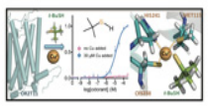
Phosphorus, Sulfur, and Silicon and the Related Elements



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Discovery of overlooked enzyme in onion and its application

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ABSTRACT

When onions (Allium cepa) are chopped, cells are broken, propanthial S-oxide (lachrymatory factor; LF) is released and it makes our eyes water. LF had long been believed to be formed non-enzymatically from 1-propenesulfenic acid (PSA), a putative reaction product of alliinase acting on trans-(+)-S-(1-propenyl)-L-cysteine sulfoxide (1-PRENCSO). During the course of our study for the discoloration of Allium plants, however, we got a clue that some unknown enzyme responsible for the LF formation should be present in the crude onion alliinase preparation. In this study, we report the discovery of this new enzyme called lachrymatory factor synthase (LFS), and its application such as non-lachrymatory onions.

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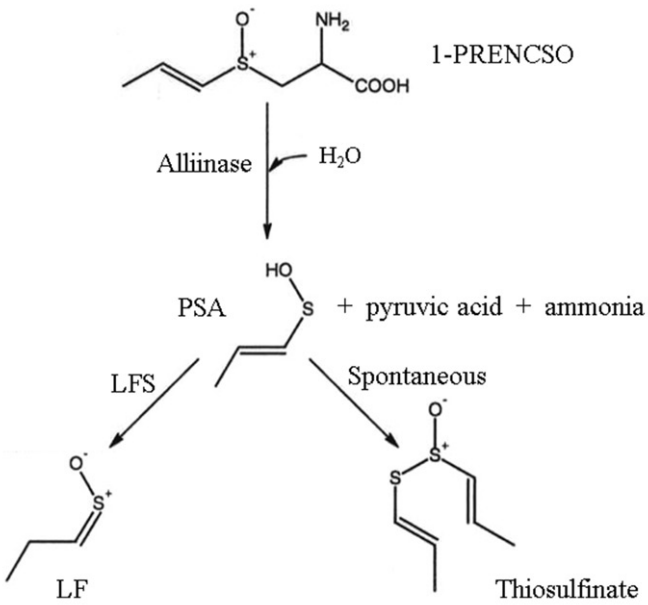
GRAPHICAL ABSTRACT



Introduction

The discoloration of garlic and onion is a well-known phenomenon, and a three-step reaction scheme was proposed for "pinking" of onion.[1,2] Although chemical constituents proposed by earlier studies for the discoloration reactions were generally in agreement, the detailed reaction mechanisms and the chemical structures of the pigments or those of the intermediates had not been fully elucidated. We, therefore, attempted to resolve those remaining issues by establishing a model reaction system that comprised only well defined, highly purified constituents.

During the course of our study for the discoloration, we got a clue that some unknown factors responsible for the lachrymatory factor (LF) formation should be present in the crude onion alliinase. Brodnitz identified the LF as propanethial *S*-oxide more than 45 years ago.[3] Like other sulfur compounds, such as thiosulfinate, the LF had long been believed to form spontaneously from 1-propenelsulfenic acid, a putative reaction product derived from *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (PRENCSO) by alliinase.[4] In this study, we report the discovery, isolation and cDNA cloning of a novel enzyme, which we named Lachrymatory

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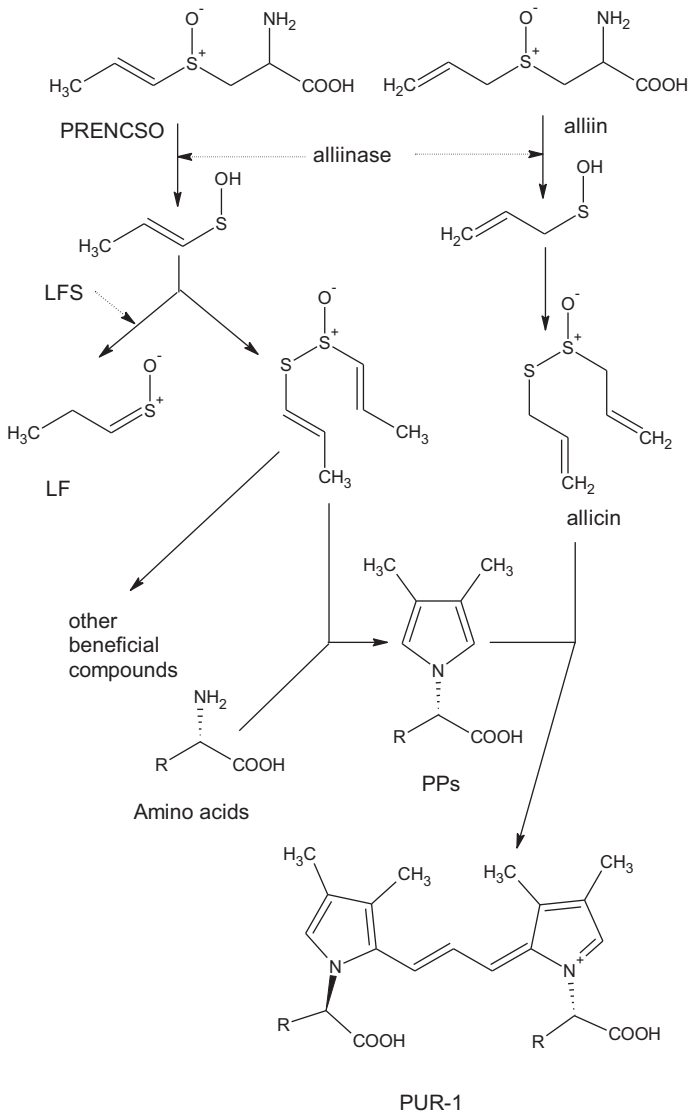

Figure 1. Reaction mechanism of blue-green discoloration of a mixture of chopped onion and garlic.

Factor Synthase (LFS), and consequences of suppressing the enzyme activity in onion.

Results and discussion

Model reaction system of discoloration

Since the vivid-blue color was produced easily by mixing juices from heated onion and unheated garlic, we isolated compounds responsible for color formation, and succeeded in inducing the pigment using a model reaction system comprising only four compounds. The first compound was PRENCSO, which was isolated from heat-treated onion. The second and the third compounds were allicin and alliinase isolated from raw garlic. The fourth compound was an amino acid, which was present in both onion and garlic.[5] Alliin was used instead of allicin because allicin is formed from alliin by the action of alliinase. Using this model reaction system, we determined the structures of a pigment precursor, PP-Val, and a pigment, PUR-1.

Structure of pigment precursors (PPs) and a reddish-purple pigment (PUR-1)

Pigment precursors were prepared by mixing PRENCSO, alliinase, and an amino acid. When we used valine for the amino acid, PP-Val was formed. The PP was isolated from reaction mixture, and the structure was determined as 2-(3,4-dimethylpyrrolyl)-3-methylbutanoic acid.[6]

Next, we prepared pigment from a heat-treated solution containing PP-Val and allicin. When heated, the mixture turned reddish purple first, then to blue to dark blue, and finally, formed green-colored precipitate. We isolated a reddish purple pigment (PUR-1) from the mixture and, its structure was determined as (1*E*)-1-(1-((1*S*)-1-carboxy-2-methylpropyl)-3,4-dimethyl-1H-pyrrol-2-yl)-prop-1-enylene-3- (1-((1*S*)-1-carboxy-2-methylpropyl)-3,4-dimethyl-1H-pyrrol-2-ylidenium).[6] From these results, it was suggested that PP molecules had been connected by an allyl group of allicin to form conjugated pigments. Proposed reaction mechanism leading to the pigment formation in the model reaction system is shown in Figure 1.

Effect of crude alliinase source on pigment and LF formation

By using our model reaction system comprising PRENCSO, alliin, glycine, and alliinase, we found that crude onion alliinase was less efficient in blue color formation than crude garlic enzyme showing equal alliinase activity. Moreover, the amount of pigment formation did not increase if the activity of crude onion alliinase added to the model reaction system was increased by about 2-fold. According to the previous studies of *Allium* chemistry, treating PRENCSO with alliinase should give rise to both the LF and di-1-propenyl thiosulfinate. Since the LF was thought not to be involved in the color forming reactions, we confirmed whether the amount of LF would differ by the source of the crude alliinase. Although the crude onion alliinase yielded LF, the LF was not formed at all when PRENCSO was mixed with crude garlic alliinase. From these results, we speculated that some unknown factors responsible for the LF formation should be present in the crude onion alliinase.

Isolation of LF forming factor and identification of lfs cDNA

We used a reaction system consisting of PRENCSO, purified garlic alliinase, and a fractionated crude onion alliinase to evaluate the LF forming activity. If the unknown factors were present in the fractionated sample, LF would be detected. We succeeded in isolating the active protein which was named LFS.

We used the RACE technique with degenerate primers deduced from the N-terminal sequence of LFS to obtain a complete cDNA sequence (GenBank accession no. AB089203). The full-length cDNA consisted of 737 base pairs, with a predicted gene product of 169 amino acids.

LF forming activity of LFS

When we expressed the LFS gene in *Escerichia coli*, the resulting recombinant protein exhibited the expected LF forming activity. The LF was detected only when all three factors, namely, PRENCSO, purified alliinase, and recombinant LFS were mixed in the reaction system, and the system lacking any of these factors did not give LF at all. This result provides a direct proof that LFS is essential in producing LF from PRENCSO.[7]

Production of non-lachrymatory onions

The discovery of LFS led to the proposal that non-lachrymatory onions unable to produce LF should be possible by silencing LFS enzyme activity. In the absence of LFS, by stopping conversion of 1-propenesulfenic acid to the LF, the unstable sulfenic acid would be predicted to undergo spontaneous self-condensation to thiosulfinates and other compounds responsible for the onion's characteristic flavor and their bioactivity. We succeeded in suppressing the endogenous *lfs* gene expression by using RNA interference silencing in onions. LFS enzyme activity as well as endogenous *lfs* transcripts levels was reduced significantly. In consequence, LF was decreased and thiosulfinates levels were increased dramatically and simultaneously.[8] Moreover, we determined the structure of one of the thiosulfinates, increased in the non-lachrymatory onion, as *S*-3,4-dimethyl-5-hydroxythiolane-2-yl 1-propenethiosulfinate, and showed its inhibitory activity against cyclooxygenase-1 in vitro.[9]

Mutagenesis was also used to silence the endogenous gene. By irradiating heavy-ion beams, we succeeded in generating non-lachrymatory onions in which alliinase enzyme activity as well as endogenous *alliinase* transcripts levels was decreased significantly.[10]

Conclusions

These non-lachrymatory onions would be a useful resource for understanding not only organosulfur chemistry of the genus Allium but also the role of specific sulfur secondary metabolites in plant biology and in human health.

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