The mutagenic activity of agaritine – a constituent of the cultivated mushroom *Agaricus bisporus* – and its derivatives detected with the Salmonella/mammalian microsome assay (Ames Test)

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Mutagene Aktivität von Agaritin – einem Inhaltsstoff des Zuchtchampignons *Agaricus bisporus* und seiner Abbauprodukte bestimmt mit dem *Salmonella*/Säuger-Mikrosomen Test (Ames Test)

Zusammenfassung. Gereinigtes Agaritin [N'-(γ-L(+)-glutamyl)-*p*-hydroxymethylphenylhydrazin]; (ein im Zuchtchampignon *Agaricus bisporus* vorkommender Inhaltsstoff), *p*-Hydrazinobenzoesäure (eine hypothetische Vorstufe von Agaritin) sowie einige Abbauprodukte von Agaritin wurden mit Hilfe des *Salmonella*/Säuger-Mikrosomen-Tests (Ames Test) auf mutagene Aktivität untersucht. In Übereinstimmung mit Literaturdaten zeigte Agaritin eine deutliche direkt wirkende mutagene Aktivität mit *Salmonella typhimurium* TA1537 (30 Revertanten/μmol) und TA97. Inkubation von Agaritin bei alkalischem pH führte zu einem pH-abhängigen Anstieg der Mutagenität. Inkubation von Agaritin mit γ-Glutamyltransferase (GT) während 10 h (Zimmertemperatur; pH 8.2) führte sogar zu einer 8- bis 16fachen Zunahme der Mutagenität. Synthetisches *p*-Hydroxymethylphenylhydrazin (das vermutliche Produkt des durch die GT katalysierten Abbaus von Agaritin) zeigte allerdings eine nur etwa 3- bis 6mal höhere mutagene Aktivität als Agaritin. Das hypothetische ultimate Mutagen, das Hydroxymethyldiazonium-Ion, welches auch in *A. bisporus* vorkommt, zeigte die höchste Mutagenität (TA1537: ca. 300 bis 1000 Revertanten/μmol).

Summary. Purified agaritine (N'-(γ-L(+)-glutamyl)-*p*-hydroxymethylphenylhydrazine) isolated from *Agaricus bisporus*, *p*-hydrazinobenzoic acid (its presumptive precursor) and some agaritine-degradation products were tested for mutagenic activity with the *Salmonella*/mammalian microsome assay (Ames test). Consistent with the literature, agaritine showed a distinct direct-acting mutagenicity with the strain TA1537 (30 revertants/μmol) and with TA97. Incubation of agaritine at alkaline pH increased the mutagenic effect. Pre-incubation of agaritine with γ-glutamyl transferase (GT) during 10 h at room temperature (pH 8.2) even enhanced the mutagenicity by a factor of 8 to 16 depending on the strain. In accordance with this finding, synthetic *p*-hydroxymethylphenylhydrazine (the presumptive product of the GT catalyzed degradation) showed also a distinct direct-acting mutagenicity, but the increase was only about 3- to 6-times compared with agaritine. The hypothetical ultimate mutagenic metabolite of agaritine, the *p*-hydroxymethylbenzenediazonium ion, a compound occuring naturally in *A. bisporus*, showed the highest mutagenic activity (with TA1537 approximately 300 to 1000 revertants/μmol).

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Introduction

Agaritine (Fig. 1) is a hydrazine derivative isolated from the edible mushroom *Agaricus bisporus* or *A. brunnescens* and from other species of the genus *Agaricus* [1, 2]. The sporophores on the fruit body, which contain the hydrazine derivative, show a γ-glutamyl transferase (GT) activity. By the activity of this enzyme the glutamyl substituent of agaritine can be released, leading to the formation of *p*-hydroxymethylphenylhydrazine, which can be further transformed into the *p*-hydroxymethylbenzenediazonium ion (Fig. 1; [3]). In several publications, data concerning the mutagenic activity of agaritine in vitro have been presented [4–11]. In mice, agaritine did not show any carcinogenic activity [8]. A comparative mutagenicity study of agaritine, its precursors and its decomposition products has not yet been published. In the present study therefore, the mutagenic activities of these compounds were compared using the Ames *Salmonella*/mammalian microsome assay.

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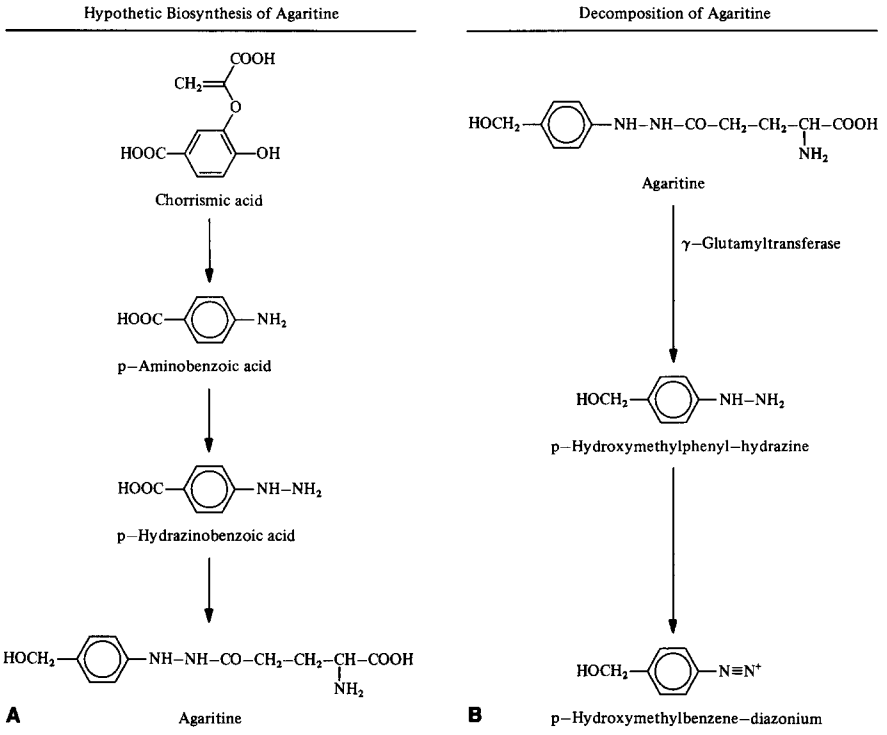

Fig. 1A, B. Hypothetical biosynthesis (**A**) of agaritine and its degradation (**B**) to the diazonium compound

Materials and Methods

Chemicals

Agaritine (A; Fig. 1A, B) was isolated from *Agaricus bisporus* following the method of Fischer et al. [21]. The final product contained 76.5% (w/w) of the pure compound. *p*-Hydroxymethylphenylhydrazine (Fig. 1 B) was obtained from Dr. L. Hoesch (Inst. Plant Physiol., Univ. Zürich, Switzerland). The *p*-hydroxymethylbenzenediazonium (Fig. 1 B) derivative of A was synthezised as the borontetrafluoride (BF_4) salt following the methods of Ross et al. [24]. *p*-Hydrazinobenzoic acid (Fig. 1 A) was purchased from Fluka (Buchs, Switzerland).

Incubation of agaritine with γ-glutamyltransferase (GT)

Agaritine (35 mM) was dissolved together with GT from bovine kidney (1 unit per 1 μmol A; Calbiochem, La Jolla, CA, USA) in 0.05 *M*-Tris-HCl buffer, pH 8.2 and incubated at 25 °C for 10 h. Appropriately diluted samples were tested for mutagenic activity.

Incubation of agaritine in buffers at different pH

Agaritine (35 m*M*) was dissolved in 0.05 m*M*-Tris-HCl buffer (pH 5.0, 7.0, 8.2 and 9.0) and incubated for up to 32 h at 25 °C. Aliquots were taken after different incubation periods and immediately frozen (−20 °C). The mutagenic activity was determined by preincubation of 0.1-ml aliquots together with *Salmonella typhimurium* TA1537 (20 min, 37 °C) in buffer.

Mutagenicity test

The *Salmonella*/mammalian microsome assay was performed following the methods of Ames et al. [12] using the preincubation modification of Yahagi et al. [13]. *Salmonella typhimurium* strains TA1535, TA1537, TA97, TA98, TA100, and TA102 were obtained from B.N. Ames (University of California, Berkeley, USA).

As an activation system 0.5 ml of 10% rat liver homogenate (S9) per plate was added. It was prepared from a batch of frozen S9 (kept in liquid nitrogen at −196 °C) from livers of 200 g male Sprague-Dawley rats treated with Aroclor 1254 [12]. The liver slices were homogenized in 0.01 *M*-Tris-HCl buffer with 0.25 *M*-sucrose. The S9 preparation contained 46 mg/ml of protein as was determined by the method of Lowry et al. (1951). For the experiments without activation 0.25 *M*-sucrose in 0.01 *M*-Tris-HCl buffer, pH 7.4 instead of S9 was added.

The following standard mutagens were included as positive controls:

– Without activation; 7.5 μg of nitrofluorene per plate for TA98, 2.5 μg of sodium azide for TA1535 and TA100, 50 μg of 9-aminoacridine for TA1537, 0.5 μg of ICR-191 for TA97 and 0.05 μg of mitomycin-C for TA102.

– With activation; 5 μg of emodin for TA1537 and 500 μg of cyclophosphamide for TA1535. All chemicals besides sodium azide, mitomycin-C and cyclophosphamide (50 μl H_2O) were dissolved in 50 μl of dimethylsulfoxide. They were obtained from Fluka AG (Buchs, Switzerland), with the exception of mitomycin-C and emodin (Sigma, St. Louis, MO, USA).

For the determination of the colony-forming units, the cell suspension was diluted 10^6 times with 0.85% NaCl soln: Aliquots of 0.3 ml were mixed with 6.0 ml of top agar supplemented with an additional amount of histidine (0.05 m*M*-biotin, 60 μg/ml of L-histidine-hydrochloride), and 2.0 ml of this suspension was spread over the surface of each of three replicate minimal medium plates.

All plates were incubated for 2 days at 37 °C. The revertant colonies were marked and counted. In cases of more than 200 colonies per plate, characteristic sectors of $^1/_3$ to $^1/_{32}$ containing between 80 and 120 colonies were chosen. The mutation factor (F) was calculated as the ratio between the total number of revertants on the treated plates and the number of spontaneous revertants (solvent control). In addition, the background growth of the bacteria was checked under a stereo-microscope.

Results

Comparison of the mutagenic activity of agaritine and related compounds

The mutagenicity data of the different compounds are summarized in Table 1. They all showed a direct acting mutagenic activity which decreased upon addition of rat liver homogenate (Table 1; with activation only data obtained with TA1537 are shown). *S. thyphimurium* TA1535 was negative for all compounds. The strains with the most pronounced sensitivity were TA1537 and TA97 indicating a frameshift acticity. The mutagenic potency of the different compounds increased in the given order: *p*-hydrazinobenzoic acid, agaritine (A), *p*-hydroxymethylphenylhydrazine, *p*-hydroxymethylbenzenediazonium borontetrafluoride. The diazonium derivative had a mutagenic potency in the range of 300 to 4000 revertants per µmol. It showed strong bactericidal effects at concentrations above 0.5 µmol/plate. Already 0.7 µmol per plate inhibited the background growth of all strains used, whereas agaritine at doses up to 15 µmol showed no cytotoxic effects. To test the possible influence of the borontetrafluoride anion, $NaBF_4$ was assayed at a concentration of 7 µmol per plate. This amount, corresponding to 10-times the dose of the diazonium compound with the maximum mutagenic effect, did not induce any mutagenic effects in *S. typhimurium* TA100, TA102, TA1535, and TA1537 with and without activation.

Incubation of agaritine (A) with γ-glutamyl transferase (GT)

Preincubation of A with GT (1 U/µmol A) resulted in a distinct increase of the mutagenic activity towards TA1537 (comparison with A preincubated at pH 8.2 without GT; Fig. 2) and the other strains used (comparison with A not preincubated at pH 8.2; Table 1). Because *p*-hydroxymethylphenylhydrazine is the first product of the enzymatic degradation of A, the mutagenicity of this compound was also determined. As can be seen from Table 1 the mutagenic activity of *p*-hydroxymethylphenylhydrazine was 2- to 5-times lower compared with the activity obtained after incubation of A together with GT.

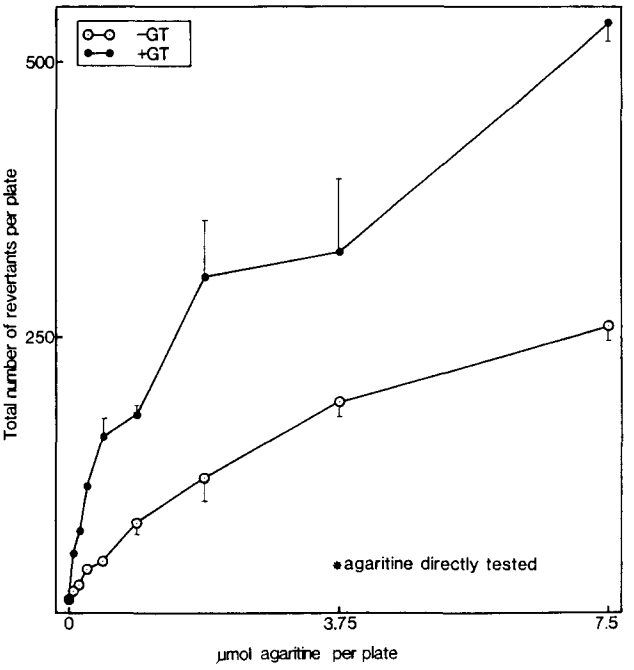

Fig. 2. Mutagenic activity of A after incubation with (●–●) and without (⊙–⊙) addition of GT (1U/µmol A) in 0.05 *M*-Tris-HCl buffer, pH 8.2 for 10 h (25 °C). After the enzyme treatment different aliquots were preincubated with *S. typhimurium* TA1537 (20 min, 37 °C) in buffer (pH 7.4). For comparison the directly measured mutagenic activity of A (3.75 µmol) is shown (no preincubation at pH 8.2)

Table 1. Comparative mutagenicity (revertants/µmol)[a] of agaritine, of one of its hypothetic precursors, and of some possible degradation products

Salmonella strains	TA97	TA98	TA100	TA1535	TA1537[d]		TA102
Compound	−S9	−S9	−S9	−S9	−S9	+S9	−S9
p-Hydrazinobenzoic acid	117	4	13	0	1	0	0
Agaritine	234	0	21	0	30	2	0
Agaritine incubated with GT	1865	30	348	0	430	181	451
p-Hydroxymethylphenylhyrazine	n.d.[b]	12	60	n.d.	191	77	n.d.
p-Hydoxymethylbenzenediazonium borontetrafluorid	3650	500–2500[c]	1500–3000	0	300–1000[c]	150–200[c]	740

[a] The number of induced revertants per µmol was determined within the rectilinear part of the dose-effect curve. The following mean numbers of spontaneous revertants per plate were counted for the different strains: TA97 177±65; TA98 34±7; TA100 165±35; TA1535 55±26; TA1537, −S9 13±6, +S9 13±5; TA102 597±136

[b] Not determined

[c] In the case of a non-rectilinear dose-effect relationship, the range of the mutagenicity (revertants/µmol) is presented

[d] For strain TA1537 the mutagenic activity with addition of rat liver homogenate is also shown

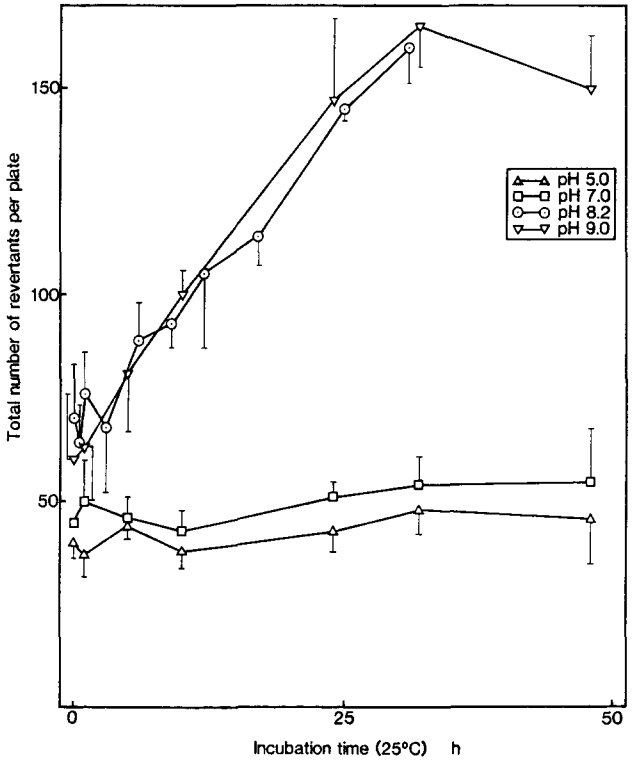

Fig. 3. A (35 m*M*) dissolved in 0.05 *M*-Tris-HCl buffer [pH 5.0 (△–△), 7.0 (□–□), 8.2 (○–○), 9,0 (▽–▽)] was incubated at 25 °C. Aliquots were collected after different incubation times and frozen immediately at −20 °C. The mutagenic activity (0.1 ml of the aliquots per plate) was assayed with TA1537 (preincubation in buffer for 20 min, 37 °C)

Incubation of agaritine at different pH

The mutagenic activity of A is enhanced after incubation of the compound at alkaline pH for 16 h at 25 °C, in comparison to the directly determined A mutagenicity (Fig. 2). Indeed, there was a time- and pH-dependent increase of the mutagenic activity of A also without the addition of GT (Fig. 3). This spontaneous degradation to products with an enhanced mutagenic activity occurred only at alkaline pH values (pH 8.2 and 9.0), whereas at pH 5 to 7 no such increase could be observed. Between 0 and 32 h the mutagenicity of A dissolved in Tris-HCl buffer, pH 9 increased continuously and finally, after incubation for 32 h, A induced approximately 3-times more revertants than at the beginning of the incubation.

Discussion

As already known from the literature [9], agaritine (A) shows a direct mutagenic activity with *S. typhimurium* TA1537 and accordingly also with TA97. These were, in comparison to the other strains used (TA98, TA100, TA102) the most sensitive strains (TA1535 was negative). TA97 and TA1537 detect certain frameshift mutagens such as 9-aminoacridine [14], ICR-170 [15] and ICR-191 [14].

The animal carcinogen 1,2-dimethylhydrazine [16] was also positive in the *Salmonella*/mammalian microsome assay ([17, 18]; *S. typhimurium* TA100 – a strain susceptible to base pair substitution mutagens; with and without activation). 2-Hydroxymethylhydrazine being also carcinogenic [19] showed mutagenic effects with TA1535 and TA100 [20].

The degradation step of A leading to mutagenic metabolites is most probably the cleavage of the γ-glutamyl residue catalyzed by a γ-glutamyltransferase (GT; Fig. 1B; [3]). The incubation of A with this enzyme at pH 8.5 enhanced the mutagenicity by a factor of approximately 2 (Fig. 2). The activation seems also to occur spontaneously at alkaline pH (pH 8.2 and 9; Fig. 3) because the mutagenic activity of A incubated under these conditions continuously increased.

For a discussion of the comparative mutagenicity data presented in Table 1 the following considerations have to be made:

1) The precursor of A, the *p*-hydrazinobenzoic acid showed the lowest mutagenic activity with the strains TA97, TA100, and TA1537. The reduction of the polar carboxy group therefore seems to enhance the mutagenic potency of the hydrazino derivative.

2) The fact that A shows a relatively weak but distinct mutagenic activity could be explained by the spontaneous cleavage of the γ-glutamyl moiety.

3) The observation that A yielded a higher specific mutagenicity after incubation with GT compared with the synthetic *p*-hydroxymethylphenylhydrazine which is the product of the enzyme activity, could be due to the relative instability of the hydrazine derivative (oxidative degradation).

4) The highest mutagenic activity of all compounds tested was obtained with the *p*-hydroxymethylbenzene-diazonium ion (TA97, TA100, TA102, TA1537). TA1535, which was negative for A and the other related compounds listed in Table 1, remained also negative upon addition of diazonium ions. The similar mutagenic pattern of the different compounds towards the different tested strains suggests a common genetic mechanism. It is therefore assumed that they are all converted to the same ultimate mutagen, which is most probably the diazonium ion.

The mutagenic potency of the diazonium derivative of A (500 to 3650 revertants per μmol; Table 1) is comparable with ethyl methanesulfonate or β-propiolactone [15], which are in fact strong direct acting mutagens.

Because the normal A content in A. bisporus is in the range of 100 to 600 mg per kg fresh weight [21] it is possible to consume 10 to 60 mg of the compound during a meal consisting of 100 g of mushrooms. This

amount would give rise to 1×10^4 to 2×10^5 revertants if the whole amount of A were to be converted into the diazonium derivative. For comparison, the addition of only 1 μg of the very potent mutagen and carcinogen aflatoxin B_1, which is a promutagen and is activated by cytochrome P-450 dependent mono-oxygenases, 1×10^4 revertants of TA100 were induced [22]. The degradation of A could be possible because GT, the enzyme capable of cleaveing its γ-glutamyl moiety is an ubiquitous membrane bound mammalian enzyme. If, however, the diazonium derivative is formed and will reach the DNA of potentially dividing cells of the digestive tract is not yet known (see next section). The fact that addition of S9 led to a reduction of the mutagenicity is indicative for a detoxification by mammalian cell constituents.

Admittedly, it is not possible yet to estimate the human risk. Nevertheless, the high content of compounds like agaritine, with a distinct mutagenic activity, in edible and widely used mushrooms points to an urgent need for studies dealing with the effects of agaritine in vivo, especially with its organ distribution and metabolism. Preliminary experiments with the radiolabeled diazonium compound, which was orally administered to rats, showed indeed the formation of DNA adducts in the cells of the stomach muscosa of the treated animals [23].

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