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The content of agaritine in spores from *Agaricus bisporus*

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Abstract

Agaritine (L-glutamic acid, 5-(2-(4-(hydroxymethyl)phenyl)hydrazide)) was identified and quantified in spores of *Agaricus bisporus* by high resolution liquid chromatography with mass spectrometric detection using negative electrospray ionisation. The spores were collected from mushrooms purchased at the open marked in Oslo, and the agaritine was extracted in pure water before analyses. On average the agaritine content was $0.304 \pm 0.003\%$ of the spores.

Keywords: *Agaricus bisporus*; Basidiospores; Spores; Agaritine; Mushroom toxins; HPLC-MS–MS

1. Introduction

Button mushroom (*Agaricus bisporus* Lange Imbach) is one of the most widely cultivated species of edible mushrooms. The *Agaricus* species contains five known aromatic hydrazine derivates (Toth, 1991), of which the most abundant is agaritine, a phenylhydrazine derivate of glutamic acid. Most hydrazines are shown to have carcinogenic potential (Toth, 2000), and long-life feeding studies with fresh, freeze dried or dry baked *A. bisporus*, have demonstrated that the mushroom was carcinogenic to Swiss albino mice, resulting in malignant tumour development in various tissues of the animals (Toth & Erickson, 1986; Toth, Erickson, Gannett, & Patil, 1997). When the phenylhydrazine derivates, occurring in *A. bisporus*, were administered in drinking water or by garvage as pure compounds in cancer tests in mice, all but agaritine resulted in tumours (Toth, Raha, Wallcave, & Nagel, 1981). It was, however, later reported that agaritine is unstable in aqueous solutions under oxidative conditions (Hajslová et al., 2002).

A very limited number of investigations regarding the toxin contents in mushroom spores have been conducted. This is in contrast to moulds that have been more extensively investigated for toxin contents. Several mycotoxins, such as aflatoxins (Wicklow & Shotwell, 1983) aurasperone C and fumigaclavine (Palmgren & Lee, 1986), trichothecene mycotoxins (Sorenson, Frazer, Jarvis, Simpson, & Robinson, 1987), fumonisins and AAL-toxin (Abbas & Riley, 1996) and citrinin (Størmer, Sandven, Huitfeldt, Eduard, & Skogstad, 1998) have been detected in conidia. The presence of ochratoxin A in dust collected from households and from cowsheds (Richard, Plattner, May, & Liska, 1999; Skaug, Eduard, & Størmer, 2001) indicates that fungal spores containing mycotoxins may pose a respiratory problems for humans as well as for animals. In particular, this may be true for the brown strains of *A. bisporus* and also *Lentinus edores*, where agaritine, also, is found at low levels, because these mushrooms are harvested fully mature with open caps.

Orellanine, a nephrotoxin responsible for serious intoxications has been quantified in spores from *Cortinarius orellanus* Fries and *C. rubellus* Cooke (Koller, Høiland, Janak, & Størmer, 2002). Orellanine was also, in this investigation, found to inhibit the growth of *Bacillus subtilis*, which indicates a biological role. To the best of our knowledge, quantification of toxins in spores from Basidiomycota, despite their frequent association with poisoning and despite an increasing interest in natural toxins and

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their effect on human health, had, prior to this study, not been reported. Recently, we have determined content of ibotenic acid and muscimol in spores from *Amanita muscaria* (Størmer, Janak, & Koller, 2004).

In this work we propose a method, based on high resolution liquid chromatographic separation of agaritine in spores' extract with mass spectrometric detection (LC-MS–MS), for quantification of agaritine in spores from *A. bisporus*. The agaritine content in other parts of the mushrooms has been determined previously by others (Andersson et al., 1999; Schulzová, Hajslová, Peroutká, Gry, & Andersson, 2002) using high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Collection of fungal material and spore isolation

Agaricus bisporus was purchased at the open marked in Oslo, Norway. The stems were removed from the mushrooms, and the caps at different maturities were placed onto glass plates for 24 h to collect the spores. No moisture was observed during the spore drop. The sampled spores contained approximately 95% basidiospores, as evaluated by microscopic examination. The spores were stored at −20 °C.

2.2. Extraction of agaritine

The spore material from at least fifty caps was diluted to 5 mg/mL in pure water. The solution was filtered and stored at −20 °C prior to analysis.

2.3. Agaritine standard

A stock solution of agaritine in pure water was prepared and stored at −20 °C.

2.4. Liquid chromatography–mass spectrometry (LC-MS–MS)

2.4.1. Instrumentation

Set-up for LC-MS–MS consisted of an HTC PAL Autosampler from CTC Analytics AG (Zwingen, Switzerland), a Surveyor MS Pump, a liquid chromatographic pump, a Surveyor PDA Detector and a TSQ Quantum, a triple stage quadrupole mass spectrometer, all from Thermo-Finnigan (San Jose, CA, USA).

2.4.2. Method

Samples (20 μl) were injected by the autosampler on a LC column, Symmetry C18 (2.1 × 150 mm, 5 μm) from Waters (Milford, MA, USA) and separation of Agaritine was achieved with a mobile phase gradient, using 10 mM ammonium acetate with pH adjusted to 3.43 by acetic acid (A) and methanol (B). Mobile phase composition A/B 1:1 was kept for 1 min after injection and then programmed to 90% of B in 4 min and held for 4 min.

2.4.3. MS–MS detection

Negative ES ionisation was optimised for agaritine parent ion ($m/z = 266.1$) at a spray voltage of 2800 V, capillary temperature at 370 °C, tube lens offset at −56 V and source collision induced dissociation at 24 V. Selective reaction monitoring (SRM) of agaritine was performed for product ions of $m/z = 128.1$ using collision energy at 15 V and collision gas pressure at 1.0 bar. Agaritine was determined using chromatographic separation with detection by SMR; solute identity was confirmed by repeating analyses using product scan for detection of qualifier ions generated from parent mass of $m/z = 266.1$. Ions with $m/z = 128.1$ and $m/z = 248.1$, detected as a major ions in the run, correspond to fragments from the agaritine structure, (see Fig. 1).

2.4.4. Agaritine standards

Solutions of agaritine were prepared at a concentration of 10, 25, 50, 100, and 200 μg/ml in 3% formic acid in water and kept in the refrigerator. Just before use, 150 μl of a 10 mM acetate buffer with pH adjusted to 3.43 was added to 450 μl of the standard solution.

2.4.5. Sample adjustment

As for standards, pH was adjusted, taking 450 μl of the sample solution and 150 μl of the 10 mM acetate buffer, in order to standardise conditions for ion suppression in MS–MS.

Standard solutions as well as the sample, were analysed in 3 replicates. Agaritine was quantified using the external standard quantification method.

3. Results

Fresh cultivated mushrooms were purchased on the open marked in Oslo. The caps were placed onto glass plates to collect the spores. Agaritine was identified and quantified in spores by high resolution liquid chromatography with mass spectrometric detection, (LC-MS–MS). High selectivity of the determination achieved by MS–MS detection using single reaction monitoring (SRM), is demonstrated in Fig. 2, showing a chromatogram of agaritine in spore extract. The agaritine content in spores from A. bisporus was determined to be on average, 0.304 ± 0.003%.

4. Discussion

In one investigation, the content of agaritine in whole fruit bodies of *A. bisporus* was found to be, on average, 272 mg kg^{-1}. The highest amounts were found in the skin of the cap and in the gills, 322 and 254 mg kg^{-1}, respectively, and the lowest, 213 mg kg^{-1}, in the stem of the mushroom. There were slight differences in levels of agaritine between mushrooms of different sizes, with the youngest and smaller fruit bodies containing higher amounts of agaritine than larger fruit bodies ready to be harvested (Schulzová et al., 2002).

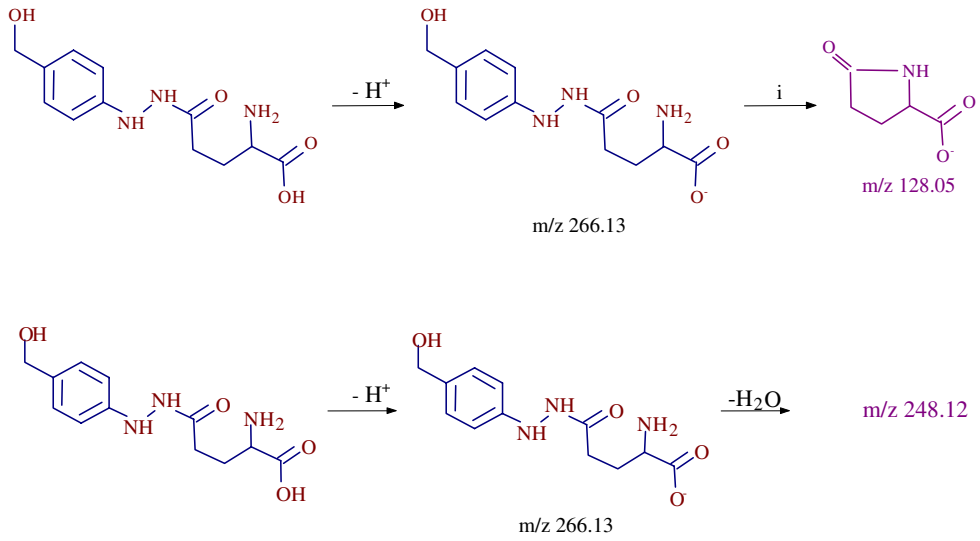

Fig. 1. Mass spectrometric fragmentation of agaritine from *Agaricus bisporus*.

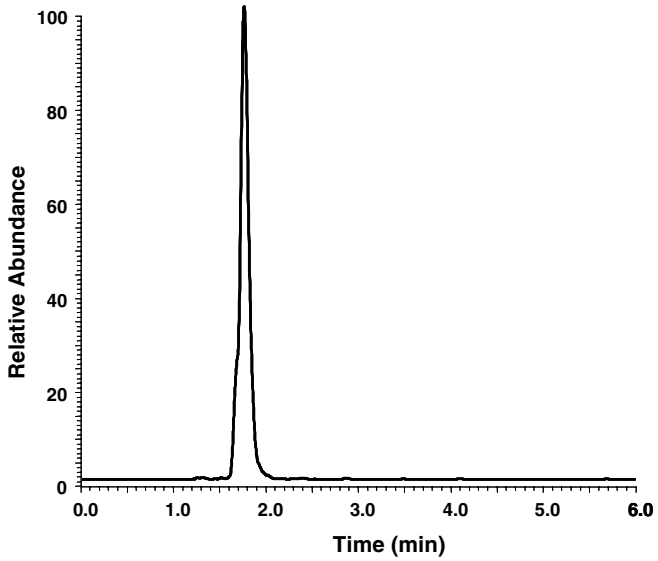

Fig. 2. Chromatographic separation of agaritine in an extract of spores from *Agaricus bisporus*.

Speroni, Beelman, and Schisler (1983) demonstrated that the level of agaritine increased as much as 7-fold throughout the life cycle of the fruiting body. They also found that agaritine was inhibitory in vitro to the growth of *Spicellum roseum* and *Trichoderma viride*; two mould competitors to *A. bisporus*. They speculated that agaritine might be a secondary metabolite that gives the mushroom a competitive advantage and this could indicate a biological role for agaritine.

The orellanine contents of basidiospores of *C. orellanus* and *C. rubellus* were previously determined to be 0.3% and 0.1%, respectively. The orellanine content in dried caps from the two mushrooms were determined to be 0.9% in *C. orellanus* and 0.8% in *C. rubellus* (Koller et al., 2002). In *A. muscaria*, we found the content of ibotenic acid to be 0.005% in spores, and 0.02% in fresh caps (Størmer et al., 2004). Both the orellanine and ibotenic acid contents in the spores were lower than in the whole basidiocarp.

We have determined the agaritine content in the spores to be 0.3%. This is about 10 times higher than what has been reported for the whole mushroom (Andersson et al., 1999; Schulzová et al., 2002), but considering the mushroom water content to be 80–90%, we might conclude that the spores from *A. bisporus* has approximately the same agaritine content as the mushroom itself.

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