecule (*(E)*-S-(1-propenyl)cysteine *S*-oxide) present in the cytoplasm of onion cells is cleaved by alliinase to a corresponding sulfenic acid that undergoes spontaneous self-condensation to *(E,E)*-S-(1-propenyl)-S-(1-propene)thiosulfinate and further nonenzymatic conversions into secondary thio-compounds. Alternatively, the sulfenic acid serves as a substrate for LFS that catalyzes its conversion into *(Z)*-propanethial *S*-oxide, the lachrymatory factor of onions.

Figure 2.

Structure of LFS in comparison to the abscisic acid receptor, PYL10. (a) Ribbon diagram of LFS structure. The overall architecture of LFS closely resembled an α -grip fold of START proteins with characteristic seven-stranded antiparallel β -sheet (strands $\beta 1$ – $\beta 7$) that enfolds long C-terminal α -helix ($\alpha 3$). (b) Overlay of LFS (gray) and PYL10 (PDB accession code 3R6P). The color scheme represents the difference in RMSD values calculated for the main chain atoms. The PYL10 ligand (abscisic acid) is colored orange and represented by stick atom/bond style. The most profound differences occur in the position of $\beta 3$ – $\beta 4$ strands and connecting the loop. Cut-away views of LFS (c) and PYL10 (d) surfaces reveal differences in volume of the ligand-binding pockets. Position of abscisic acid bound to PYL10 is shown in orange, whereas the cavity of LFS is colored gold.

Figure 3.

Location of crotyl alcohol within LFS. (a) Chemical structure of two geometric isomers of crotyl alcohol. Blue arrows indicate location of a rotatable bond in the ligand molecule. (b) Position of crotyl alcohol isomers within the enzyme structure. The ligands are shown as balls and sticks. Hydrophobic side chain of residues that limit access to the binding pocket are highlighted in color and labeled according to their primary sequence position. (c) Cutaway views of LFS surface revealing orientation of *E*- and *Z*-isomers of crotyl alcohol within the intermolecular cavity. Water molecules permeating into the channel that connects the exterior of the protein with the binding site are shown as red spheres. Panels d and e represent positions of *Z*- and *E*-crotyl alcohols (shown as balls and sticks) in the binding pocket of LFS. Labels indicate selected side chains of amino acids located within 5 Å from the position of the ligands. Hydrogen bonds are denoted as dashed lines. Distances between atoms of interest are provided in Å.

Figure 4.

Architecture of the active site of LFS. (a) Comparison of the chemical structures of substrate and product of the enzymatic reaction. Blue arrows indicate a change in the location of rotatable bonds in these compounds. (b) Putative position of (*E*)-1-propenesulfenic acid within the active site obtained by a molecular docking approach. Two of the most preferable solutions are indicated as balls and sticks and colored light blue and green. Distances between the catalytic residues (tinted in tan) and docked substrates are shown in Å. Inactivating mutations of Glu88 and Arg71 to glutamine and leucine are marked by the overlapping side chains for the substituting amino acids (colored orange).

Figure 5.

Putative catalytic mechanism of LF formation in the onion. Arginine-assisted deprotonation of sulfenic acid by glutamate triggers formation of a double bond between O–S and subsequent rearrangement to *S*-oxide with concomitant creation of a carbanion intermediate. This strong nucleophile subtracts a proton from a tyrosine residue. Consequently, the final product of the reaction contains a newly acquired hydrogen atom (shown in blue) bonded to C4.

Table 1**X-Ray Data Collection and Refinement Statistics**

	Apo-LFS	Apo-LFS	Holo-LFS (crotyl alcohol)
PDB accession code	5VGL	5VGL	5VGS
beamline	24-ID-C	24-ID-C	24-ID-E
wavelength (Å)	0.9791	1.8785	0.9791
data collection			
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	39.07, 40.13, 98.51	39.01, 40.16, 98.44	38.93, 40.10, 97.63
resolution (Å)	40.13–1.40 ^a (1.48–1.40) ^b	98.44–1.98 (2.03–1.98) ^b	40.10–1.90 ^a (1.94–1.90) ^b
R_{sym} (%)	11.2 (78.9) ^b	6.5 (28.7) ^b	15.3 (90.0) ^b
R_{pim} (%)	4.5 (34.6) ^b	2.6 (17.4) ^b	7.8 (51.3) ^b
$I/\sigma I$	8.4 (1.8) ^b	27.2 (5.1) ^b	6.6 (1.5) ^b
completeness (%)	97.8 (94.5) ^b	97.3 (72.4) ^b	99.4 (95.7) ^b
redundancy	4.1 (4.0) ^b	11.0 (3.8) ^b	5.2 (4.4) ^b
refinement			
resolution (Å)	31.11–1.40		37.09–1.90
no. reflections	30,780		12,511
$R_{\text{work}}/R_{\text{free}}$ (%)	15.2/18.9		16.7/21.5
no. atoms	1585		1440
proteins	1310		1228
ligand			10 (9A4/9A7) ^c
water	275		202
mean <i>B</i> -factor (Å ²)			
protein	14.2		21.8
ligand			23.1 (9A4/9 A7) ^c
water	25.7		28.0
RMSD			
bond lengths (Å)	0.008		0.009
bond angles (deg)	0.938		0.994
validation ^d			
Ramachandran plot favored/outliers (%)	99.3/0		99.3/0
rotamer outliers (%)	0		0
clash score	1.5		0.4

^aReported data set was collected on a single crystal.^bHighest-resolution shell is shown in parentheses.^cLigand's accession code.^dAs defined by MolProbity.