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Analysis of indole derivatives in methanolic extracts from mycelium of *Agaricus bisporus* cultured *in vitro* on liquid Oddoux medium

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ABSTRACT

Methanolic extracts obtained from biomass of *Agaricus bisporus* (J.E. Lange) Imbach cultured *in vitro* were analyzed for qualitative and quantitative composition of non-hallucinogenic indole compounds in order to compare their amount with fruiting bodies of these species. Extracts demonstrated to contain six indole compounds. Contents of individual compounds ranged from 0.01 to 21.33 mg/100 g d.w. in biomass from *in vitro* cultures. The quantitatively dominating compounds included: 5-hydroxytryptophan (12.50 mg/100 g d.w.), L-tryptophan (14.00 mg/100 g d.w.) and serotonin (7.00 mg/100 g d.w.). Total content of the remaining indole compounds under analysis in the study was 55.32 mg/100 g d.w.

KEY WORDS: *Agaricus bisporus*, *in vitro* culture, L-tryptophan, serotonin

Introduction

The project consisted of experiments utilizing edible mushroom: *Agaricus bisporus* – White bottom mushroom (Basidiomycota), mainly because this species is widely used for commercial purposes in Poland and Europe, and among all mushroom species, it is the most frequently consumed mushroom in Polish and European society due to its taste and nutritional qualities. In addition, choice of mushroom was influenced by practical aspects – a possibility for mass production. Currently, fruiting bodies of *A. bisporus* are irradiated by UV light during process of production to increase vitamin D content (Roberts 2008, Koyalamudi *et al*. 2008). Fruiting body of *A. bisporus* also contains ergothioneine compound. This substance plays an important antioxidative and anti-mutagenic role, as well as chemo- and radioprotective (Ey *et al*. 2007). *A. bisporus* is also a highly valued source of laccase, vitamins (especially riboflavin, vitamin D3) and bioelements such as selenium, magnesium, copper, iron, calcium, zinc and potassium (Baross *et al*. 2008, Roberts 2008, Reczyński *et al.* 2013).

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The group of indole compounds that are not yet fully researched belongs to non-hallucinogenic indole type. Taking into consideration the significance of such indole derivatives as L-tryptophan, 5-hydroxytryptophan, 5-methyltryptamine, serotonin, melatonin, tryptamine – which are known as neurotransmitters or their precursors, it makes sense to examine the presence of them in edible mushrooms (Muszyńska *et al*. 2007, 2009, 2011 a, b, c, 2012a, b). A notable aspect regarding mycelium of higher mushrooms is its ability to accumulate easily absorbed substances but there is a lack of information in respects to types and degree of accumulation of such compounds introduced to culture media. Due to this, these mushrooms can be used for a research of indolic compounds accumulated in the biomass from *in vitro* cultures. The difficulty in obtaining research material (due to temporary and unpredicted occurrence of fruiting bodies from natural sites) were the reason to use biomass from *in vitro* cultures for further experiments (Muszyńska *et al.* 2012a, b). Morever, in enclosed laboratory conditions, it is easier to control accumulation of chosen metabolites.

Material and methods

Fungal material

The studies were conducted with young fruiting bodies of *Agaricus bisporus* (White button mushroom) from commercial origin. After taxonomic identification based on Knudsen and Vesterholt (2008) (representative samples of mushrooms were deposited in the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum, Kraków, Poland), some of young sporocarps were used to derive *in vitro* cultures, from which obtained mycelium formed material for further analysis.

In vitro culture

The pieces of fruiting bodies were defatted with 70% ethanol for 15 s then sterilized in 15% hypochlorite solution for 5 min (manufactured by Unilever, Hungary). After several rinses with sterile redistilled water, mycelium fragments were transferred to Petri dishes containing agar-solidified medium with composition according to Oddoux (1957).

Experimental *in vitro* culture

After growing on solid medium, the pieces of mycelium were placed into an Erlenmeyer flask (500 mL) containing 250 mL of liquid modified Oddoux medium at initial biomass of 0.1 g. The cultures were incubated at the temperature 25 ± 2° C under 16 h light (900 lx/8 dark) and shaken at 140 rpm (shaker ALTEL, Łódź). The agitated liquid cultures of *A. bisporus* were maintained for two weeks and were subcultured afterwards. The obtained biomass was separated from the liquid medium using Büchner funnel with a filter paper, rinsed with redistilled water and immediately dried by lyophilization (lyophilizer Freezone 4.5, Labconco; temperature: - 40° C).

Dry, lyophilized materials (5 g of each species) were placed in a glass percolator and extracted with petroleum ether under dark conditions to remove oil fractions. Oil fractions were discarded and the remaining biomass was dried and

extracted again with methanol in a percolator for 24 h. The extracts were concentrated by distillation in a vacuum evaporator under reduced (200 mBa) pressure at 40° C. To remove the remaining lipids, concentrated extracts were frozen, while polysaccharides were removed using Chihara method. The residues were quantitatively dissolved in methanol, filtered through Whatman No. 3 paper and purified by TLC. For the purification of the extracts, we used TLC aluminium-backed silica gel 60 plates (Merck, Art. No 1.055540001), onto which the methanol extracts were loaded. Chromatograms were developed in mobile phase found to be optimal for separation of indole compounds: n-propanol/ethyl acetate/water (7:1:2 v/v/v). Spots containing indole compounds were identified under a UV lamp at $\lambda = 280$ nm. TLC chromatogram of extract from mycelium of *A. bisporus* is presentend in Fig. 1. The obtained fractions were extracted from chromatograms with methanol, filtered through syringe driven filter unit (Millex, Milipore Corporation, USA) than concentrated by distillation in a vacuum evaporator under reduced pressure at 40° C. The extracts, quantitatively dissolved in 1.5 mL of methanol, were subjected to HPLC analysis.

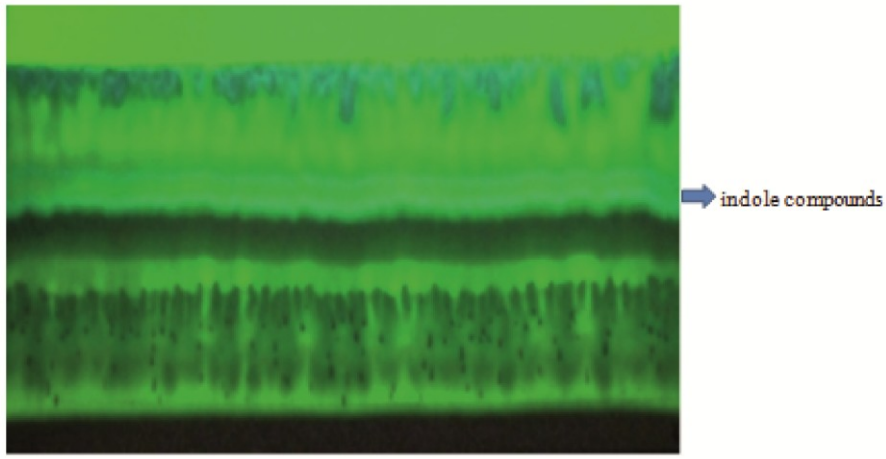

Figure 1. TLC chromatogram of extract from mycelium of *Agaricus bisporus* identified under a UV lamp at $\lambda = 280$ nm.

High performance liquid chromatography analysis (HPLC)

The obtained extracts were analyzed for contents of L-tryptophan, 5-hydroxytryptophan, 5-methyltryptamine, serotonin, melatonin, tryptamine, kynurenine sulfate, indoleacetic acid, indoleacetonitrile, indole and indoleacetamide. The analysis was performed according to the procedure by Kysilka and Wurst (1985) with our modifications (Muszyńska *et al*. 2009). HPLC analyses were performed with Hitachi apparatus equipped with L-7100 pump, reversed phase column: Purospher® RP-18 (4 x 200 mm, 5 µm) at 25° C. The solvent system used for analysis was composed of:

methanol/water/ammonium acetate (15:14:1 v/v/v) at flow rate of 1 ml/min. Detection was carried out with a UV detector, using λ=280 nm. The identification of indole compounds was made by comparing the retention times of sample peaks with those of the standards. The presence of tested metabolites in the sample was evident as an increase in peak height for the appropriate retention time. Quantitative analysis was carried out using the calibration curve method. The results are expressed in mg/100g of dry weight, calculated by internal normalization of the chromatographic peak area. A representative chromatogram is presented in Figure 2.

For each mushroom, three samples were used for the determination of the quality attribute and all the analyses were carried out in triplicate. The results were expressed as the mean values and standard deviation (SD). The experimental data were submitted for analysis of variance for completely random design to determine the least significant difference at the level of 0.05.

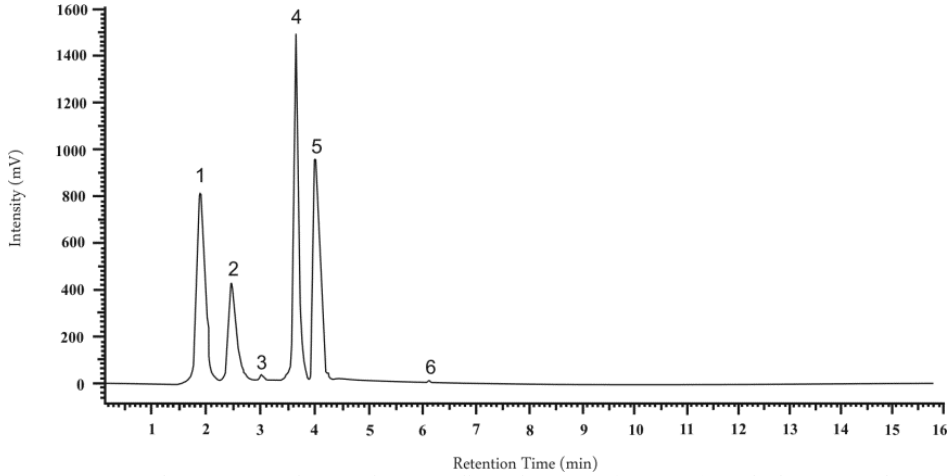

Figure 2. HPLC chromatogram of extract from mycelium of *Agaricus bisporus*: (1) -5-hydroxytryptophan, (2) - serotonin, (3) - tryptamine, (4) -5-methyltryptamine, (5) - L-tryptophan, (6) - melatonin.

Results

After several attempts to establish an optimal sterilization method, we were successful in the initiation of *A. bisporus* mycelia *in vitro* culture from hymenial part of fresh, young fruiting bodies. The best biomass growth was obtained during 3-week growth cycles in shaking liquid cultures using modified Oddoux medium. The biomass growth in the initiated cultures averaged at 8.1 g d.w./L of medium. Maximum mycelium biomass growth of *A. bisporus* was observed at initial medium pH of 6.2 and at temperature of 25º C. *In vitro* cultures maintained under laboratory conditions and optimized for maximum growth, can provide a uniform mycelium which may be a reproducible and efficient source of metabolites. The obtained biomass increments and dynamics of mycelium growth did not differ from the results that we obtained for *Boletus badius* (Fr.) Kuhn. ex Gilb, *Cantharellus cibarius* Fr. and *Tricholoma equestre* (L.) Kumm. and for *Calocera viscosa* (Pers.) Fr. cultures studied earlier (Muszyńska *et al*.

2009, 2011c, 2012b). The HPLC procedure applied to determine qualitative and quantitative content of non-hallucynogenic indole compounds offered an optimum conditions for most effective separation of the analyzed metabolites. Methanolic extracts obtained from biomass of *A. bisporus* cultured *in vitro* were analyzed for qualitative and quantitative composition of non-hallucinogenic indole compounds and their amount was compared with ones obtained from fruiting bodies of these species. The extracts were shown to contain six indole compounds: L-tryptophan, 5-hydroxytryptophan, serotonin, melatonin, tryptamine and 5-methyltryptamine. Contents of individual compounds varied ranging from 0.01 to 21.33 mg/100 g d.w. in biomass from *in vitro* cultures. The quantitatively dominating compounds included: 5-methyltryptamine (21.33 mg/100 g d.w.), L-tryptophan (14.00 mg/100 g d.w.), 5-hydroxytryptophan (12.50 mg/100 g d.w.) and serotonin (7.00 mg/100 g d.w.). The total content of the remaining indole compounds was 55.32 mg/100 g d.w. The contents of the remaining indoles: melatonin and tryptamine in mycelium from *in vitro* cultures were low, below 1.00 mg/100 g d.w. The contents of indole compounds in the methanolic extracts of mycelium of *A. bisporus* and in fruiting bodies are presented in Table 1.

Table 1. Contents of indole compounds under study (mg/100 g d. w.) in extracts from mycelium and fruiting bodies of *Agaricus bisporus*. Data are presented as the mean ± SE of 3 series.

Indole compounds	*Agaricus bisporus* mycelium from cultures *in vitro* (mg/100 g d.w.)	*Agaricus bisporus* fruiting bodies (mg/100 g d.w.) (Muszyńska *et al.* 2011 a)
L-tryptophan	14.00 +/- 0.300	0.39 +/- 0.003
5-Hydroxytryptophan	12.50 +/- 0.671	a
Serotonin	7.00 +/- 0.070	5.21 +/- 0.055
Melatonin	0.01 +/- 0.006	0.11 +/- 0.006
Tryptamine	0.48+/- 0.050	0.02 +/- 0.002
Indole-3-acetic acid	a	0.19 +/- 0.017
5-Methyltryptamine	21.33 +/- 0.755	a

a - Content lower than 0.001 mg/100 g d. w.

Discussion

The fruiting bodies of *A. bisporus* indicated presence of five indolic compounds: melatonin, tryptamine, L-tryptophan, serotonin, indolo-3-acetic acid (contents from 0.06 to 5.21 mg/100 g d.w.). Serotonin was quantitatively dominant compounds in extracts from fruiting bodies of this species (5.21 mg/100 g d.w.) (Muszyńska *et al.* 2011a). However, the mycelium from *in vitro* cultures showed a greater content of these indolic compounds. Serotonin contents were of the same order of magnitude but were slightly greater in the extracts from *in vitro* culture (7.00 mg/100 g d.w.). On the other hand, L-tryptophan contents were almost 30 times greater in the material from *in vitro* cultures compared with the fruiting bodies (14.00 and 0.39 mg/100 g d.w.,

respectively). In addition, extracts from *in vitro* cultures were characterized by the presence of 5-hydroxytryptophan and 5-methyltryptamine but the absence of indole-3-acetic acid evidenced in the fruiting bodies. To the best of our knowledge, this is the first time to identify and quantify indole compounds from *in vitro* culture of *A. bisporus*, the most popular edible mushroom. The mycelial culture seems to be a valid model for investigation of indole compounds accumulation and to study their metabolism in mushrooms. High content of serotonin and its precursors L-tryptophan and 5-hydroxytryptophan in the fruiting bodies and in the mycelium cultured *in vitro* of *A. bisporus,* demonstrate also a potential for the use of this material as a source of this physiologically important compound for humans. Serotonin is a long known compound playing the role of a regulator of sleep, body temperature, mood, maturation and regeneration and an inhibitor of cell aging, thereby contributing to general strengthening of the immune system and is used also as an antidepressant. Further optimization of conditions for *in vitro* cultures may allowan alternative method for commercial cultivation of this species. This is desirable since it may be expected that mycelium cultured *in vitro* may also be a source of other important metabolites, possessing both culinary and medicinal values, characteristic of fruiting bodies.

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Streszczenie

Lecznicze i przeciwutleniające właściwości grzybów są doskonałym połączeniem, które stanowi o ich wartości dietetycznej i umożliwia korzystanie z nich zarówno, jako żywności jak i dodatku żywieniowego. Celem niniejszej pracy była analiza zawartości fizjologicznie aktywnych związków indolowych w mycelium z kultur *in vitro Agaricus bisporus* (pieczarka dwuzarodnikowa). L-tryptofan, egzogenny aminokwas i jego pochodne, takie jak np. 5-hydroksytryptofan, muszą być dostarczane z pokarmem w codziennej diecie. Związki te mają działanie przeciwdepresyjne, są bezpośrednimi prekursorami serotoniny, a w przeciwieństwie do niej przekraczają barierę krew – mózg. Są też biogenetycznymi prekursorami innych związków indolowych, które pełnią funkcję neuroprzekaźników, co uzasadnia oznaczanie ich zawartości w grzybach jadalnych. Materiał do badań stanowiły owocniki *A. bisporus* pochodzenia komercyjnego. Z owocników *A. bisporus* wyprowadzono kultury *in vitro* na podłożu stałym Oddoux (1957). Eksperymentalne kultury *in vitro* prowadzono na płynnym, wytrząsanym podłożu Oddoux. Co dwa tygodnie prowadzenia kultur pasażowano je na świeżą pożywkę. Biomasę mrożono i suszono metodą liofilizacji. Otrzymaną biomasę z kultur *in vitro* analizowano jakościowo i ilościowo metodą HPLC na obecność niehalucynogennych związków indolowych.

Po raz pierwszy zidentyfikowane i ilościowo oznaczone zostały związki indolowe w kulturach *in vitro Agaricus bisporus* na płynnym podłożu wg Oddoux. Analiza wykazała, że ekstrakty metanolowe otrzymane z grzybni zawierają sześć związków indolowych: L -tryptofan, 5 - hydroksytryptofan, serotoninę, melatoninę, tryptaminę i 5-metylotryptamię. Zawartości poszczególnych składników w biomasie z kultur *in vitro* były zróżnicowane w zakresie od 0,01 do 21,33 mg/100 g s. m. Dominującymi ilościowo związkami były: 5-hydroksytryptofan (12,50 mg/100 g s. m.), L-tryptofan (14,00 mg/100 g) i serotonina (7,00 mg/100 g). Całkowita zawartość związków indolowych w badanym materiale wynosiła 55,32 mg/100 g s. m. Biomasa z kultur *in vitro* badanego gatunku jest dobrym źródłem 5-hydroksytryptofanu i L- tryptofanu. Kultury *in vitro A. bisporus* mogą być wykorzystane jako model do badań nad akumulacją i metabolizmem związków indolowych.