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Ibotenic acid in *Amanita muscaria* spores and caps

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Summary: Ibotenic acid (α-amino-3-hydroxy-5-isoxazole acetic acid) was separated from spores and caps from *Amanita muscaria* by reversed phase high performance liquid chromatography and identified by flow injection analysis with mass spectrometric detection. The keto and enol tautomers of ibotenic acid were separated and their ratio of 96:4 in favour of the enol form was determined. On average the ibotenic acid content was 0.0054±0.0010% of the spores and 0.017±0.010% in fresh caps. Muscimol, the decarboxylated product from ibotenic acid, was neither detected in spores nor in caps. 50 nanomol of ibotenic acid, muscimol or extracts from spores or caps did not inhibit the growth of *Bacillus subtilis*.

Keywords: *Amanita muscaria*, basidiospores, ibotenic acid, muscimol, neurotoxin, tautomerism

Introduction

Fly agaric (*Amanita muscaria* (L.: Fr.) Pers.) is one of our most handsome and mysterious toadstools. It grows in symbiosis with arboreal trees, such as birch, pine or fir in Europe and America. Poison extracted from this mushroom has traditionally been used for the destruction of flies and other insects – hence its name. Ibotenic acid, a-amino-3-hydroxy-5-isoxazoleacetic acid, is the principal toxin in *A. muscaria*. It is a neurotoxic substance found in *A. muscaria* and *A. pantherina* (DC.: Fr.) Krombh. Because of the acidic property of the isoxazole moiety, it is similar to glutamic acid and mimics its effect in animals. It causes motor depression, ataxia, and changes in mood, perceptions and feelings. Muscimol, 3-hydroxy-5-amino methylisoxazole, which is the decarboxylated product from ibotenic acid, shows structurally resemblance to GABA (g-amino butyric acid) and imitates the action of GABA, as an inhibitory neurotransmitter in the central nervous system in animals and humans.

Several mycotoxins have been detected in the conidia of moulds, for instance aflatoxins (Wicklow & Shotwell, 1983) aurasperone C and fumigaclavine (Palmgren & Lee, 1986), trichothecene mycotoxins (Sorensen *et al.*, 1987), fumonisins and AAL-toxin (Abbas & Riley, 1996), and citrinin and minor amounts of ochratoxin A (Størmer *et al.*, 1998). The presence of ochratoxin A in dust collected from households and from cowsheds (Richard *et al.*, 1999, Skaug, Eduard & Størmer, 2001) indicates that fungal spores containing mycotoxins may pose a respiratory problem for humans as well as for animals. Occupational respiratory diseases associated with exposure to mushroom spores have been reported in recent years, but no toxins have been described. (Kamm *et al.*, 1991; *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinus edodes*, Moinard *et al.*,1991; *Poria megalopora*, Matsui *et al.*, 1992; *Lentinus edodes*, Ishii *et al.*, 1994; *Pholiota nameko*, Inage *et al.*, 1996; *Pholiota nameko*.) Working on a mushroom farm carries a significant risk for chronic cough from inhalation of spores from *Hypsizigus marmoreus* (Tanaka *et al.*, 2002).

Orellanine, a toxin responsible for serious intoxications has recently been quantified in spores from *Cortinarius orellanus* Fries and *C. rubellus* Cooke (Koller *et al.*, 2002). Prior to this study no report had described quantification of toxins in spores from Basidiomycota, despite their frequent association with poisoning and the increased interest on natural toxins and human health. In this work we have determined the amount of ibotenic acid in spores and caps from *A. muscaria*.

Materials and methods

Collection of fungal material and spore isolation- *Amanita muscaria* was collected in three different localities in Akershus and Oslo County in 2001, so that variation due to age and locality difference could be properly assessed. The stems were removed from the

mushrooms, and the caps 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 caps and spores were and stored at -20°C. Spore volume can be calculated as that of an ellipsoid (Gross, 1972, Meerts, 1999) with spores of *A. muscaria* species with average length and width 10.5x8.5 µm having a spore volume of approximately 400 μm^3. Assuming a density of 1 g/cm^3, this corresponds to a spore weight of 400 pg.

Extraction of ibotenic acid and muscimol- The spore material was diluted to 5 mg/ml corresponding to $1.3x10^7$ spores/ml in water containing 0.1% formic acid and stored 10 min at room temperature. The solution was filtered, and 1.5 ml of the clear solution was freeze dried, dissolved in 0.5 ml water and used in the analysis. Prolonged storage, freezing and thawing, or 15 min with ultrasound treatment, did not increase the content of ibotenic acid released from the spores. At least three caps from each locality were powdered in liquid N_2 and added twice its weight with water. After centrifugation for 15 min at 15000 g and 4°C, the clear supernatant was removed and stored at -20°C.

High-pressure liquid chromatography (HPLC)- The HPLC equipment used for analysis consisted of a Perkin-Elmer series 4 HPLC pump, a Hewlett Packard 11040 photodiode array detector, and a 7125-075 Rheodyne injector with a variable volume loop. Waters (115x13 mm) C-18 preparative column was used for the ibotenic acid analysis. The mobile phase, flow rate of 0.5 ml/min, consisted of water-acetonitrile-methanol (65:20:15 v/v) containing sodium dodecylsulfate (2.1 mM) and phosphoric acid (4 mM) (Tsunada *et al.* 1993). All analyses were carried out at laboratory temperature. Ibotenic acid in extracts was tentatively confirmed by retention of standard at 8.2 min, and the amounts of ibotenic acid in the sample was determined by comparison with a standard curve. Zorbax SB-Aq (4.6x150 mm) was used for the muscimol analysis with flow rate of 1.0 ml/min and the same mobile phase as described above with a retention time of 12.8 min (sensitivity 1 nmol).

Liquid chromatography-Mass spectrometry (LC-MS)- Mass spectra were obtained with a VG Platform quadruple mass spectrometer (Fisons Instruments, VG Bio-tech, Altrincham, UK) equipped with an atmospheric pressure electro spray ionisation source. Characteristic ions for identification of ibotenic acid were determined by flow injection analysis (FIA) with mass spectrometric detection using electro spray in positive mode. Standard solution of the ibotenic acid at a concentration of 0.1 µg/ml in methanol-water (1:3 v/v) was injected into VG Platform at a flow of 10 µl/min. Total ion current mass spectra were recorded for mass ions in the range of 50–250 at a 3.75 kV and 0.35 kV for Capillary and HV lens, respectively, at a Skimmer Lens voltage of 5 V and at a Cone voltage optimised in the range of 10-70 V. The temperature of the electro spray source was set to 80°C. Positive ionisation of ibotenic acid was examined at a pH of 2.0 adjusted by formic acid and at a pH of 6.1 adjusted by ammonium/ammonium acetate solution. To confirm the findings of ibotenic acid in spores and caps, water extracts of samples acidified with formic acid (0.1% v/v) were injected by Rheodyne 7125 injector using 20 µl loop on an XTerra™ MS C_{18} column (5 µm; 2.1x100 mm) (Waters) provided with Sentry™ Guard Column (2.1x10 mm). Composition of the mobile phase delivered by gradient pump (Perkin-Elmer) at a flow of 0.5 ml/min was programmed from water/methanol (19:1 v/v) to acetonitrile/water/methanol (18:1:1 v/v) in 1 min and then it was held for 7 min. Analysis was monitored by variable UV detector (Hewlett-Packard 1050) at 254 nm and ibotenic acid was selectively detected by VG Platform quadruple mass spectrometer equipped with an electro spray atmospheric pressure ionisation source. Parameters controlling mass spectrometric detection in single ion recording mode for masses at 53.7 and 91.0 were set as given above. Analysis was performed twice with cone voltages 50 V and 60 V, respectively. Chromatograms were acquired and result evaluated using Masslynx™ 3.5 software (Micro mass UK Lim.).

Results

The amount of ibotenic acid in spores of *Amanita muscaria*, based on the detemination from three different localities, varied from 0.0047 to 0.0061%with an average of 0.0054±0.0010 (Table 1). The amounts of ibotenic acid in caps (wet weight) from the same localities varied from 0.0078 to 0.0260%

Table 1 Ibotenic content of spores and caps from *A. muscaria*:

Location	% IBO in spores[a]	% IBO in fresh caps
1	0.0047	0.0078
2	0.0061	0.0260
3	0.0055	0.0160

[a]Assuming no water content

with an average of 0.017±0.010% (0.17% dry weight based on the assumption that 90% of the mushroom content is water) from the measurement from the three different localities. Muscimol was not detected in extracts from either spores or caps.

LC-MS experiments were performed in order to confirm the findings of ibotenic acid in the spores and caps of *A. Muscaria*. Using FIA with mass spectrometric detection at a positive electro spray ionisation, ibotenic acid gave an intense molecular ion M^+ at m/z=158.0 and even more intense ion $(M\text{-}OH)^+$ at m/z=140.9 at both pH=2.0 and pH=6.1. The presence of ibotenic acid was confirmed in all samples from both spores and caps using these specific masses. LC-MS using monitoring of the molecular ion M^+ resulted in two poorly resolved and tailing peaks with retention time of 0.98 min and 1.12 min respectively for both the standard of ibotenic acid as well as the extracts of the spores and caps. Selective ion monitoring revealed two clear peaks that were identified as keto and enol tautomers of ibotenic acid. The peak less retained on C-18 reverse phase column gave in electro spray atmospheric pressure ionisation predominantly ion at m/z=91 which corresponds to an adduct of the enol form of the protonated ibotenic acid with a sodium ion. The next intense ion in this peak is the one with m/z=82 that correspond to sodium adduct with a dehydroxylated form of the enol form of ibotenic acid. The more retained peak eluting in between 1.06 min and 1.23 min with a maximum at 1.12 min has been identified as the keto form of the ibotenic acid. The mass spectrum recorded at the maximum of the peak revealed an intense ion at m/z=53.7 (triple protonated molecular ion of the ibotenic acid), and a minor ion at m/z=71 (double charged: protonated and dehydroxylated molecular ion of the ibotenic acid) and at m/z=102 (molecular cluster with two sodium ions). The prevailing enol tautomer was evaluated to make about 95% of the compound.

Using selective ion monitoring at m/z=53.7 and at m/z=91.0, tautomers of ibotenic acid were identified in caps of the mushrooms. Ratio of keto and enol tautomers of ibotenic acid in the extracts was evaluated to be about 4:96 in favour of the hydroxy form. Neglecting minor ions (m/z=82 for enol- form, and m/z=71 and m/z=102 for keto- form) should not result in relative deviation higher then 10%.

We measured the effect of spore and cap extracts upon growth of *Bacillus subtilis*. Extracts from 0.5 mg spores or 12.5 mg caps (wet weight) did not inhibit the growth of *B. subtilis*. The extracts tested stimulated bacterial growth. 50 nmol of either ibotenic acid or muscimol had no effect upon growth of the organism.

Discussion

In two earlier investigations the content of ibotenic acid and muscimol in the caps (wet weight) from *A. muscaria* were determined to 0.0519% and 0.0253% (Tsunoda *et al.*, 1993) and 0.099% and 0.038% respectively (Gennaro, Giascosa & Angelino, 1997). We determined the ibotenic acid in samples from three different localities with an average of 0.017% (Table 1). These values are lower than earlier described. Different environments and different strains of the mushroom may explain the discrepancy. The amount of toxin present may also be dependent on the age of the collected species, which may also be reflected in the variation in our values. Muscimol was not detected in our samples, probably due to the pre-treatment of the caps with liquid nitrogen followed by extraction, which did not allow the decarboxylase enzymes to act upon ibotenic acid.

The ibotenic acid content in the spores was in the range from 0.0047% to 0.0061%, indicating that the toxin content was less variable than we determined in the cap extracts. This may be due to the less variation in the water content in the spores which we assume are in the dry state and that the presence of ibotenic acid is not depending on the spore age.

Regarding keto and enol tautomerism, imidazolols are reported to prefer the hydroxy structure (Elguero *et al.*, 1976). Our results show that the enol tautomer of ibotenic acid comprises 95% of the compound in the pH range 2-6, i.e. at physiological pH.

The orellanine content of basidiospores of *Cortinarius orellanus* and *C. rubellus* was previously determined to be 0.3% and 0.1% respectively. Orellanine (25 nmol) inhibited growth of *B. subtilis*. The amount of spore extracts corresponding to the presence of 25 nmol orellanine inhibited bacterial growth and indicate that the toxin could have a function in the germination process (Koller *et al.*, 2002).

Average ibotenic acid content per *A. muscaria* spore is 0.005%. The function of ibotenic acid in the spores is dubious due to the small content, and no inhibition of *B. subtilis* growth was observed with 50 nmol ibotenic acid, muscimol or spore extracts. The growth was rather stimulated in the presence of the spore extract. These could be nutrients or trace elements that enabled bacterial growth. It is possible that other extraction methods could release compound(s) that would inhibit the growth of *B. subtilis*. We have not been aware of any report concerning a bacteriostatic effect of ibotenic acid.

Spores from *Aspergillus ochraceus* and *Penicillium verrucosum* contain ochratoxin A, and spore extracts from these organisms inhibited growth of *B. subtilis* (Skaug *et al.*, 2001) which indicates that these toxins could play a role in the germination process (Størmer & Høiby, 1996). It has been suggested by the same authors that ochratoxin A and citrinin from spores could affect the iron uptake of other competing organisms and the presence of citrinin in the range of 8-24% of the spore weight in *P. verrucosum* make it obvious that this compound has an important role in the survival and/or the germination process. It was suggested that citrinin could protect the spores against UV radiation (Størmer *et al.*, 1998).

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