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The Sulfur Chemistry of Shiitake Mushroom

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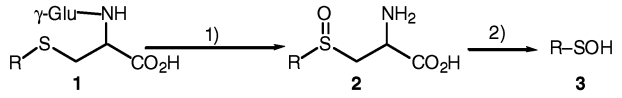
Plants and fungi rich in sulfur, from the well-studied plants of genus *Allium* (Chinese chive, garlic, onion, etc.) to the edible mushrooms of the family *Tricholomataceae*, are known for their culinary value and their application in traditional medicine.[1,2] The pungent aroma and flavor associated with the alliums probably serve a defensive purpose and arise from a complex mixture of sulfur species formed following cell disruption from odorless precursors.[3,4] Although the sulfurous allium end products differ greatly in their chemical structure and reactivity, they are all derived from a family of γ-L-glutamyl-cysteine sulfoxide precursors.[5−8] In a two-step enzymatic process (Figure 1), precursors **1** are first activated by the removal of their γ-glutamyl moieties, before or after oxidation, and the resultant L-cysteine-sulfoxides **2** undergo α,β-elimination catalyzed by cysteine−sulfoxide (C−S) lyases (called alliinases in garlic and onion),[9] generating highly reactive sulfenic acid intermediates **3**.[10,11] Onion is unusual in possessing a potent lachrymatory factor which is generated by the action of a specific enzyme on sulfenic acid **3c**.[12] The multifarious flavor-containing species (thiosulfinates, etc.) from all allium plants are thought to form spontaneously from sulfenic acids **3**. Closely related biochemistry is thought to be important in shiitake mushrooms (*Lentinula edodes*)[10] and other fungi of the genera *Tricholoma*[8] and *Marasmius* (e.g., *M. alliaceus*) which have a garlic-like odor. In parallel with the allium enzymatic mechanism,[11] sulfenic acid intermediates **3** are presumed to be enzymatically generated from sulfoxide precursors **2**, although with markedly different substrate specificity, leading in shiitake to fungicidal cyclopolythiepane flavorants.[9]

One source of uncertainty in determining the mechanism is a lack of information on the composition of the whole system. In a typical analysis, plant samples are homogenized, and solvent extracts are subjected to GC−MS or LC−MS analysis, and to distillation or chromatography to isolate individual sulfur-containing species. This approach has been very successful in developing an understanding of the complex chemistry of precursor and flavorant species of the allium plants, and most of the species identified have also been synthesized.[3]

A disadvantage of the conventional approach is that the species taken up by solvent extraction may only represent a fraction of the total present, and those forms that are not extracted remain uncharacterized.

We describe herein the results of a fundamentally different and direct method of identifying and studying these organosulfur compounds by using sulfur K-edge X-ray absorption spectroscopy (XAS).[13] The major advantages of XAS are that it can be used on intact solid samples as small as seeds or single cells and it can be used directly on aqueous homogenates, including those quick-frozen in liquid nitrogen, without requiring prior extraction, derivatization, separation, or heating. For K-edges the near-edge portion of the X-ray absorption spectrum is dominated by dipole-allowed transitions to unoccupied levels possessing significant p-orbital character. XAS is thus very sensitive to electronic structure and is a particularly useful probe of sulfur since the spectra of various chemical forms are generally quite distinct, and both qualitative and quantitative analysis of different general organic sulfur forms (e.g. thiols, sulfides, sulfoxides, sulfones, etc.) can be achieved.[14−16] Figure 2 compares the sulfur K-edge spectra of intact shiitake mushroom (Figure 2g) plus selected standard compounds, together with a least-squares deconvolution of the mushroom data. Spectra were also recorded of standards that contain different functional sulfur forms separated by methylene atoms (not illustrated), similar to the precursor molecule **2e** in the mushroom (Figure 1). These, together with density functional theory calculations of the near-edge spectra,[17] indicated that the individual sites in the multisulfur species should give spectra clearly related to those of the isolated functional forms of sulfur (e.g. the spectrum of sulfoxide components is essentially similar to that of simple sulfoxides). Figure 3 compares the sulfur K near-edge spectra of intact and homogenized onion (*Allium cepa*), garlic (*A. sativum*), and Chinese chive (*A. tuberosum*) samples with those of shiitake mushroom (*L. edodes*).[18]

For the intact tissues of all four species, the spectra clearly show the presence of the sulfoxide precursor (ca. 2473.5 eV), the more reduced sulfur forms including thiols, disulfides, and sulfides (2469−2472 eV), and small quantities of sulfate (2479.6 eV). An additional peak (2477 eV) seen for shiitake corresponds to sulfone. The biochemical transformation of the sulfur due to cell breakage (e.g., see Figure 1) is obvious for the allium species, with the sulfoxide peak (ca. 2473.5 eV) decreasing and the more reduced forms of the flavorant molecules developing.[19] In marked contrast, and in disagreement with what is expected from the literature, the shiitake mushroom showed only very subtle spectral changes on cell breakage. The magnitude of these changes is similar to that of differences observed in different parts of the fungus and are likely not significant. No changes were observed in the sulfur spectra on incubation of the juiced tissue at room temperature for more than



1) γ-glutamyl transpeptidase [transferase]; oxidase; 2) C-S lyase; R: a) CH_3–, *A. tuberosum*; b) CH_2=$CHCH_2$–, *A. sativum*; c) CH_3CH=CH–, *A. cepa*; d) $CH_3S(O)CH_2$–, *M. alliaceus*; e) $CH_3SO_2CH_2S(O)CH_2S(O)$–, *L. edodes*

Figure 1. Generic mechanism postulated for generation of sulfenic acids **3** from precursors **1** and **2**.

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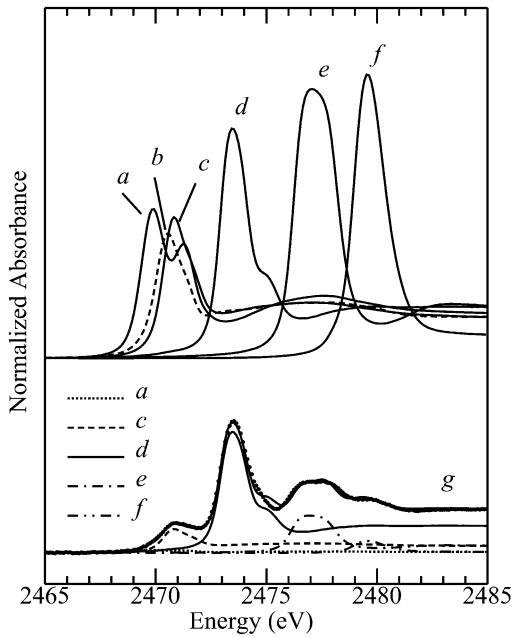

Figure 2. Sulfur K near-edge X-ray absorption spectra of intact shiitake mushroom and relevant standard species in aqueous solution at neutral pH [(*a*) oxidized glutathione, (*b*) reduced glutathione (shown as a broken line for clarity), (*c*) methionine, (*d*) methionine sulfoxide, (*e*) methionine sulfone, (*f*) sulfate (scaled by 0.5), and (*g*) shiitake mushroom (points)] together with a least-squares deconvolution using the standards *a*, *c*, *d*, *e*, and *f* (which gave proportions for these functional forms of 2, 19, 60, 16, and 3%, respectively). The calculated 3.75:1 sulfoxide-to-sulfone ratio is consistent with the 3:1 ratio of these groups in **2e**.

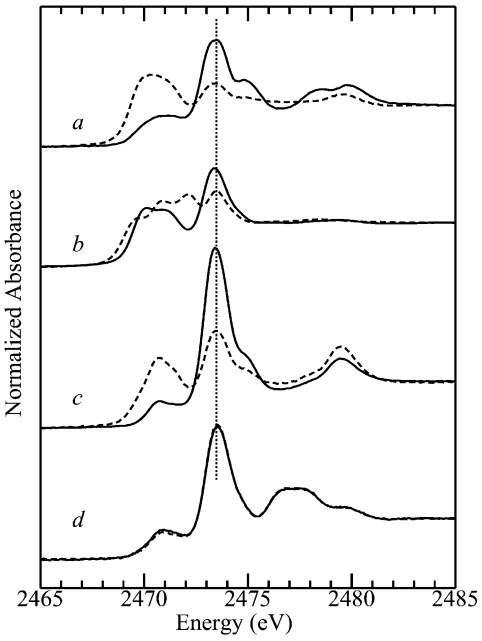

Figure 3. Sulfur K near-edge spectra of intact (solid line) and juiced (broken line) plant tissues (*a*) onion, (*b*) garlic, (*c*) Chinese chives, and (*d*) shiitake mushroom. The dotted line marks the position of the major absorption peak of the sulfoxide precursor molecules.

24 h. A variety of different treatments of the mushroom tissues were also performed, including grinding, crushing, or extensive cooking in a microwave oven. None of these treatments resulted in any significant changes to the sulfur spectra, indicating that no significant modification of the sulfur species occurs in shiitake mushrooms on cell breakage and that the sulfur species present are in fact remarkably stable. To explain these observations we note that in contrast to garlic, where abundant precursors **2** are cleaved by equally abundant C−S lyases, in shiitake it is necessary for γ-glutamyl transpeptidases [transferases] to first cleave oxidized **1e** (lentinic acid) giving **2e**. Only then would **2e** be cleaved to **3e** by shiitake C−S lyases, but these are known to be only one-tenth as abundant as garlic C−S lyases.[20] It is also known that cleavage of **2e** by shiitake C−S lyases, rapid at first, ceases after a short period of time before decomposition of **2e** is complete,[10] suggestive of product or suicide inhibition of shiitake C−S lyases by **3e** or its products. The sulfoxide compounds in shiitake mushroom are thus not likely to act as a deterrent to herbivores as they are not apparently noxious, and exactly why the mushroom makes such large quantities of exotic sulfur species remains a mystery.

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References

(1) Augusti, K. T. *Indian J. Exp. Biol.* **1996**, *34*, 634−640.
(2) Wasser, S. P.; Weis, A. L. *Int. J. Med. Mushrooms* **1999**, *1*, 351−370.
(3) Block, E. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1135−1178.
(4) Yasumoto, K.; Iwami, K.; Mitsuda, H. *Mushroom Sci.* **1976**, *9*, 371−83
(5) Stoll, A.; Seebeck, E. *Experientia* **1947**, *3*, 114−15
(6) Virtanen, A. I.; Spåre, C. G. *Suomen Kemistilehti* **1961**, *34*, 72
(7) Yasumoto, K.; Iwami, K.; Mitsuda, H.. *Agr. Biol. Chem.* **1971**, *35*, 2059−2069.
(8) Gmelin, R.; N'Galamulume-Treves, M.; Höfle, G. *Phytochemistry* **1980**, *19*, 553−557.
(9) (a) Morita, K.; Kobayashi, S. *Chem. Pharm. Bull.* **1967**, *15*, 988−993. (b) Iwami, K.; Yasumoto, K. *Agr. Biol. Chem.* **1980**, *44*, 3003−3004. (c) Block, E.; DeOrazio, R.; Thiruvazhi, M. *J. Org. Chem.* **1994**, *59*, 2273−2275.
(10) Yasumoto, K.; Iwami, K.; Mitsuda, H. *Agr. Biol. Chem.* **1971**, *35*, 2070−2080.
(11) Gmelin, R.; Luxa, H.-H.; Roth, K.; Höfle, G. *Phytochemistry* **1976**, *15*, 1717−1721.
(12) Imai, S.; Tsuge, N.; Tomotake, M.; Nagatome, Y.; Sawada, H.; Nagata, T.; Kumagai H. *Nature* **2002**, *419*, 685.
(13) Sulfur K-edge X-ray absorption spectra were collected on beamline 6−2 (1T wiggler field) at the Stanford Synchrotron Radiation Laboratory (SSRL) using the SPEAR storage ring operating at 60−100 mA and 3 GeV. Data collection and analysis were carried out as previously described.[14−16] As before, the energy scale was calibrated with reference to the lowest-energy peak of a sodium thiosulfate standard, assumed to be 2469.2 eV, and all spectra were recorded at room temperature.
(14) Pickering, I. J.; Prince, R. C.; Divers, T.; George, G. N. *FEBS Lett.* **1998**, *441*, 11−14.
(15) Yu, E. Y.; Pickering, I. J.; George, G. N.; Prince, R. C. *Biochim. Biophys. Acta* **2001**, *1527*, 156−160.
(16) Pickering, I. J.; George, G. N.; Yu, E. Y.; Brune, D. C.; Tuschak, C.; Overmann, J.; Beatty, J. T.; Prince, R. C. *Biochemistry* **2001**, *40*, 8138−8145.
(17) Density functional Slater transition potential calculations used the StoBe code (IGLO-III basis set used for S, DZVP for all other atoms) [Triguero, L.; Pettersson, L. G. M.; Agren, H. *Phys. Rev. B* **1998**, *58*, 8097−8110].
(18) Fresh Shiitake mushrooms, onion bulbs, garlic bulbs, and whole Chinese chives were purchased from local markets. For intact tissues thin sections were prepared and were mounted under 6-μm thick polypropylene film to retard desiccation during exposure to the X-ray beam. Juices of each specimen were prepared with an electrical food "juicer".
(19) As expected from the instability of these compounds no indications of sulfenic acid intermediates are observed
(20) Kumagai, H.; Kono, H.; Sakurai, H.; Tokimoto, K. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2560−2566.

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