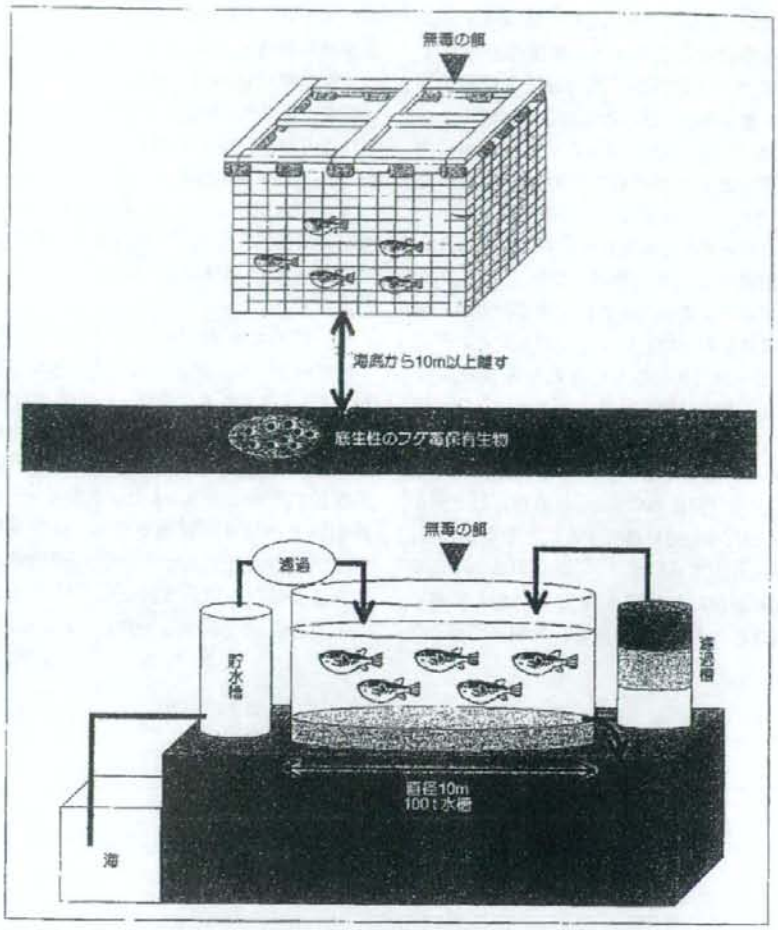

図2　トラフグの囲い養殖法

底生性の有毒餌生物を遮断した上で無毒の餌を与えて飼育する養殖法を'囲い養殖法'と称している。これにより無毒のフグを生産することが可能である。網生け簀養殖(上)と陸上養殖(下)の2つの形態がある。

かった。

一方，毒を持たない一般の魚は致死量未満のTTXを餌に混ぜて投与しても，すぐに排泄もしくは分解して蓄積することはないが，無毒の養殖トラフグや養殖クサフグに，同様にTTXを与えると，肝臓や卵巣に効率良くそれを蓄積する[5,6]。他方，養殖トラフグにPSPを投与すると，蓄積はするがその量はTTXの場合の2～5割程度と少ない。TTXを持つ海産フグはTTXを選択的に蓄積する能力があるといえる。

以上の知見から，有毒フグのTTXが内因性ではなく餌由来であることは明らかである。フグに共生もしくは寄生するTTX産生細菌がフグ体内でTTXを産生し，フグがそれを直接取り込んで毒化

するというルートも考えられるが，食物連鎖による生物濃縮を経ないとすると，細菌の産生するTTX量は天然フグの極めて高い毒量を説明するには少なすぎるように思われる。さらに無菌でない囲い養殖フグ5,000個体以上が無毒であったことを考慮すれば，フグ腸内で細菌が産生したTTXのフグのTTX蓄積に対する寄与は，あったとしてもごくわずかで無視しうるものであろう。

2．各組織へのTTXの移行・蓄積

フグの毒化は細菌から始まる食物連鎖で説明することが可能となった。しかしながら，有毒餌生物を介してフグ体内に取り込まれたあとのTTXの存在形態や動態に関しては，いまだに不明な部分が少なくない。最近筆者らは，天然では主に肝臓にTTXを保有するトラフグの無毒養殖個体の筋肉にTTXを注射投与すると，血液を介して速やかにTTXが皮や肝臓に移行することを見出した。PSPを皮に保有する淡水フグ(*Tetraodon turgidus*)の無毒養殖個体にPSPを投与した場合も同様の移行が観察されるが，この場合，肝臓への移行量はわずかで，最終的に体内に残存した毒の9割以上が皮に移行・蓄積した[6]。一方，海産フグ肝臓の培養組織が*in vitro*で蓄積したTTXを取り込むとの報告もある[9]。無毒の一般魚ではこのような現象はみられず，明らかにフグの肝臓や皮には特異なTTX/PSP蓄積機構が存在する。関連して，海産フグの血漿中にはTTX結合性タンパク質の存在が見出されており[10]，このような高分子物質がTTXの輸送や蓄積に深く関わっているものと推察される。

3．TTXの生理機能

前述のように，フグをはじめ非常に多様な生物がTTXを保有する。これらの生物においてTTXの体内分布を知ることにより，TTXの機能を推定することが可能である。海産フグやヒラムシは卵の毒量が非常に高い。またフグやイモリは，皮に腺組織または分泌細胞を持ち，外的刺激によりTTXを分泌することから，彼らは卵や自身を外敵の捕食から守る防御物質としてTTXを保有している可能性がある[6][11]。一方，ヒョウモンダコは後

表2　TTX保有生物と非保有生物のTTXに対する抵抗性

生物	種	MLD (MU/20g)*
フグ以外のTTX保有生物		
魚類	ツムギハゼ	300以上
両生類	ニホンイモリ	10,000以上
甲殻類	スベスベマンジュウガニ	1,000
フグ類		
フグ科・有毒	クサフグ	700～750
	ヒガンフグ	500～550
	トラフグ(養殖)	300～500
フグ科・通常無毒	シロサバフグ	15～18
	クロサバフグ	19～20
	ヨリトフグ	13～15
ハコフグ科・無毒	ハコフグ	0.9～1.3
一般魚類	イシガキダイ	0.8～0.9
	イシダイ	0.8～1.8
	メジナ	0.3～0.5
ほ乳類	マウス	1

*腹腔内投与におけるTTXの最少致死量(MLD；MU/20g体重)

部唾液腺に，ヒモムシは口吻にTTXをもち，餌生物の捕獲にそれを利用していると考えられる[6]。関連して，最近筆者らはその機構は不明であるが，養殖フグにフグ毒添加飼料を与えて飼育すると免疫機能が活性化することを見出している[12]。

TTXを保有する有毒海産フグ，ツムギハゼ，スベスベマンジュウガニ，およびニホンイモリはTTXに対して極めて高い抵抗性を示す[5][6]。すなわちこれらの生物に腹腔内投与した場合のTTXのMLDは，マウスの300～1,000倍(イモリでは10,000倍以上)に達する(表2)。これに対し，無毒の海産フグは中程度(MLDがマウスの13～15倍)，一般魚はマウスと同程度の低い抵抗性を示す。有毒フグやイモリのTTX抵抗性発現メカニズムのひとつとして，彼らがTTX耐性型のナトリウムチャンネルを保有していることが上げられる[13]。TTX保有イモリを捕食するある種のヘビやPSPで毒化する二枚貝は，TTXまたはPSPに曝されることでナトリウムチャンネルをコードする遺伝子に変異を起こし，TTX/PSP抵抗性を獲得するとの報告もある[14][15]。

ごく最近筆者の共同研究者らは，無毒トラフグ人工稚魚にTTXを投与すると行動生態が天然魚に近くなり，捕食魚による食害を受けにくくなることを見出した。このことはフグの中枢神経系においてTTXが情報伝達の制御に関わっている可能性を示唆している。脳神経系におけるナトリウムチャンネルの変異やTTXの有無がフグの生理・生態に大きな影響を及ぼしている可能性がある。

Ⅳ　TTXによるヒトの中毒事例

1．フグによる中毒

我々日本人の多くは，フグ，特にその肝(きも)には致死的な毒があることを知っている。にもかかわらず昔から，'秘伝の毒抜き'を施すなどして多くの食通があえて肝を食してきた。しかしながらこれによる食中毒もあとを絶たず，厚生省（現厚生労働省）は1983年12月に「フグの衛生確保について」の通知を出し，あらゆるフグの肝を食用に供することを禁止した（現時点では無毒養殖フグの肝も食用不可)。この通知以降，専門店での事故はほとんどなくなったが，素人が自分で釣ったフグや知人から譲り受けたフグを自ら調理し，肝まで食べて中毒する事例がいまだにあとを絶たない。厚生労働省の統計によれば，2002～2006年の5年間において，フグによるTTX中毒の患者数は223名で，うち13名が死亡している（表3)。中毒の一例[1]を記すと次のようである。1996

表3　動物性自然毒による食中毒発生状況
(厚生労働省統計 2002～2006年の合計値)

原因食品	原因毒	事件数	患者数	死者数
フグ(フグ科)	テトロドトキシン	166	223	13
シガテラ毒魚 (バラフエダイ，バラハタ等)	シガテラ毒	17	67	0
エゾバイ科貝 (エゾボラモドキ，ヒメエゾボラ等)	テトラミン	16	38	0
ハコフグ	パリトキシン様毒	3	6	0
ナガヅカ	ジノグネリン	1	4	0
ウミガメ	不明	1	1	0
不明		19	31	1
計		223	370	14

年10月，長崎県長崎市で48歳の男性が天然コモンフグの肝臓を軽く調理し，筋肉と一緒に4切れ以上を食べて中毒した。食後30～60分で手足がしびれ始め，その1時間後には呼吸不全に陥り，チアノーゼが現れた。ただちに入院したが，まもなく呼吸麻痺で死亡した。食べ残しの筋肉と肝臓について毒性を調べたところ，筋肉は無毒であったが，肝臓の毒力は715～4,260 MU/gで，わずか数ないし10数グラムの摂取でヒトの推定致死量（10,000 MU）に達することが明らかとなった。さらに機器分析により毒の本体はTTXと同定され，問題の男性はコモンフグ肝臓のTTXを摂取したことにより死亡したと結論された。

中国や台湾では，日本ほど頻繁にはフグを食べないが，天然フグの喫食により多くの食中毒事例が発生している[16]。日本の事情とは異なる例，すなわち，からすみ（ボラ卵巣の塩蔵品）の模造品として売られていたフグの卵巣，あるいはカワハギの肉として販売されていた有毒フグの乾燥魚肉を食べて中毒した例などもみられる。一方，東アジア地域以外の国では，一般にフグを食べる習慣がなく，中毒事例はそれほど多くない。米国では海産フグの摂取により10名の死亡例が報告されているが，そのうち4例は1908～1925年にハワイで発生したもので，いずれもモヨウフグ属の1種が原因種とされている[4]。一方バングラデシュでは，1998年11月にタキフグの卵巣の喫食による中毒事件が発生している。8名が摂食2時間後に，唇のしびれ，麻痺，嘔吐，呼吸困難などを呈し，このうち病院に搬送される途中2名，さらに入院後に3名が死亡したという。オランダ人船員2名が南アフリカ産フグの肝臓を食べ，20分以内に死亡したという記録もある[4]。

2．小型巻貝による中毒

中国や台湾では昔から，アラレガイ，ハナムシロガイ類縁種などの小型巻貝類を食べる習慣があり，それによる食中毒が頻発している[16][17]。中国本土の場合，公式記録として残っているだけでも，1985年から2004年にかけての20年間に少なくとも28件の事例が発生しており，患者総数は187名，うち死者は21名にのぼる。一例を記すと次のようである。2001年6月に浙江省温

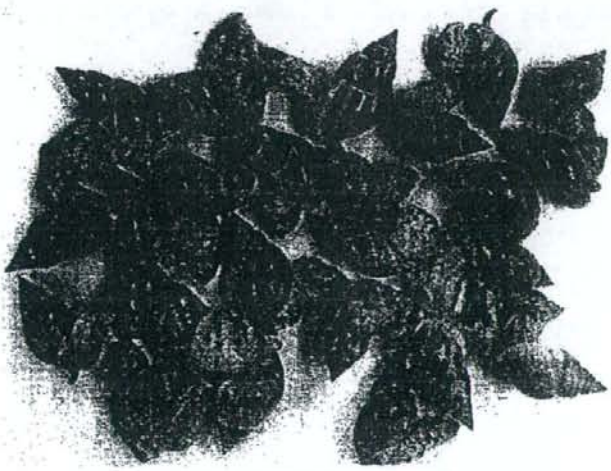

図3　扁肉食性巻貝キンシバイ

2007年7月に長崎市で本巻貝による食中毒事件が発生した。被害者は，しびれ，麻痺，呼吸困難などを呈したあと，一時呼吸停止に陥った。

嶺市において，ハナムシロガイ類緑種の喫食による中毒事件が発生した。患者は12歳～73歳の温嶺市民31名で，麻痺，吐き気，嘔吐，失語，昏睡などの症状を呈した。原因となった貝は近くの海岸で採られたもので，ボイルの上，1人10～100個食べていた。食べ残しの貝10個体の調査では，可食部で最大688 MU/g (平均307 MU/g) という高い毒性が認められ，原因物質はTTXと同定された。

小型巻貝によるTTX中毒は，中国・台湾だけの問題ではない。長崎市でも2007年7月にキンシバイという小型巻貝(図3)による食中毒事件が発生した。患者は60歳の女性で，しびれ，麻痺，呼吸困難などを経たあと，一時呼吸停止に陥る極めて重篤な症状を呈した。事件直後に食べ残しの貝を入手して調べたところ，筋肉や中腸腺から最高4,290 MU/gに達する高濃度のTTXが検出された。小型巻貝の毒蓄積機構は明らかでないが，それらは魚の死骸などを食べることから，'腐肉食性の巻貝' と呼ばれており，産卵後に死んだフグの有毒内臓を食べてTTXを蓄積している可能性もある。

3．その他の生物による中毒

前述のフグや小型巻貝以外にTTX中毒を引き起こす生物として，ボウシュウボラを上げることができる[4]。1979年12月に静岡県清水市（現静岡市清水区）で同巻貝の中腸腺を食べて41歳の男性が中毒し，ただちに入院して人工呼吸器の助けを借りた。男性は2日間意識不明であったが，5日後には完全に回復した。食べ残しの中腸腺からは17,000 MUと，ヒトの推定致死量を超えるTTXが検出された。1982年に和歌山県，1987年に宮崎県でも同様の中毒が発生している。

一方，日本で問題になることはないが，タイやカンボジアなど東南アジアの一部の国ではカブトガニの卵を食材として利用しており，それによる食中毒がまれに発生する[4]。患者の症状はTTXないしPSP中毒と類似する。マルオカブトガニの卵や肝膵臓から両毒が検出されており，これらの毒のいずれか，もしくは両毒の共存によりカブトガニ中毒が引き起こされるものと考えられる。

V　TTXとは異なる毒を持つフグによる中毒

1．PSPを持つフグ

東南アジアの河川や湖沼には淡水産の小型のフグが生息している。日本にも輸入されて観賞用に売られているが，食用にされることはない。しかしながらタイやカンボジアでは時にこれによる食中毒が発生し，死者も出ている。筆者らは1990年代にタイ産淡水フグ2種，*Tetraodon leiurus* および *T. suvatii* の毒組成を調査した。その結果TTXは全く見当たらず，代わって主に皮から多量のPSPが検出された[3][18]。PSPはある種の有毒植物プランクトンが産生する1群の神経毒で，このプランクトンが異常発生すると，その海域に棲む通常は無毒の二枚貝が毒化し，食中毒を引き起こすことがある[1]。PSPの主要成分であるサキシトキシン(STX) はTTXとは化学構造が異なる別の毒であるが，分子の大きさや毒力，中毒症状はTTXとほぼ同じで，0.5mg程度の極微量でヒトを死に至らしめることができる[1]。淡水フグではブラジル産の *Colomesus asellus* やカンボジア産の *T. turgidus* も主に皮にPSP主体の毒を持つ[8]。フィリピン産の数種の海産フグや日本近海産のホシフグも主要毒成分としてSTX群を保有することが明らかにされている[3]。米国フロリダでは2002年～2004年にかけ，インディアン・リバー・ラグーン産の *Sphoeroides* 属のフグにより28の中毒事例が発生している。このフグは筋肉に多量のPSPを保有する。

2．パリトキシン様毒を持つフグ

バングラデシュには2種の淡水フグが生息している。これらに市場価値はほとんどないが，地方の漁村では他の魚種と混獲されたフグを食べ，死者を伴う中毒が頻発しているという。中毒事例を調査したところ，呼吸困難や麻痺など1部の症状はTTXもしくはPSP中毒と共通するものであっ

STX (サキシトキシン)

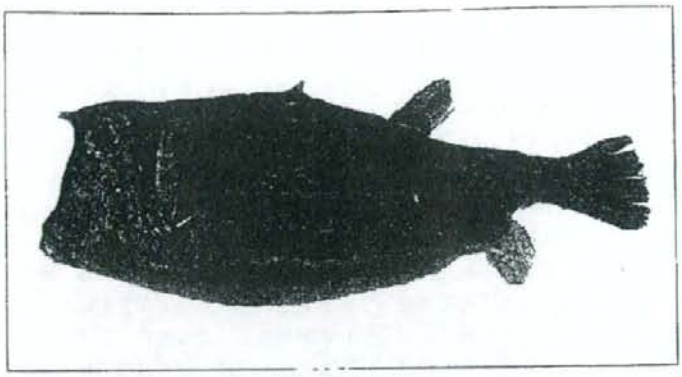

図4 ウミスズメ
2001年～2007年にかけ，三重，宮崎，長崎の各県でハコフグ類の喫食によるアオブダイ中毒様食中毒が相次いで発生した。写真は一部の事件で原因魚種として疑われているハコフグ科の魚，ウミスズメ。

たが，ミオグロビン尿症(黒色の尿が出る)や血清クレアチンホスホキナーゼ（CPK）値の上昇を伴う激しい筋肉痛がみられる点，ならびに発症から死亡もしくは回復に至るまでの時間経過が比較的長いという点では，アオブダイ中毒（日本でまれに起こる特異な食中毒）に酷似していた[19]。バングラデシュ産淡水フグも他の淡水フグ同様PSPを保有することが知られているが，食用となる筋肉の毒量はほとんどの試料で数MU/gと低く，これだけで死者を伴う食中毒を説明するのは難しい。そこでアオブダイ中毒の研究で用いる分析法をバングラデシュ産淡水フグに適用したところ，同中毒の原因物質と考えられているパリトキシン（PTX）様毒が検出された。PTXはTTXやSTXのさらに上をいく強力な毒で，腔腸動物パリソアやある種の付着性渦鞭毛藻が産生することが知られている[1]。日本では，2001年11月～2007年8月にかけ，三重，宮崎，長崎の各県でハコフグ類（図4）の喫食により同様の中毒が5件発生し，9名が中毒，うち1名が死亡している[19]。

Ⅵ おわりに

上述のように，日本あるいは中国，台湾などでは，フグや巻貝によるTTX中毒が多発している。中毒症状は原因食品の摂取後すぐに現れ，致死時間は平均6時間程度と非常に短い。従って中毒した場合は，ただちに設備の整った病院に運ぶことが肝要である。今のところTTXに対する解毒剤や特効薬はなく，体外への毒の排出を促進し人工呼吸器の使用により呼吸循環系を適切に管理する以外，根本的な治療法はない。近年，モノクローナル抗TTX抗体が開発され[20]，研究用の試薬として活用されているが，臨床的な効果はほとんどないと考えてよい。

一方TTXはナトリウムチャンネルの特異的ブロッカーとして，主に神経生理学分野の研究において極めて重要な役割を演じてきた。中国では臨床用医薬(末期ガン患者の鎮痛薬)としても用いられているという。日本でも以前は神経痛とリウマチの止痛薬として臨床応用されていた。

魚介毒の中には，PTX様毒やシガテラ毒[1]など海洋環境の変化により分布の広域化や保有生物の

CPK（血清クレアチンホスホキナーゼ）　PTX（パリトキシン）

多様化が危惧されているものもある。TTXのみならずそのような魚介毒についても，その性状や保有生物における蓄積機構・生理機能を熟知することで，中毒を未然に防ぐ手立てを案出・確立することができよう。さらにTTXの試薬としての応用や無毒養殖フグ作出の例のように，毒そのもの，もしくは毒保有生物を有効利用する技術を開発することが可能となるであろう。

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The metabolisms of agaritine, a mushroom hydrazine in mice

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Abstract

The mushroom hydrazine agaritine was measured in mouse plasma and urine using LC/MS/MS, which is highly specific. Agaritine concentration peaked 20 min after oral administration to mice (4.0 and 40 mg/kg). The concentration gradually decreased and returned to the basal level in 100 min. The maximum concentration, the time to the maximum concentration, and the half life were 0.37 μg/ml plasma, 0.33 h, and 0.71 h, respectively after administration of agaritine at 40 mg/kg body weight. One agaritine metabolite was found in the plasma and the urine from agaritine-administered mice. The structure of metabolites of agaritine by γ-GT was next investigated using LC/MS. HMPH proved to be generated from agaritine. The oxidative stress marker 8-OHdG was detected in agaritine-administered mouse urine. After administration, the 8-OHdG level immediately tripled, and then decreased to the control level over 48 h. Its level then elevated again and remained high for 11 days. These results suggest that agaritine quickly metabolizes and disappears in the plasma, whereas DNA damage lasts for a long time after a single administration of agaritine to mice.
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Keywords: Agaritine; Mushroom; Plasma; Urine; LC/MS/MS; Phenylhydrazine; Metabolism

1. Introduction

Agaricus mushrooms (Agaricus spp.) including the cultivated mushrooms, Agaricus bisporus, contain substantial amounts of aromatic hydrazines. The most abundant is genotoxic agaritine, [β-N-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine] (Kelly et al., 1962; Gigliotti and Levenberg, 1964). This L-glutamic acid-containing phenylhydrazine is susceptible to oxidation. Toth and Erickson demonstrated that the administration of uncooked mushrooms to mice induced a significant increase in the number of bone and forestomach tumors in both sexes, and in the occurrence of lung tumors in males (Toth and Erickson, 1986; Toth et al., 1998). Bladder implantation of methanol extracts of fresh mushrooms induced cancer in the bladder epithelium (Hashida et al., 1990). Toth et al. reported that backed mushrooms could induce tumors (Toth et al., 1997). Ethanolic and aqueous extracts from A. bisporus demonstrated mutagenicity in the Ames test (von Wright et al., 1982). The carcinogenicity and mutagenicity of these mushrooms can be attributed to agaritine and 4-(hydroxymethyl)phenylhydrazine (HMPH) or the 4-(hydroxymethyl)benzenediazonium ions (HMBD), both of which are believed to be formed by the enzymatic degradation of agaritine, resulting in the loss of the γ-glutamyl group (Toth et al., 1978, 1981; Toth and Nagel, 1981; Walton et al., 1997). It has been previously reported that HMPH and HMBD are highly unstable and exhibit carcinogenicity (Toth et al., 1978, 1981; Ross et al., 1982; Walton et al., 1997). HMPH and HMBD are thought to be potent components that exert genotoxicity. However, they are virtually impossible to detect, because of their instability. The formation of HMPH was speculated by a UV absorption shift from 273 to 325 nm in the presence of sodium glyoxylate (Gigliotti and Levenberg, 1964).

Ross et al. revealed the recovery of agaritine from the gastro-intestinal tract of 3 mg-agaritine-administered mice using the HPLC–UV method. They also showed the agar-

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itine contents in the stomach, small intestine, caecum, and large intestine were successfully determined, but failed to detect agaritine in the blood because of the low sensitivity of the UV method (Ross et al., 1982). Walton et al. reported an investigation of the agaritine content in blood and urine using a scintillation counting method after ^{14}C-labeled agaritine had been administered to rats and mice. In the mouse, radioactivity in the blood peaked after 30 min, and then gradually decreased over a period of 12 h (Walton et al., 2000). The ^{14}C-labeled-agaritine-based experiment, however, has a limitation in that the radioactivity detected in the blood cannot distinguish between intact agaritine and degradative products including ^{14}C-labeled moieties. The fate of agaritine has thus up to now remained unclear.

More recently, we have established a new method for agaritine determination in mouse plasma and urine using HPLC coupled with tandem mass spectrometry (LC/MS/MS) that has high sensitivity and specificity (Kondo et al., 2006a,b). In the present study, the agaritine content in the plasma and the urine from agaritine-administered mice was determined using this method in addition to LC/MS/MS investigation of agaritine metabolism and DNA adduct (Singh and Farmer, 2006), the structure of the degraded products, DNA damage by agaritine in mice. This should facilitate the assessment of the health risk to humans.

2. Materials and methods

2.1. Chemicals and LC/MS/MS conditions

Agaritine and agaritine-COOH ([β-N-(γ-L(+)-glutamyl)-4-carboxyphenylhydrazine]) were synthesized according to the methods of Wallcave et al. and Hoesch et al. with minor modifications (Wallcave et al., 1979; Hoesch and Datta, 1987). Briefly, reaction mixtures in the reduction reaction of carboxylic acid to hydroxymethyl group in p-hydrazinobenzoic acid moiety were recrystallized from acetone/diethyl ether (1:4) to remove byproducts. Total yield was 17%.

The purities of the synthetic compounds were >95% by HPLC (254 nm) and >95% by ^1H NMR (500 MHz, DMSO-d_6 and DMSO-d_6-D_2O). The synthetic agaritine and agaritine-COOH were stored at −80 °C under a N_2 gas atmosphere. Standard stock solutions were prepared in methanol and stored at below −20 °C before use. The both compounds were stable in methanol for 1 week at −20 °C.

The structures of synthetic agaritine and agaritine-COOH were examined using 2-D NMR and high-resolution (HR) mass analysis. The results of ^1H, ^{13}C, and 2D NMR (HMBC and HMQC) were recorded using an ECA 500-MHz FT NMR spectrometer (JEOL, Japan).

Chemical shifts (δ) are described in ppm using tetramethylsilane (TMS) as a reference. Coupling constants (J) are given in Hz. HR-FABMS was performed in positive mode to identify them described previously (Kondo et al., 2006).

LC/MS/MS measurements were performed using a PE SCIEX model API 3000 triple–quadrupole mass spectrometer coupled to an Agilent 1100 HPLC system, including a G1315 photodiode array detector and a 3-μm Shiseido Capcell Pak AQ column (2.0 × 250 mm). Gradient conditions ranged from 99% water containing 0.01%AcOH–1%MeOH to 90% MeOH–10% water containing 0.01% AcOH. The analyte was detected using electrospray ionization in negative mode. Multiple-reaction-monitoring (MRM) was performed using characteristic fragmentation ions (m/z 266 > 248 and 266 > 122) for agaritine. A full scan analysis (m/z 50–1000) was carried out to analyze agaritine metabolites in plasma and urine. The parameters for the LC/MS/MS analysis of agaritine were as follows. Ionspray voltage (IS) = −4500 V; collision gas = 6; focusing and entrance potentials (FP and EP) = −60 V and −20 V, and temperature (T) = 500 °C, respectively. A switching valve led the column eluents to the mass spectrometer while the analytes were being eluted. The data were acquired and calculated using Analyst 1.4.1 software (PE SCIEX). Metabolite ID 1.3 was used to analyze agaritine metabolites.

2.2. Animals

Male ddY mice (7 weeks, 26–30 g, average = 28.07 ± 0.81 g) were purchased from SLC (Shizuoka, Japan) and were housed for one week. Each cage contained three or four mice. The animals were kept on a 12 h light/dark cycle, at room temperature of 24 ± 1 °C, humidity of 55 ± 5%, with free access to food (CRF-1, Oriental Yeast Co Ltd.) and water. The animals were used according to the guidelines of National Institute of Health Sciences and the Ministry of Health, Labour and Welfare of Japan.

2.3. Agaritine in plasma and pharmacokinetic study

The synthetic agaritine in MilliQ water (4.0 and 40.0 mg/kg) was administered to male ddY mice (8 weeks) by gavage after overnight starvation. The 33 mice were used for the time course of agaritine content in mouse plasma. Three mice were anesthetized with diethyl ether and the blood samples were withdrawn from them to prepare the plasma every 20 min until 180 min after agaritine-administration. MilliQ water was administered to the control mice. The pharmacokinetic parameters were calculated using the PK program @PKANS (D Three, Japan).

Mouse plasma from agaritine-administered mice was prepared as follows; blood was collected 20 min after oral administration of agaritine to mice and immediately placed on ice before centrifugation (10,000 rpm, 2 min). After centrifugation, the plasma (200 μL) was deproteinized by acetonitrile (750 μL), and then evaporated the solvent. The residue was dissolved with mobile phase (600 μL).

2.4. Agaritine in urine

For the analysis of agaritine and its metabolites in mouse urine, 20 mice were divided into five groups (one control and four agaritine-administration groups). Each group of four mice was housed in a metabolic cage immediately after oral administration of agaritine or MilliQ water to mice, and then urine was collected in 50 ml Falcon tubes every 12 or 24 h. Urine and fecal matter were separated by metabolic cages. Falcon tubes were covered with aluminum foil to avoid oxidative degradation.

2.5. Oxidative stress

Forty mice were divided into 5 control and 5 agaritine administration groups. Each group comprised 4 mice. The synthetic agaritine was dissolved in MilliQ water prior to this experiment. Mice were put in metabolic cages immediately after agaritine or MilliQ water administration. Mouse urine was then collected at indicated times (12, 24, 48, 72, 216, 264 h). Urine samples were diluted to 1:10 for ELISA. Creatinine contents were also measured by an enzymatic method. Oxidative damage to mice was evaluated by quantifying an oxidative stress marker 8-OHdG (8-hydroxy-2′-deoxyguanosine) according to the manufacturer's instruction. This marker was measured using a high sensitivity 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). MDA (malondialdehyde) levels were also measured using HPLC after the reaction of MDA with thiobarbituric acid (TBA).

2.6. Agaritine degradation by γ-glutamyltranspeptidase (γ-GT)

Sodium glyoxylate (23.5 mg, Sigma, St. Louis, MO) and bovine γ-GT (29.2 mg, Sigma EC 2.3.2.2) were added to agaritine (3.6 mg) in 2.0 ml of

PBS. The mixture was allowed to stand for 180 min at room temperature. UV spectra were recorded at intervals. The reaction mixture was membrane-filtered (Millipore, Microcon YM-3, Bedford, MA) to separate the low molecular weight fraction from the high molecular weight fraction. The low molecular weight fraction was analyzed using LC/MS coupled with a photodiode array detector (PDA). Similarly, agaritine-COOH (0.086 mg) in 2.0 ml PBS was subjected to the same reaction using sodium glyoxylate (4.6 mg) and γ-GT (4.5 mg).

2.7. Agaritine degradation in microsomes

Pooled male human liver microsomes and male CD-1 mouse liver microsomes were purchased from Charles River Laboratories Japan. Microsomes (1 mg/ml) were incubated with NADPH regenerating system (Oriental Yeast Co Ltd.), uridine 5′-diphosphoglucuronic acid (UDPGA, Sigma), or 3′-phosphoadenosine 5′-phosphosufate (PAPS, Sigma) at 37 °C for 60 min. Decrease in agaritine content was monitored using LC/MS/MS.

3. Results

3.1. Determination of agaritine in mouse plasma and urine

To date, agaritine in blood samples has been determined by a scintillation counting method after administration of ^{14}C-radio labeled agaritine due to its higher sensitivity than the UV method (Walton et al., 2000). The total amounts of intact agaritine and its metabolites were given as a level of radioactivity. More recently, we have developed a sensitive and specific method for determining agaritine in mushroom samples using LC-coupled electrospray ionization tandem mass spectrometry (LC/MS/MS) (Kondo et al., 2006a,b). Determination of agaritine in mouse plasma and urine was also carried out using this method. As shown in Fig. 1B, there were no interference peaks on the chromatograms of the blank mouse plasma. The recovery of agaritine from the plasma spiked with 0.25 and 2.50 μg/ml of the synthetic agaritine was 77% (n = 11) and 90% (n = 3), respectively, and the spiked sample provided a distinct agaritine peak on the chromatogram (Fig. 1C). Application of our LC/MS/MS method to the mouse urine samples showed agaritine to be similarly distinctly identified without any interference peaks in the urine samples (data not shown). Multiple-reaction-monitoring (MRM) proved possible using two characteristic fragmentation ions (m/z 266 > 248 and 266 > 122) for agaritine. We used MRM (m/z 266 > 248) for the mouse plasma and urine samples because of their lower background.

We have next investigated the time course of the agaritine content in the mouse plasma after a single administration of the synthetic agaritine to mice. Agaritine appeared in the plasma 5 min after administration and the agaritine level peaked after 20 min. It then gradually decreased to the basal level over 100 min, as shown in Fig. 2. The limit of quantification using this method was 0.005 μg/ml plasma. Two different doses were administered: 4 and 40 mg/kg body weight. The time-courses were virtually the same. The pharmacokinetic parameters were calculated from these experiments. The maximum concentrations of agaritine in blood were 0.06 μg/ml (4.0 mg/kg p.o.) and 0.37 μg/ml (40.0 mg/kg p.o.), and the time to the maximum concentration was very fast (0.33 h) in both cases. The AUCs (area under the blood concentration–time curve) showed 0.06 μg ml^{-1} h (4.0 mg/kg p.o.) and 0.50 μg ml^{-1} h (40.0 mg/kg p.o.), which were different from the AUC

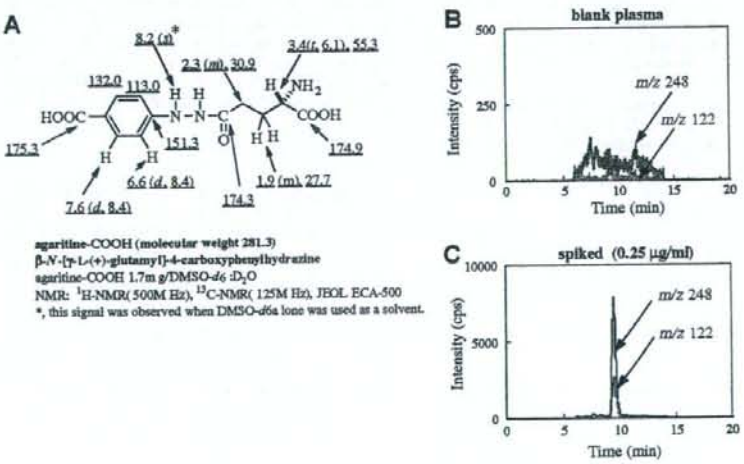
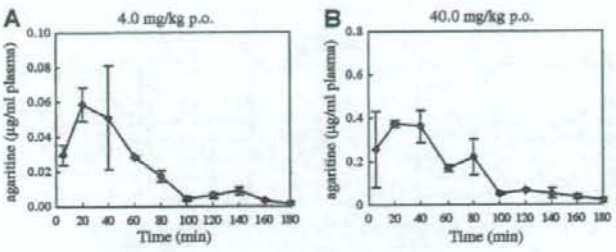


Fig. 1. Structure of agaritine-COOH and typical chromatograms of agaritine: (A) Structures and ^{1}H and ^{13}C NMR chemical shifts (δ). NMR measurements of agaritine-COOH in DMSO-d_6/D_2O were carried out using ECA-500; (B) MRM chromatogram of blank plasma; (C) MRM chromatogram of the plasma spiked with the synthetic agaritine (0.25 μg/ml). Two fragment ions were monitored simultaneously (m/z 266–248 in blue, m/z 266–122 in red). MRM (m/z 248) was used to determine agaritine content in mouse plasma. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)



C **Pharmacokinetic parameters of agaritine in plasma**

parameter	mouse		Walton et al.
	4.0 mg/kg	40.0 mg/kg	25 mg/kg
C_{max}, μg/ml	0.06	0.37	8.18
t_{max}, h	0.33	0.33	0.49
$t_{1/2\ max}$, h	0.61	0.71	0.99
k, h^{-1}	1.14	0.98	3.1
AUC, μg/ml*h	0.06	0.50	20.97

*Walton *et al.*: *Nutr Cancer*, **37**, 55–64 (2000)

D **Agaritine content in mouse urine**

Time after administration (h)	agaritine (μg/ml)
0-12	0.042 ± 0.021
12-24	N.D.
24-48	N.D.

Fig. 2. Time course of agaritine content in the plasma and agaritine content in the urine collected from agaritine-administered mice. Thirty three mice were used. (A and B) Synthetic agaritine (4.0 mg/kg or 40.0 mg/kg) was orally administered to 8-week male ddY mice. After administration, blood was withdrawn every 20 min from three mice to prepare plasma samples. Agaritine concentration in the plasma peaked 20 min after administration. Values are means ±SD from three mice. (C) Pharmacokinetic parameters of agaritine in mice. C_{max}, maximum concentration; t_{max}, time to maximum concentration; $t_{1/2}$, half life, k, absorption rate constant; AUC, area under the blood concentration–time curve. (D) Agaritine concentration in the urine. Twenty mice were housed in 5 groups in metabolic cages (each group comprised 4 mice). The 0–12 h urine after agaritine administration contained agaritine. Values are means ±SD from 5 groups of mice.

value 20.97 μg ml^{-1} h (25 mg/kg p.o.) reported by Walton et al. The results are summarized in Fig. 2.

We also analyzed the agaritine content in the urine. Agaritine was detected in the urine collected between 0 and 12 h after agaritine administration (0.042 μg/ml urine). In the urine between 12 and 48 h or later after administration, no agaritine was detected as shown in Fig. 2D Taken together, these results suggest that agaritine is rapidly metabolized in mice.

3.2. Agaritine metabolites

Agaritine metabolites in the plasma and urine samples were investigated using LC/MS (total ion scans) coupled with DAD (total wavelength scans). The plasma samples (control and agaritine administration groups) were analyzed and the data acquired were compared to each other using Analyst 1.4.1. Fig. 3A and B show total ion chromatograms (TIC) and total wavelength chromatograms (TWC) of the plasma from the control group and from the agaritine-administered group, respectively. The TIC showed no difference between the two groups. To identify metabolites from the TIC of the two groups, we used Metabolite ID software (Applied Biosystems). However, no agaritine metabolites were found. On the other hand, one metabolite peak was detected in the TWC of the plasma samples 20 and 60 min after agaritine administration as shown in Fig. 3B. The metabolite peak became clearer on the chromatograms taken at UV 270 nm (Fig. 3C) and its UV spectrum was presented in Fig. 3D.

The urine samples were similarly investigated. The urine collected between 0 and 12 h after agaritine administration showed a distinct metabolite peak. The urine samples collected between 12 h and 48 h or later after administration did not show any such peak (Fig. 3E). The UV spectrum of the peak (λ_{max} 282 nm) was the same as that of the metabolite peak shown in the plasma, suggesting that the same metabolites are formed from agaritine in both mouse plasma and urine (Fig. 3F). However, the structure of the compounds remains to be solved, since the product was present only in trace quantities.

3.3. Oxidative stress

Agaritine is a phenylhydrazine derivatives, but the hydrazine moiety of agaritine (–NH–NH–) is masked by glutamic acid. Phenylhydrazine (Ph-NHNH2) can produce free radicals through free iron release, which causes anemia (Ferrali et al., 1997), suggesting that agaritine or its metabolites could subject mice to oxidative stress. We therefore investigated the effect of agaritine on oxidative stress in mice by measuring an oxidative stress marker 8-OHdG

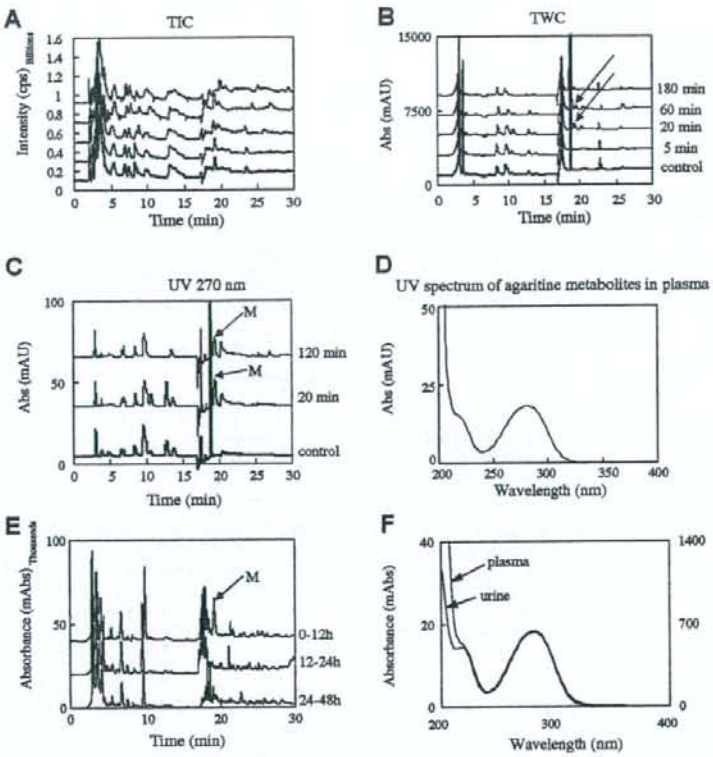


Fig. 3. Analyses of agaritine metabolites in mouse plasma and urine. LCMS coupled with PDA were used to investigate agaritine metabolites. (A and B) TIC and TWC of the plasma prepared from agaritine-administered ddY mice. The plasma samples after administration were analyzed (5, 20, 60, 180 min); the agaritine metabolite peaks were indicated by the arrows. (C) Chromatogram at 270 nm of the plasma samples (M = metabolite). (D) UV spectrum of agaritine metabolite in the plasma. (E) Chromatogram at 270 nm of the urine samples 12, 24, 48 h after administration. (F), UV spectrum of agaritine metabolite in the urine.

(8-hydroxy-2′-deoxyguanosine). As shown in Fig. 4, 8-OHdG levels in the urine collected between 0–12 h after administration almost tripled, then transiently decreased to the control level at 48 h after administration. The level then increased again and remained high for 11 days, suggesting that a single administration of agaritine may induce continuous oxidative DNA damage. In contrast, levels of malondialdehyde (MDA), another oxidative stress marker, in the urine were not significantly different between the control and the agaritine administration groups. The results of the two markers were not paralleled.

The changes in daily urine output between control and agaritine-administered mice were examined over 2 weeks. There was no significant difference in the daily urine volume, but there was a distinct tendency for the agaritine-administered group of mice to show lower urine output than the control mice during the experiments (Fig. 4A). The body weights of both groups steadily increased every week.

3.4. Degradation of agaritine in vitro

Agaritine is thought to be degraded by an enzyme γ-glutamyltranspeptidase (γ-GT), present at high levels in the kidney, to form 4-(hydroxymethyl)phenylhydrazine (HMPH), which can be a potent carcinogens. However, due to its instability, there have been no reports describing the direct detection of HMPH. We investigated agaritine degradation in mouse and human liver microsomes using LC/MS. Our results showed no decrease in intact agaritine, and no metabolites were found during one hour-incubation of agaritine in those microsome systems, suggesting that agaritine is stable in the liver microsome systems (data not shown).

In contrast, agaritine was degraded by γ-GT to form a conjugated product with sodium glyoxylate, which showed UV absorption at 325 nm (Fig. 5). This result is in good agreement with the data previously published else where (Gigliotti and Levenberg, 1964). The enzymatic reaction

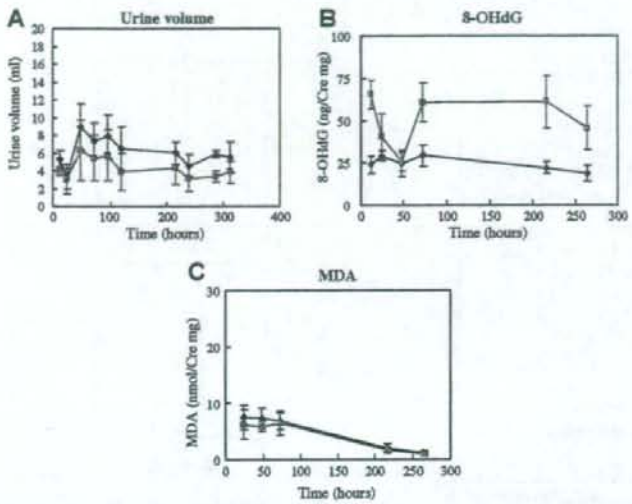


Fig. 4. Effect of agaritine on oxidative stress. Forty mice were divided into 5 control and 5 agaritine administration groups. Each group comprised 4 mice. Mouse urine was collected using metabolic cages. (A) Urine volume per day during the experiments; (B) 8-OHdG level in the urine. Oxidative stress marker 8-OHdG was measured in the urine collected from agaritine-administered mice. The urine was collected 12, 24, 48, 72 h, 216, 264 h after administration; (C) MDA level in the urine. Values are means ±SD ($n = 5$ groups). ▯ (black), control; ■(red), agaritine administration group. 8-OHdG and MDA levels are described as ng or nmol per 1 mg creatinine. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

completed within 180 min. However, there is as yet no data on where the absorption comes from. No investigations have been carried out. The reaction mixture was therefore separated through a membrane filter to form two fractions, low and high molecular weight fractions. The UV spectra were then recorded separately. The low molecular weight fraction showed UV absorptions at 292 and 325 nm, suggesting that a UV-absorbing substance, newly formed by the enzymatic reaction, was derived from a low molecular compound, not from a high molecular weight compound like γ-GT. Agaritine-COOH was similarly examined, showing that this compound was also degraded by γ-GT.

Moreover, when the low molecular weight fraction was subjected to LC/MS analysis, no peak with the absorption at 325 nm was detected (data not shown). It is possible that the high pressure in the HPLC system may decompose the product. Flow injection analysis (FIA) was carried out and one absorption peak at 325 nm was observed (Fig. 6). This peak had the same UV spectrum as that in Fig. 5 and, in addition, showed an m/z of 195 in LC/MS analysis, indicating that HMPH was indeed produced from agaritine by γ-GT though hydrazone structure.

4. Discussion

Agaritine is a major hydrazine rich in Agaricus spp. of mushrooms. This compound is believed to be degraded to metabolites such as HMPH and HMBD, which show carcinogenicity (Toth et al., 1978, 1981; Toth and Nagel, 1981; Walton et al., 1997). However, there has been no direct evidence of HMPH and HMBD formation from agaritine, and agaritine metabolism is still unclear. Ross et al. failed to detect agaritine in blood (Ross et al., 1982) due to the lack of sensitivity of their method. Walton et al. reported agaritine metabolism in the mouse and rat. Agaritine content in the blood was measured using liquid scintillation counting. After the administration of a single dose of radio-labeled agaritine, radioactivity peaked in 0.49 h and then gradually decreased over the next 12 h (Walton et al., 2000). The radioactivity in the plasma showed the total amounts of agaritine and its metabolites. They, however, failed to detect intact agaritine in the plasma or urine. We have measured agaritine in blood and urine samples using the LC/MS/MS method, which is highly specific. Agaritine appeared 5 min after agaritine administration to ddY mice, peaked in 20 min and then decreased to base level. The pharmacokinetic parameters calculated were similar to those reported by Walton et al. However, the maximum concentration of agaritine ($C_{max} = 0.37$ vs. 8.18) and area under the blood concentration–time curve (AUC = 0.50 vs. 20.97) were different. We analyzed agaritine in mouse plasma whereas Walton et al. measured radio-labeled agaritine in blood. The recovery rates of agaritine from mouse plasma were 77% and 90% as mentioned previously (Kondo et al., 2006a). We also analyzed agaritine in mouse whole blood, which showed a similar pattern of recovery (data not shown). Thus, the agaritine content we detected in the plasma is equal to

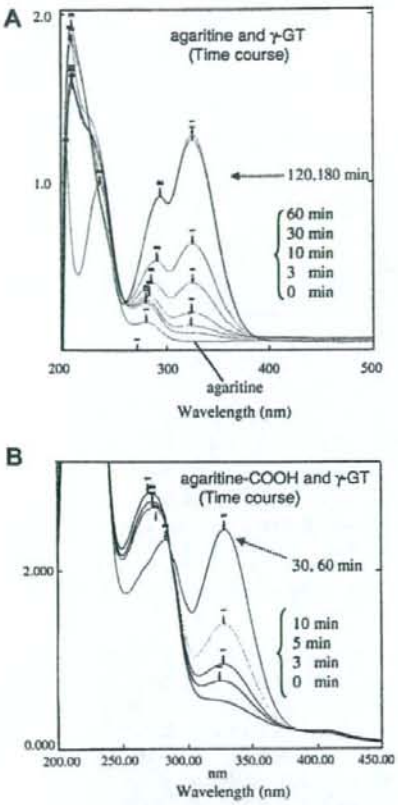


Fig. 5. Reactions of agaritine and agaritine-COOH with γ-glutamyltransferase (γ-GT). (A) Changes in the UV spectrum of agaritine during the enzymatic reaction. In the presence of sodium glyoxylate, agaritine was reacted with γ-GT and UV spectra were monitored at intervals. Sodium glyoxylate trapped the free hydrazine which was produced by the enzymatic reaction. (B) Changes in the UV spectrum of agaritine-COOH during the enzymatic reaction.

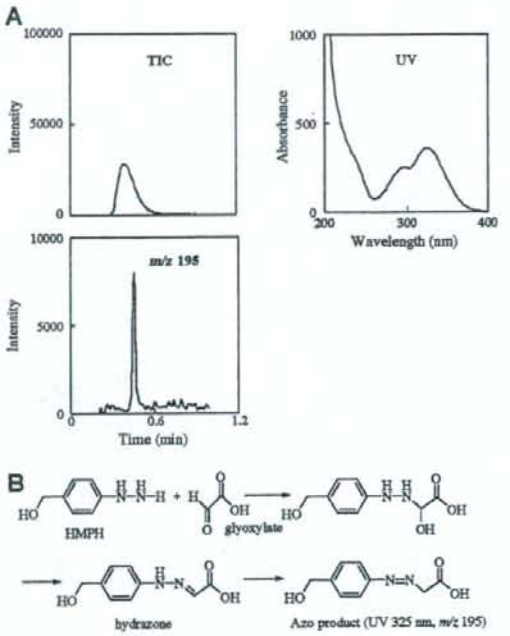


Fig. 6. Reaction product of agaritine with γ-glutamyltransferase (γ-GT) in the presence of glyoxylate. Flow injection analysis was carried out. (A) TIC of reaction product (left), UV spectrum of the peak (right), and mass chromatogram at an m/z of 195 of reaction product (bottom). (B) Reaction scheme. HMPH was generated from agaritine by γ-GT and then trapped by glyoxylate to form hydrazone followed by an azo compound that showed UV absorption at 325 nm.

agaritine contents in blood fluids. The higher values of C_{max} and AUC in Walton's report may have been based on agaritine metabolites. Another possible explanation is that the differences may depend on mouse strains. We have measured intact agaritine in blood and urine and analyzed the pharmacokinetic parameters for the first time. Assuming that the volume of mouse blood is 2 ml, the absorption ratio of agaritine is 0.053%. Agaritine contents did not change in the absence and presence of glucuronidase, indicating that there was no conjugated agaritine in the blood and urine (data not shown).

We next investigated agaritine metabolites. Agaritine is believed to be metabolized to free hydrazine HMPH by γ-GT in vitro. However, HMPH was not detected in the plasma and urine. HPLC analysis of agaritine-fed mice urine revealed that three unidentified metabolites formed, none of which was HMPH (Walton et al., 2000). In the presence of γ-GT, agaritine was thought to be metabolized to HMPH in vitro, which trapped by sodium glyoxylate. However, there is no evidence as to how agaritine is metabolized in vivo and what compounds are produced. We analyzed the plasma and urine samples taken from agaritine-administered mice using LC/MS/MS coupled with PDA, and found that only one metabolite of agaritine appeared in 20 min to 180 min in the plasma (Fig. 3C) and within 12 h in the urine (Fig. 3E) after administration. Both have the same UV spectra. Since they were only trace amounts of the product, a complete identification could not be made. However, typical ^{1}H NMR signals related to benzene-ring (δ 6.7 and 7.0 ppm) disappeared. Even partial structures of agaritine would be no longer kept. Agaritine metabolites might tightly bind to DNA and induce DNA damage though free radical reactions and finally complicated products may be excreted.

Agaritine is a phenylhydrazine derivative that can produce free radicals. This compound may affect oxidative stress to mice administered with agaritine. Thus, the oxidative stress marker 8-OHdG was measured in the mouse urine.

Surprisingly, 8-OHdG levels in the urine of agaritine-fed mice nearly tripled in the 12 h after administration. After a transient drop in 8-OHdG to the basal level over the next 48 h, the 8-OHdG level increased again and stayed high for 11 days (Fig. 4). Although another marker MDA showed no difference between the control and agaritine-administration groups, a single administration of agaritine induced DNA damage through OH radicals for 11 days. Formation of 8-[4-(hydroxymethyl)phenyl]deoxyadenosine (8-HMP-dAdo) or 8-[4-(hydroxymethyl)phenyl]deoxyguanosine (8-HMP-dGuo) was reported in a simple in vitro system containing DNA bases and HMBD (Hiramoto et al., 1995). Therefore, we investigated these DNA adducts and was not able to detect 8-HMP-dAdo and 8-HMP-dGuo in mouse urine. Although phenyl radicals produced from HMBD might react with DNA base in vivo, concomitant OH radicals could mainly react with DNA base (Hiramoto et al., 1995; Gannett et al., 1997; Lawson et al., 1995).

Walton et al. reported that radioactivity was detected in mouse liver and kidney 120 h after a single administration of ^{14}C-agaritine (25 mg/kg). Together with our results, this suggests that agaritine metabolites may play a role in oxidative damage to DNA.

Although the fate of agaritine after its administration in vivo remains unknown, γ-GT in in vitro is believed to convert agaritine into free hydrazine HMPH in an indirect manner. We confirmed that agaritine is degraded to a product that shows UV absorption at 325 nm in the presence of sodium glyoxylate, possibly an adduct of HMPH, within 180 min as mentioned previously elsewhere (Gigliotti and Levenberg, 1964; Ross et al., 1982; Walton et al., 2000). This UV absorption at 325 nm was not observed in γ-GT and sodium glyoxylate or in γ-GT and agaritine or in sodium glyoxylate and agaritine. In fact the absorption is attributable to agaritine. In addition, this low-molecular metabolite has two distinct absorption peaks at 292 and 325 nm. In a further study, we attempted to analyze this product using LC/MS and revealed that HMPH is indeed generated from agaritine. HMPH and/or HMBD converted from HMPH by liver drug-metabolizing enzymes may be potent carcinogens. Agaritine is a mutagenic compound. Kidney homogenates enhanced this mutagenicity, whereas liver microsomes alone did not, due to the low γ-GT activity of liver. On the other hand, the treatment of agaritine with kidney homogenate followed by liver homogenate strongly augmented its mutagenicity (Walton et al., 1997).

We also have data that completely degraded agaritine with heat in MilliQ water still retains weak mutagenicity and that Agaricus blazei Muril mushrooms show stronger mutagenicity than agaritine alone (unpublished data). Agaritine and unstable free hydrazines such as HMPH probably are degraded by heat. These results indicate that another mutagenic compound may be present in Agaricus mushrooms. One of which was agaritine-COOH present in Agaricus mushrooms (agaritine 1350 μg/g dry), agaritine-COOH 24.8 μg/g dry in our data vs. agaritine-COOH 42 μg/g wet in (Chauhan et al., 1985). This compound also reacts with γ-GT as agaritine does as we already showed in the present study. Therefore, the mutagenicities of Agaricus mushrooms are attributed to agaritine and agaritine-COOH, both of which can produce free hydrazines, leading to the formation of free radicals.

In summary, agaritine was absorbed immediately after agaritine administration and disappeared in 100 min from mouse plasma. It was excreted within 12 h in urine. DNA damage occurred for 11 days after a single administration of agaritine. Our findings may assist with evaluating the carcinogenic effects of Agaricus mushrooms in humans.

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