# Marked Formation of Thiazolidine-4-carboxylic Acid, an Effective Nitrite Trapping Agent in Vivo, on Boiling of Dried Shiitake Mushroom (*Lentinus edodes*)

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Thiazolidine-4-carboxylic acid (TCA, thioproline) is a condensation product of cysteine and formaldehyde. TCA is an effective nitrite trapping agent in the human body and may block endogenous formation of carcinogenic *N*-nitroso compounds. In this study, we investigated the levels of TCA in five edible mushrooms. The amounts of TCA in dried shiitake mushroom (*Lentinus edodes*) were 134 ± 137 (uncooked) and 843 ± 427 μg (boiled) per 100 g (dry weight basis), respectively. Uncooked samples of raw shiitake mushroom contained no detectable TCA, but 406 ± 248 μg/100 g (dry weight basis) was formed during boiling. This TCA formation was effectively inhibited by addition of *N*-ethylmaleimide, an SH-trapping reagent. Dried samples soaked in water contain a specific system for generating formaldehyde via the formation of lenthionine from lentinic acid. The contents of TCA in other raw edible fungi examined, such as *Agaricus bisporus* (champignon), *Collybia velutipes* (enokitake in Japanese), *Lyophyllum aggregatum* (shimeji), and *Tricholoma matsutake* (matsutake), were <3.8 ppm and with formaldehyde concentrations similar to that of raw shiitake mushroom.

Thiazolidine-4-carboxylic acid (TCA, thioproline) is a cyclic sulfur-containing amino acid that is a condensation product of cysteine and formaldehyde (Schubert, 1936; Ratner and Clarke, 1937). It was detected in a rat liver homogenate incubated with cysteine and is known to be formed nonenzymatically (Cavallini et al., 1956). Therefore, endogenous formation of TCA has been considered to be a detoxification pathway of formaldehyde (Debey et al., 1958). In rats, TCA has been shown to protect the liver against the hepatotoxic effects of ethanol, carbon tetrachloride (Ligny and Manouiloff, 1972), bromobenzene (Siegers et al., 1978), acetoaminophene (Strubelt et al., 1974), tetracycline (Pérès and Dumas, 1972), and thiourea (Mackenzie and Harris, 1957). It also has antiaging effects in *Drosophila* (Miquel et al., 1982) and mice (Miquel and Economos, 1979), and its antitumor effect has been examined clinically (Brugarolas and Gosalvez, 1980).

Ohshima et al. (1983, 1984) and Tsuda et al. (1983, 1984) found that *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *cis*/*trans*-*N*-nitroso-2-methylthiazolidine-4-carboxylic acids are significant *N*-nitroso compounds in human urine. NTCA is nonmutagenic (Tahira et al., 1984; Umano et al., 1984) and may be noncarcinogenic like *N*-nitrosoproline, which was negative in several carcinogenicity assays (Mirvish et al., 1980; Lijinsky and Reuber, 1982), and is also commonly present in human urine (Tsuda et al., 1986, 1987). The rates of nitrosation of TCA in vivo and in vitro are several hundredfold faster than those of proline (Tahira et al., 1984; Ohshima et al., 1984; Tsuda et al., 1988). NTCA is rapidly excreted in the urine without metabolic change (Ohshima et al., 1984; Tsuda et al., 1988). On the basis of these findings, we have proposed that TCA is an effective nitrite trapping agent in the human body and may block the endogenous formation of carcinogenic *N*-nitroso compounds (Tsuda et al., 1988; Tahira et al., 1988).

We found a marked increase of urinary NTCA in subjects who ate a popular Japanese dish called tarachiri, consisting of boiled cod (tara) and vegetables such as Japanese radish (daikon), Chinese cabbage, mushrooms, and carrots (Tsuda et al., 1988). This increase was found to be because TCA was produced during cooking by a condensation reaction of cysteine and formaldehyde, which are present in the cod and vegetables, and then converted to NTCA under acidic conditions in the stomach by reaction with nitrite originating from nitrate in vegetables and excreted into urine. The edible mushroom *Lentinus edodes* (its Japanese name is shiitake), which is eaten in large quantities in Japan and China, has an unusually high amount of formaldehyde (Yada et al., 1970). We investigated the levels of TCA in shiitake mushrooms and other commonly eaten fungi including champignon.

Here we report that dried shiitake mushroom produces a large amount of TCA due to a specific formaldehyde generation system.

## MATERIALS AND METHODS

**Chemicals.** L-Cysteine hydrochloride, *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosamide, a reagent for diazomethane generation, and *N*-ethylmaleimide (NEM) were purchased from Tokyo Kasei Co. (Tokyo, Japan). L-Thiazolidine-4-carboxylic acid (L-thioproline) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were standard commercial products. NTCA was prepared from TCA as described previously (Tahira et al., 1984).

Raw and dried shiitake mushrooms (raw S mushroom and dried S mushroom, respectively) and other fungi were purchased from a local market. The weight of dried S mushroom was usually about 20% of that of raw S mushroom. Water contents of raw fungi used ranged from 88 to 92%.

**Preparations of Supernatants of Raw Shiitake Mushroom.** Samples of 5 g of S mushroom were homogenized uncooked with 50 mL of distilled water or after the mushrooms were boiled for 5 min. The homogenates were centrifuged twice at 3000 rpm for 10 min, and the combined supernatants were used for nitrite treatment and formaldehyde determination. Twenty milliliters of both uncooked and boiled supernatants was gram equivalent to raw S mushroom.

**Preparations of Supernatants of Dried Shiitake Mushroom.** Dried S mushroom (5 g) was soaked in 50 mL of distilled water for 30 min at room temperature and then homogenized immediately (uncooked) or after the mushrooms

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**Table I. Amounts of Thiazolidine-4-carboxylic Acid and Formaldehyde Found in Edible Fungi**

| mushroom | prefecture of production | thiazolidine-4-carboxylic acid, μg/100 g of dry fungus[a,b] | | formaldehyde, mg/100 g of dry fungus[a] | |
|---|---|---|---|---|---|
| | | uncooked | boiled[c] | uncooked | boiled[c] |
| shiitake (*L. edodes* Sing.) | | | | | |
| raw | Ibaraki | ND[d] | 688 | 4.5 | 4.5 |
| | Ibaraki | NT[e] | 218 | 3.4 | 3.4 |
| | Gunma | ND | 314 | NT | NT |
| dried | Iwate | 53 | 776 | 71.8 | 8.6 |
| | Ohita | ND | 532 | 4.0 | 8.1 |
| | Ohita | 107 | 692 | 63.3 | 4.3 |
| | Ehime | 96 | 804 | 27.6 | 9.5 |
| | Ehime | 159 | 985 | 24.4 | 14.3 |
| | Miyazaki | 245 | 839 | 37.5 | 9.7 |
| | Miyazaki | ND | 337 | 19.4 | 11.0 |
| | Saitama | 415 | 1777 | 24.3 | 12.3 |
| champignon (*Agaricus bisporus* Sing.) | | | | | |
| raw | Gunma | 111 | 377 | 2.4 | 2.4 |
| enokitake (*Collybia velutipes* Quel) | | | | | |
| raw | Nagano | 100 | 234 | 6.8 | 2.9 |
| matsutake (*Tricholoma matsutake* Sing.) | | | | | |
| raw | Kyoto | ND | 263 | 10.3 | 3.4 |
| shimeji (*Lyophyllum aggregatum* Kuhner) | | | | | |
| raw | Shizuoka | 64 | 59 | 16.0 | 1.3 |

[a] Edible part of raw or dried fungus, dry weight basis. [b] Values were not corrected for recovery (>80%). [c] Boiled for 5 min in distilled water. [d] ND, not detected (detection limit, 0.1 ppm). [e] NT, not tested.

were boiled for 5 min. The homogenates were diluted with water and centrifuged twice at 3000 rpm for 10 min, and the combined supernatants were used for experiments. Twenty milliliters of these supernatants was the gram equivalent of the dried S mushroom.

**Determination of TCA in Shiitake Mushrooms.** For determination of the TCA content of homogenates of S mushroom, the supernatant was treated with nitrite under acidic conditions to convert TCA quantitatively to NTCA. The resulting NTCA was then determined by gas chromatography–thermal energy analyzer (GC–TEA) as described previously (Tsuda et al., 1986).

*Nitrite Treatment.* Samples of 1 mL of the supernatant of S mushrooms were diluted with 3.6 mL of 0.1 M citrate hydrochloride buffer (pH 1.5), mixed with 0.4 mL of 250 mM sodium nitrite or distilled water (without nitrite treatment), and incubated at 37 °C at pH 2 for 20 min. Excess nitrite was decomposed by addition of 20% ammonium sulfamate at pH 2, and then NTCA was extracted three times with 4 mL of EtOAc containing 10% MeOH in the presence of 25 mg of hippuric acid, used to increase the recovery of NTCA. The combined EtOAc–MeOH extracts were dried over anhydrous $Na_2SO_4$ and evaporated to dryness in vacuo after addition of 0.5 mL of 10% $NH_4OH$ in MeOH to avoid acid decomposition of NTCA. The residual materials were treated with diazomethane generated from *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosamide in $CH_2Cl_2$ containing 10% MeOH. After removal of excess diazomethane by evaporation, methylated NTCA was analyzed by GC–TEA.

Recovery of TCA added into a supernatant of S mushroom (1–10 μg/mL) by this nitrosation method was >80%. The amount of preformed NTCA in S mushroom was negligible (<0.1 ppm).

**Addition of *N*-Ethylmaleimide (NEM) during TCA Determination.** Since TCA is synthesized by the reaction of cysteine with formaldehyde even at room temperature (Ratner and Clarke, 1937), NEM, an SH-trapping reagent, was added when necessary to trap cysteine and avoid artifactual formation of TCA during analysis.

**Conditions of GC–TEA Analysis.** A GC-9A gas chromatograph (Shimadzu Corp., Kyoto, Japan) was used with argon carrier gas (40 mL/min) and was connected to a thermal energy analyzer (TEA, Model 543; Thermo Electron Corp., Waltham, MA). For GC, a glass column (3.6 m × 2.6 mm i.d.), packed with 5% OV-1 on Gas-Chrom Q (80–100 mesh), was programmed from 90 to 200 °C at 4 °C/min. The GC–TEA chromatogram was recorded with a Chromatopac C-R3A (Shimadzu). The retention times of the methyl esters of *N*-nitrosopipecolic acid as an internal standard and NTCA were 20.5 and 21.0 min, respectively.

**Determination of Formaldehyde in Shiitake Mushrooms.** Formaldehyde was measured by a modification (Iwami et al., 1974) of the method of Nash (1953).

## RESULTS

**Amount of Formaldehyde in Shiitake Mushroom.** As reported previously (Yada et al., 1970), the formaldehyde level in a supernatant of raw S mushroom was <45 ppm (dry weight basis), but the level in the supernatant of dried S mushroom was much higher (40–720 ppm). The formaldehyde levels of raw samples of other edible fungi were as low as that in raw S mushroom (Table I).

**Amount of TCA in Shiitake Mushroom.** TCA was not detected in raw S mushroom (detection limit, 0.1 ppm) but increased to 406 ± 284 μg/100 g (dry weight basis) when raw S mushroom was boiled for 5–8 min. The amount of TCA in uncooked dried S mushroom samples was 134 ± 137 μg/100 g (dry weight basis) and increased markedly when the samples were boiled to 843 ± 427 μg/100 g (dry weight basis), as shown in Table I. The amounts of TCA in boiled samples of other edible fungi were <3.8 ppm (dry weight basis).

**Effects of Temperature and Concentration of Added Cysteine on the Formation of TCA in Supernatants of Dried Shiitake Mushroom.** Addition of cysteine to the supernatant of dried S mushroom at the same or 10 times the level of formaldehyde present in the supernatant greatly increased TCA formation during heating at 95 °C for 5 min (Figure 1).

**Effect of NEM on the Formation of TCA.** To determine whether TCA found in dried S mushroom was formed during analytical processes or not, the effects of addition of NEM, an SH-trapping agent, just before each step, e.g., soaking, boiling, homogenization, centrifugation,

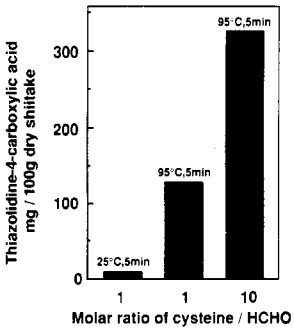


**Figure 1.** Effects of temperature and addition of cysteine on the formation of thiazolidine-4-carboxylic acid (TCA) in a supernatant of uncooked dried shiitake mushroom. Aliquots of the supernatant of a dried S mushroom containing formaldehyde (ca. 1.0 mM) were supplemented with cysteine to give molar ratios of cysteine/HCHO of 1.0 an 10.0 and then incubated for 5 min at 25 or 95 °C. The amount of preformed TCA in the supernatant of dried S mushroom used was 700 μg/100 g (dry weight basis).

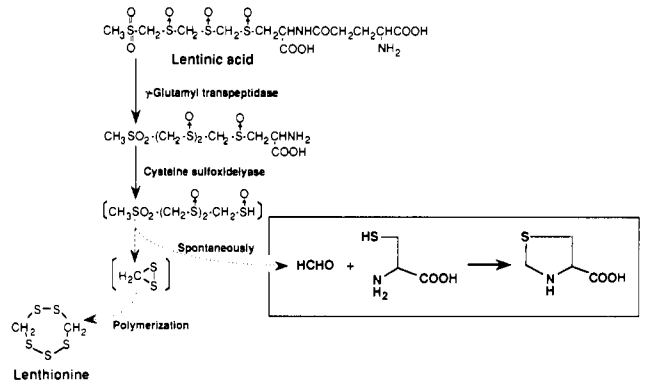


**Figure 2.** Proposed reaction scheme for formation of formaldehyde and thiazolidine-4-carboxylic acid during cooking of dried shiitake mushroom. Reaction pathways for enzymatic formation of lenthionine from lentinic acid were proposed by Yasumoto et al. (1971).

and nitrite treatment, were examined. The formation of TCA in boiled dried S mushroom was apparently inhibited by NEM addition before boiling. However, no significant difference was observed in TCA levels in uncooked dried S mushroom with or without NEM treatment. Therefore, it was suggested that TCA found in uncooked dried S mushroom was mainly formed during the processes of drying and storage.

**pH Dependency of the Formation of TCA by the Reaction of Cysteine with Formaldehyde.** When buffered solutions containing 5 mM cysteine and 5 mM formaldehyde were incubated at 37 °C for 60 min at pH 7.0 and pH 2.0, the yields of TCA were 45.5 and 11.5%, respectively. This pH dependency was consistent with the results of Ratner and Clarke (1937) and supported the conclusion that artifactual formation of TCA during nitrite treatment under acidic conditions was negligible.

## DISCUSSION

A large part of the carcinogenic *N*-nitroso compounds to which humans are exposed are of endogenous origin mainly through formation in the stomach (National Research Council, 1981). Humans are exposed to nitrite through ingestion of nitrate-rich vegetables and drinking water, in addition to nitrite taken in as food additives (Knight et al., 1987). Consequently, the salivary nitrite level can reach more than 200 ppm (Ladd et al., 1984) due to bacterial reduction of nitrate in the oral cavity. Numerous nitrosatable amine precursors, which are converted to mutagenic compounds by treatment with nitrite, have been detected in foods (Nagao et al., 1986).

On the other hand, endogenous formation of carcinogenic compounds by nitrite can be blocked by ascorbic acid due to scavenging nitrite (Mirvish et al., 1972; Ohshima and Bartsch, 1981; Leaf et al., 1987). TCA has been proposed to inhibit formation of carcinogens by nitrite in vivo because it traps nitrite, resulting in quantitative urinary excretion of nonmutagenic NTCA. Tahira et al. (1988) demonstrated that production of squamous cell carcinomas (forestomach tumors) in rats by oral administration of benzylmethylamine plus nitrite was inhibited by additional ingestion of TCA. This inhibition can be explained as the result of trapping of nitrite by TCA, so blocking the formation of carcinogenic *N*-nitroso benzylmethylamine. From this point of view, we have been investigating the detection of TCA in various foods as a precursor of NTCA in human urine (Tsuda et al., 1986, 1987). Shiitake mushroom is an edible fungi eaten in great quantities in Japan and China; its total production in Japan in 1984 was 74 000 tons of raw S mushroom and 16 600 tons of dried S mushroom (Federation of Nippon Shiitake Agricultural Cooperative Association, 1984). Raw S mushroom has only a slight smell but, when dried S mushroom is soaked in water, a characteristic aroma gradually develops. Lenthionine (1,2,3,5,6-pentathiepane, $C_2H_4S_5$, see Figure 2), a cyclic sulfur compound known to possess the characteristic aroma of S mushroom, was first identified in dried S mushroom (Morita and Kobayashi, 1966). Iwami et al. (1975) and Yasumoto et al. (1971) proposed that cyclic sulfur compounds in S mushroom originate from lentinic acid, which is derivative of γ-glutamylcysteine sulfoxide. Enzymatic formation of lenthionine from lentinic acid is reported to be accompanied by the liberation of formaldehyde, as shown in Figure 2. The amount of formaldehyde in a supernatant of dried S mushroom after 30 min of soaking in water is higher than the level in any other Japanese food, except frozen cod (100–300 ppm), as described previously (Yada et al., 1970; Tsuda et al., 1988). When dried S mushroom is ground to a powder and heated quickly in boiling water before being soaked at room temperature, the amount of formaldehyde was only 8.9 ppm, whereas it was 346 ppm when the boiling was omitted, indicating that the formation of formaldehyde was due to an enzymatic process. The formaldehyde thus formed from lentinic acid reacted nonenzymatically with cysteine present in S mushroom to produce TCA, as indicated in Figure 2. Results of the effects of heating and addition of cysteine on the formation of TCA in the supernatant of dried S mushroom (Figure 1) showed that during cooking the large amount of formaldehyde in the dried S mushroom can produce TCA in the presence of cysteine originating from other foods. Our recent finding showed that 100 g of dried S mushroom produced approximately 10 mg of TCA when cooked with 100 g of chicken liver, a source of cysteine (Tsuda et al., unpublished result). When a subject ingested 10 mg of TCA with 4 mmol of nitrate (~250 mg $NO_3^-$, average daily nitrate intake among the Japanese population), the urinary excretion of NTCA increased to 165 ± 137 μg/12 h (Tsuda et al., 1989). This excretion level of NTCA is much higher because the averaged urinary excretion level among the Japanese general population has been found to be ~20 μg/day (Tsuda et al., 1987). These results indicate that TCA generated from edible amounts of S mushroom can actually contribute to the trapping of nitrite in the human body.

Formaldehyde shows genotoxicity in various in vitro assay systems (International Agency for Research on Cancer, 1982) and has been proved to be carcinogenic to

the nasal cavity of rodents when administered by inhalation (Kerns et al., 1983). However, there is no evidence that formaldehyde is carcinogenic when administered orally to experimental animals. Furthermore, the results of in vivo assays have suggested that formaldehyde has a threshold level for exhibiting genotoxicity because of the presence of a metabolic pathway for detoxifying it (Bolt, 1987). The formation of TCA has also been considered as a mechanism for detoxifying formaldehyde (Cavallini et al., 1956).

From another point of view, TCA formation may be considered to play a role in stabilizing cysteine in foods serving as a source of "frozen" sulfhydryl agent that liberates cysteine after its uptake by cells (Debey et al., 1958).

Other edible fungi examined in this study also produced small amounts of formaldehyde, but its origin may be nonspecific. However, this formaldehyde can also participate in formation of TCA by boiling.

Shiitake mushroom contains lentinane, an immunopotentiating polysaccharide (Chihara et al., 1969), eritadenine (lentinacin, an anticholesteremic substance) (Chibata et al., 1969), and double-strand RNA (an interferon inducer) (Takahara et al., 1984). In addition to these biologically active components, we discovered that S mushroom has an effective TCA generating system and may contribute to trapping nitrite in the human body, which may result in blocking the endogenous formation of carcinogenic *N*-nitroso compounds.

## ABBREVIATIONS USED

GC–TEA, gas chromatography–thermal energy analyzer; NEM, *N*-ethylmaleimide; NPRO, *N*-nitrosoproline; NTCA, *N*-nitrosothiazolidine-4-carboxylic acid (*N*-nitrosothioproline); TCA, L-thiazolidine-4-carboxylic acid (L-thioproline).

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