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Study of protein patterns in wild mushrooms using a proteomic approach

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Abstract

Wild mushrooms consist of a wide range of species and are popular for cooking. However, wild mushrooms are both edible and inedible and their traits are quite similar in particular stages. It could be a disaster if anyone misunderstands and eats inedible mushrooms. Due to the ultimate goal of most proteomic studies, it is essential to determine which proteins are responsible for a specific function or phenotype. Thus, the objective of this study was to evaluate protein patterns of wild mushrooms using a proteomics approach. Two wild mushrooms from the Northeast of Thailand with similar traits were selected. The results from a BLAST search indicate that these mushrooms possess homogenous ITS nucleotide sequence identities with *Amanita princeps* and *Agaricus purpurellus*. Then, they were further used for proteomic analysis with a combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS). Interestingly, their protein patterns are quite different. The number of protein spots of *A. princeps* (159 spots) was higher than *A. purpurellus* (32 spots). As well as the protein spot scattering, both the pI and the molecular weights of the proteins from *A. princeps* were also wider spread than those of *A. purpurellus*. Among all of the proteins, 26 spots of *A. princeps* and 15 spots of *A. purpurellus* with a pronounced and clear expression were selected and further identified by mass spectrometry (LC-MS/MS). Since there are limited mushroom genome and proteome databases, there were 7 and 3 hit proteins in *A. princeps* and *A. purpurellus*, respectively. These proteins were involved in protein/amino acid metabolism, cell growth and development, carbohydrate/energy metabolism, and the hydrolase enzyme. The current study was preliminary. However further studies in protein patterns of a wide range of mushroom species will be necessary for identification of unique protein markers to identify edible and inedible mushrooms.

Introduction

Mushrooms belong to the fungi kingdom, and they are highly evolved fungi which produce spores in their petals for propagation. Humankind has used mushrooms for cooking for centuries due to their high nutritional value, as well as being a popular culinary flavouring[1].

Hundreds of years ago, mushrooms were dangerous for consumption. According to the records, mushrooms had approximately 140,000 known species and about 2,000 species are classified as edible, and are safe for human consumption[2]. Seven hundred species are qualified as medicinal because they contain valuable compounds that are secondary metabolites and have

medicinal properties. On the other hand, many other species are classified as inedible or poisonous mushrooms. Upon eating these mushrooms, there is a consequent risk to health or death, even if they are eaten in small amounts[3]. The major reason for people eating poisonous mushrooms is that they cannot identify the differences between edible and inedible mushrooms. The consequence of eating poisonous mushrooms is death, and this disaster happens repeatedly in many countries including Thailand. In 2008 to 2014 there were 11,817 cases of poisoning by eating poisonous mushrooms in Thailand and 57 deaths were reported[4].

Considering that accidental poisoning is a major public health problem, the largest number of poisoning cases is caused by eating poisonous mushrooms[2]. There has been much research at high cost to find out how to accurately and quickly determine and distinguish mushroom types. So far a number of molecular biological techniques have been used extensively, such as the amplified fragment length polymorphism (AFLP) technique and the random amplification polymorphic DNA (RAPD) technique[5,6]. There are methods to increase the number of DNA fragments by PCR to perform DNA sequencing for mushroom classification, but these techniques still have limitations if the edible and inedible mushrooms are close species. In addition, specific DNA sites are used to analyze the sequence of ribosomal DNA at the internal transcribed spacer (ITS) for classification of poisonous mushrooms[7]. Consequently a number of poisonous mushrooms have been classified.

Furthermore, proteomics is another biological analysis technique to qualify and quantify the total proteins in either particular stages or compartments of living organisms. Proteomics constitutes a powerful tool to identify proteins in different stages, defined as the "protein complement of a given genome" and thus refers to all proteins expressed by a cell or tissue[8,9]. Since its emergence, the term proteomics has come to encompass the systematic analysis of protein populations with the goal of concurrently identifying, quantifying, and analyzing large numbers of proteins in a functional context. As such, the ultimate goal of most proteomic studies is to determine which proteins are responsible for a specific function or phenotype[8,9]. Hence, proteomics is believed to be an appropriate tool for identifying unique protein markers, which would allow the unambiguous classification of poisonous mushrooms. Previously this technique was used for the analysis of protein patterns in the growing stages of edible mushrooms (*Termitomyces heimii*) to understand the relationship of protein functions in each development stage of mushroom growth[10]. However, the proteomics analysis of both edible and inedible mushrooms has been limited. Thus, the purpose of this research is to evaluate the protein patterns in wild mushrooms, *A. princeps* and *A. purpurellus*, using a proteomics technique. The obtained protein patterns might lead to an understanding of some molecular mechanisms in mushrooms and might lead to the development of protein markers to classify edible and inedible mushrooms in the future.

Methodology

Mushroom materials

Two types of mushrooms were collected from natural forests in the Northeastern Thailand. Morphological mushroom classification was undertaken, including the color of caps, gills, stalk, volva etc.

DNA extraction

Fresh mushrooms were cleaned with distilled water, cut into small pieces and ground into a powder in a crusher with added liquid nitrogen. Then, DNA was extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis, USA) according to the manufacturer's instructions. The DNA was kept at -70 °C until used.

Polymerase chain reaction (PCR) method

DNA extraction was performed according to the method of the GF-1 Nucleic Acid Extraction Kit (Vivantis, USA) and PCR was performed according to the Dörnte and Kües method[11]. One

microliter of DNA was mixed with 25 µl of the PCR reactants (10 mM Tris pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPS, 0.4 µM of each primer and 1 U of *Taq* DNA polymerase). The fungal universal primer including ITS1: 5´TCCGTAGGTGAACCTGCGG-3´ and ITS4: 5´-TCCTCCGCTTATTGATATGC-3´ were used to amplify the ribosomal DNA (Initialization, denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec and a final extension step at 72°C for 10 min)[12]. Subsequently, DNA content was analyzed by 2% agarose gel electrophoresis. Then, the interesting DNA band was cut and nucleotide sequencing was performed. The obtained nucleotide sequences were sent to BLAST and the NCBI database to identify the mushroom species.

Protein extraction

Mushroom protein extraction was performed according to the method of Lai et al[13]. Initially, the fruiting bodies of each mushroom was deep-frozen in liquid nitrogen and ground to a powder using a mortar and pestle. The ground mushroom was dissolved and lysed in 1 ml of lysis buffer (8M urea, 4% CHAPS, 2% IPG Buffer, 40 mM dithiothreitol (DTT)). Then, the suspension was centrifuged at 12,000 ×*g* for 10 min at 4 °C. The supernatant was collected to measure protein content by the Bradford method using BSA as a standard[14].

Two-dimensional gel electrophoresis (2D-PAGE)

In the first dimensional isoelectric focusing (IEF), 120 µg of mushroom protein samples were re-suspended in the rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2 mM DTT, 0.8% (w/v) IPG buffer and 0.2% bromophenol blue) to obtain 125 µl of final volume. The IEF was performed on 7 cm immobilized pH gradient gel (IPG) dry strips, pH 3-10 (Amersham Bioscience, Sweden). The IPG dry strip was allowed to rehydrate for 12 h at 4 °C. Then the IEF was carried out by an Ettan IPGPhor II unit (GE Healthcare, Sweden). The IEF was performed following these steps: Step 1 (voltage step and hold) 250Vh, Step 2 (voltage step gradient) 500Vh and Step 3 (voltage step gradient) 8500Vh. After IEF, the IPG strip was firstly equilibrated for 15 min in SDS equilibration buffer solution (6 M urea, 50 mM Tris-HCl pH8.8, 2% (w/v) SDS, 30% (v/v) glycerol) containing 0.06 M dithiothreitol (DTT). A second 15 min equilibration was performed with an SDS equilibration buffer substituting DTT with 0.1 M iodoacetamide solution (IAA). Subsequently, the protein sample was separated in the second dimension by 13.5% SDS-PAGE and visualized by Colloidal Coomassie Brilliant blue G-250 staining.

Image scan and data analysis

The pI and molecular weight of each protein spot were analyzed by Image master™ 2D platinum version 7.0 program.

Protein identification

Interesting protein spots were cut and used to perform trypsin digestion. The tryptic peptides were subject to LC-MS/MS. Then, the peptide mass fingerprint data was uploaded to the database search program MASCOT (Matrix science) for protein identification.

Results and Discussion:

Mushroom Identification

It was necessary to identify the exact type of wild mushroom prior to proteomics analysis. After mushroom collection, two types of mushrooms were identified by morphological observation and further confirmed by PCR. The ITS1 and ITS4 primers were selected for this experiment. These primers are situated between the Small Sub Unit-coding sequence and the Large Sub Unit-coding sequence of the ribosomal operon[15]. They are widely

accepted for amplification of the highly variable ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and for analysis of fungal ITS sequences[15]. The obtained PCR products were used for nucleotide sequences analysis and subsequently compared with the nucleotides in the NCBI database using the BLAST program. The results demonstrate the homogenous ITS nucleotide sequences of wild mushroom 99% identities with *Amanita princeps* (GenBank: KT213713.1) (Figure 1 A) and 97% identities with *Agaricus purpurellus* (GenBank: KF447903.1) (Figure 1 B). *A. princeps* is found as an excellent edible species in various countries, including Thailand that called as Hed Ra York Kao[16]. This wild mushroom contained high protein, high fiber and low fat content[16]. For *Agaricus*, it is well known that *Agaricus* includes various both of edible and inedible species which contain dangerous poisoning[17]. Several reports have been described *A. purpurellus* as edible[18]. On the contrary, this species has caused several cases of poisoning[19]. Morover, Priyamvada et al. reported *A. purpurellus* as a poisonous mushroom[17]. This diminutive agaric is poisonous in nature and commonly found in Europe, North America, and Asia[17]. Hence, in the present research, *A. purpurellus* have been identified as a poisonous mushroom. Due to the possibility of poisoning combined with the difficulty of distinguishing edible from poisonous species, consumers should be careful about eating any *Agaricus* specimen.

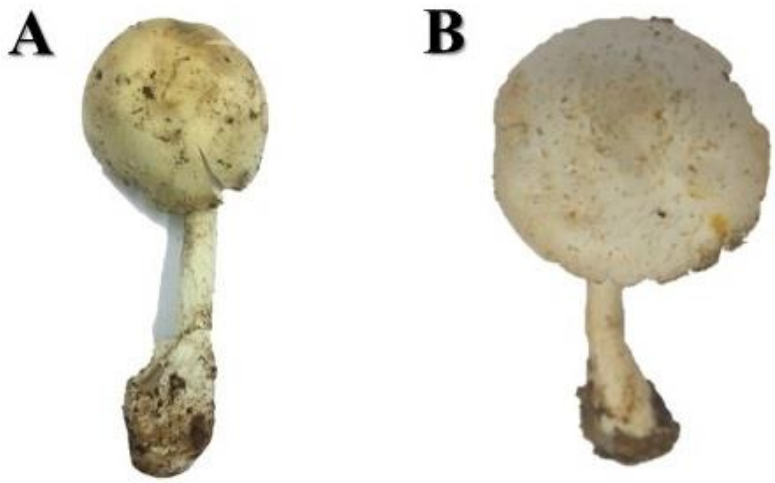

Figure 1. Morphology of edible mushroom, *Amanita princeps* (A) and inedible mushroom, *Agaricus purpurellus* (B).

Protein Patterns of Mushrooms Extracts

In this research, to study the protein patterns of *A. princeps* and *A. purpurellus*, proteins are extracted from all parts of the fruiting body. Then these proteins were resolved using 2D-PAGE. In this study, total proteins of about 159 and 32 spots were obtained in *A. princeps* and *A. purpurellus*, respectively. Among these, 26 obvious protein spots of *A. princeps* were selected for identification by LC-MS/MS (Figure 2 A). As illustrated in Figure 2 A, the protein found was dispersed at pH 5-8 ranged in size from 97 kDa down. However, the protein in *A. purpurellus* was dispersed at pH 6-8 ranging in size from 45 kDa down (Figure 2 B). The 15 protein spots that also obviously appeared in *A. purpurellus* were selected for identification by LC-MS/MS (Figure 2 B).

In terms of protein identification, all protein spots with a pronounced and clear expression were further subjected to mass spectrometry. The results show that 7 of 26 protein spots of *Amanita princeps* could be identified. In detail, protein spot number E02 was aspartic peptidase A1 of *Trametes versicolor*, protein spot number E03 was GTPase IMAP family member 4 of *Valsa mali*, protein spot number E04 was glycoside hydrolase family 5 protein of *Amanita muscaria*, protein spot number E07 was bZIP transcription factor of *Metarhizium*

robertsii, protein spot number E13 was GTPase SAR1-like protein of *Enterocytozoon bieneusi*, protein spot number E23 was nucleoside diphosphate kinase of *Cylindrobasidium torrendii*, and protein spot number E26 was rheb GTPase Rhb1 of *Schizosaccharomyces japonicas* (Table 1). These protein were grouped into 4% of cell growth and development (spot number E07), 4% of carbohydrate/energy metabolism (spot number E23), 19% of hydrolase enzyme (spot numbers E02, E03, E04, E13 and E26), and 73% of predicted proteins, hypothetical proteins, unnamed proteins and putative proteins (spot numbers E01, E05, E06, E08, E09, E10, E11, E12, E14, E15, E16, E17, E18, E19, E20, E21, E22, E24 and E25) (Figure 3).

For *Agaricus purpurellus*, a poisonous mushroom, 3 of 15 protein spots could be identified. In detail, protein spot number T01 was leucine aminopeptidase of *Leucoagaricus sp.*, protein spot number T10 was related to NADH oxidase of *Fusarium fujikuroi*, and protein spot number T14 was ubiquitin extension protein of *Nematocida sp.* (Table 2). These protein were 6% of cell growth and development (spot number T14), 7% of carbohydrate/energy metabolism (spot number T10), 7% of protein/amino acid metabolism (spot number T01), 80% of predicted proteins, hypothetical proteins, unnamed proteins and putative proteins (spot numbers T02, T03, T04, T05, T06, T07, T08, T09, T11, T12, T13 and T15) (Figure 4).

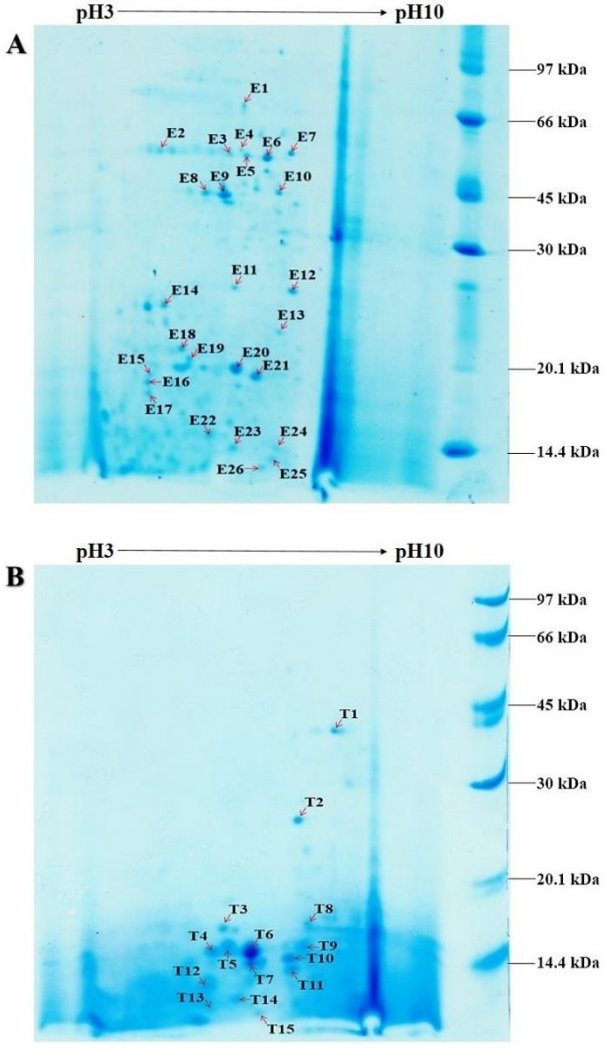

Figure 2. Protein profile of *A. princeps* (A) and *A. purpurellus* (B) separated by 2D-PAGE. The immobilized strips with pH gradient 3-10 were used for the first dimension. The second dimension separation was performed using 13.5% SDS-PAGE. Protein spots were visualized by staining with Colloidal Coomassie Brilliant blue G-250. The arrows indicate protein spots selected for LC-MS/MS analysis.

The qualitative and quantitative knowledge of mushroom proteins reveals great intra and inter species differences[20]. The protein identified in cell growth and development was found to be bZIP transcription factor (spot number E07). This protein was found in *A. princeps* and is important for control of biochemical and physiological processes, and is involved in abiotic stress tolerance in plants and all other eukaryotic organisms[21]. The processes regulated by the bZIP proteins in filamentous fungi can be classified into development, amino acid biosynthesis, unfolded protein response, various stress responses and nutrient utilization. The main nutrients utilized by fungi via bZIP include iron, nitrogen and sulfur compounds[22]. The protein in carbohydrate/energy metabolism was found to be nucleoside diphosphate kinase (spot number E23). The nucleoside diphosphate kinase (NDPK, also NDP kinase, (poly) nucleotide kinase and nucleoside diphosphokinase) is an enzyme that catalyzes the transfer of a terminal phosphate group of nucleoside triphosphate (NTP) to nucleoside diphosphate (NDP) to produce nucleotide triphosphates[23]. NDP kinase could function not only to provide NTPs as a housekeeping enzyme, but also play a crucial role in the central part of signal transduction in bacteria, fungi, plants, invertebrates and vertebrates[24]. This is an essential protein for mushroom growth. Aspartic peptidase A1 was identified in *A. princeps* (spot number E02). This protein classified into the group of hydrolase enzymes also known as the pepsin family[10]. In mushrooms, Rahmad et al[10]. revealed that this protein was highly expressed during primordial development. However, a dramatic decrease in their expression level was found during fruiting body formation. Although aspartic peptidase A1 has been reported in mushrooms, its function in mushroom development has not been clarified. The identified protein in protein/amino acid metabolism was found to be leucine aminopeptidase (LAP) (spot number T01). This protein is an exopeptidase that catalyzes the hydrolysis of amino acid residues from the amino terminus of polypeptide chains[25]. Aminopeptidases play important roles in cell maintenance, growth and development, and also cell defense[25]. The protein in the cell growth and development group was found to be ubiquitin extension protein (spot number T14). This protein may be present in fungi to control various processes of the cell[26]. NADH oxidase was found in *A. purpurellus* (spot number T10). In eukaryotes, NADPH oxidases are key enzymes in pathogen defense mechanisms. For fungi, this protein is involved in a wide variety of differentiation functions, such as sexual reproduction, formation of penetration structures, and establishment of mutualistic interactions[27].

The results reflect that a lot of proteins could not be identified. This may be because only a few mushroom proteins have been classified.

Table 1. Proteins identified from *Amanita princeps* by 2D-PAGE analysis

Spot No.	Matched protein	Experimental Mw/pI	Theoretical Mw/pI	Score	Sequence coverage (%)	Number of peptides matched	Species
E01	predicted protein	73/7.53648	82.4/5.3	50.7	4.2	2	*Laccaria bicolor*
E02	aspartic peptidase A1	57/5.55365	44.8/4.8	66.3	11.3	5	*Trametes versicolor*
E03	GTPase IMAP family member 4	56/7.20601	40.6/7.7	37.3	3.6	1	*Valsa mali*
E04	glycoside hydrolase family 5 protein	57/7.44635	46.4/5.6	51.7	2.8	1	*Amanita muscaria*
E05	hypothetical protein PDE_05880	55/7.58155	58.6/6.6	39.7	2.5	1	*Penicillium oxalicum*
E06	hypothetical protein HIM_10505	54/8.07725	64.1/9.9	40.1	3.2	1	*Hirsutella minnesotensis*
E07	bZIP transcription factor	56/8.63305	52.7/6.4	46.3	5.1	1	*Metarhizium robertsii*
E08	hypothetical protein EPUS_00189	46/6.60515	67.4/5.6	32.5	4.9	1	*Endocarpon pusillum*
E09	unnamed protein product	46/7.04077	46.8/8.8	38.7	2.2	1	*Aspergillus oryzae*
E10	hypothetical protein M378DRAFT_185173	46/8.34764	42.5/5.4	150.9	3.7	1	*Amanita muscaria*
E11	hypothetical protein	26/7.29614	60.9/7.8	30.6	3.9	1	*Tuber melanosporum*
E12	hypothetical protein J132_02423	26/8.64807	93.5/6.6	62.1	3.2	1	*Termitomyces sp.*
E13	GTPase SAR1-like protein	23/8.34764	23.3/9.1	60.9	5.5	1	*Enterocytozoon bieneusi*
E14	hypothetical protein PAXRUDRAFT_154615	25/5.64378	21.8/6.0	38.2	6.4	1	*Paxillus rubicundulus*
E15	hypothetical protein AOL_s00188g310	20/5.29828	31.5/4.9	19.5	2.5	1	*Arthrobotrys oligospora*
E16	putative septin	19/5.26824	40.1/5.0	37.3	2.6	1	*Pyrenopeziza brassicae*
E17	uncharacterized protein VP01_7109g1, partial	18/5.28326	28.2/7.4	35.1	7.3	1	*Puccinia sorghi*
E18	hypothetical protein PTT_20265	21/6.04936	25.9/5.8	15.4	3	1	*Pyrenophora teres*
E19	hypothetical protein AGABI1DRAFT_127400	21/6.24464	42.3/10.2	37.4	3.2	1	*Agaricus bisporus*
E20	hypothetical protein PTT_20265	20/7.32618	25.9/5.8	16.4	3	1	*Pyrenophora teres*
E21	hypothetical protein CONPUDRAFT_169001	19/7.80687	78.4/8.3	18.5	1	1	*Coniophora puteana*
E22	hypothetical protein V500_07911	15/6.7103	16.0/10.8	16.5	4.7	1	*Pseudogymnoascus sp.*
E23	nucleoside diphosphate kinase	14/7.26609	16.8/6.8	251.4	11.1	2	*Cylindrobasidium torrendii*
E24	hypothetical protein LEMA_P067880.1	14/8.30258	15.1/8.9	45.1	11.1	1	*Leptosphaeria maculans*
E25	hypothetical protein SNOG_15805	14/8.19742	22.1/9.1	20.3	3.4	1	*Parastagonospora nodorum*
E26	rheb GTPase Rhb1	13/7.91202	20.5/7.0	28.1	3.8	1	*Schizosaccharomyces japonicus*

Table 2. Proteins identified from *Agaricus purpurellus* by 2D-PAGE analysis

Spot No.	Matched protein	Experimental Mw/pI	Theoretical Mw/pI	Score	Sequence coverage (%)	Number of peptides matched	Species
T01	Leucine aminopeptidase	40/9.11111	36.9/5.7	48.3	2.7	1	*Leucoagaricus sp.*
T02	hypothetical protein AGABI2DRAFT_189889	26/8.36111	22.2/5.4	37.3	4.1	1	*Agaricus bisporus*
T03	unnamed protein product	16/6.78704	26.4/7.1	40.3	4.9	1	*Pneumocystis jirovecii*
T04	hypothetical protein AGABI2DRAFT_195273	15/6.53704	37.4/5.5	155.2	4.2	2	*Agaricus bisporus*
T05	unnamed protein product	15/6.87963	26.4/7.1	34.6	4.9	1	*Pneumocystis jirovecii*
T06	hypothetical protein ASPCAL12514	15/7.37037	35.4/5.7	38.9	4.1	1	*Aspergillus calidoustus*
T07	hypothetical protein AGABI2DRAFT_195273	14/7.35185	37.4/5.5	264.2	4.2	2	*Agaricus bisporus*
T08	hypothetical protein M422DRAFT_269213	17/8.5463	37.1/5.5	31.6	3.9	1	*Sphaerobolus stellatus*
T09	hypothetical protein PGTG_17769	15/8.47222	22.7/9.5	14.9	3.3	1	*Puccinia graminis*
T10	related to NADH oxidase	14/8.16667	47.6/6.6	26.2	1.6	1	*Fusarium fujikuroi*
T11	hypothetical protein AGABI2DRAFT_195273	14/8.23148	37.4/5.5	194.3	4.2	2	*Agaricus bisporus*
T12	hypothetical protein WALSEDRAFT_55002	13/6.43519	28.0/9.9	54.7	3.8	1	*Wallemia mellicola*
T13	hypothetical protein GALMADRAFT_251146	12/6.38889	16.8/7.8	45	11.3	1	*Galerina marginata*
T14	ubiquitin extension protein	12/7.06481	15.1/10.0	49.8	6.8	1	*Nematocida sp.*
T15	hypothetical protein SMAC_08853	11/7.52778	20.0/10.8	44.9	5.3	1	*Sordaria macrospora*

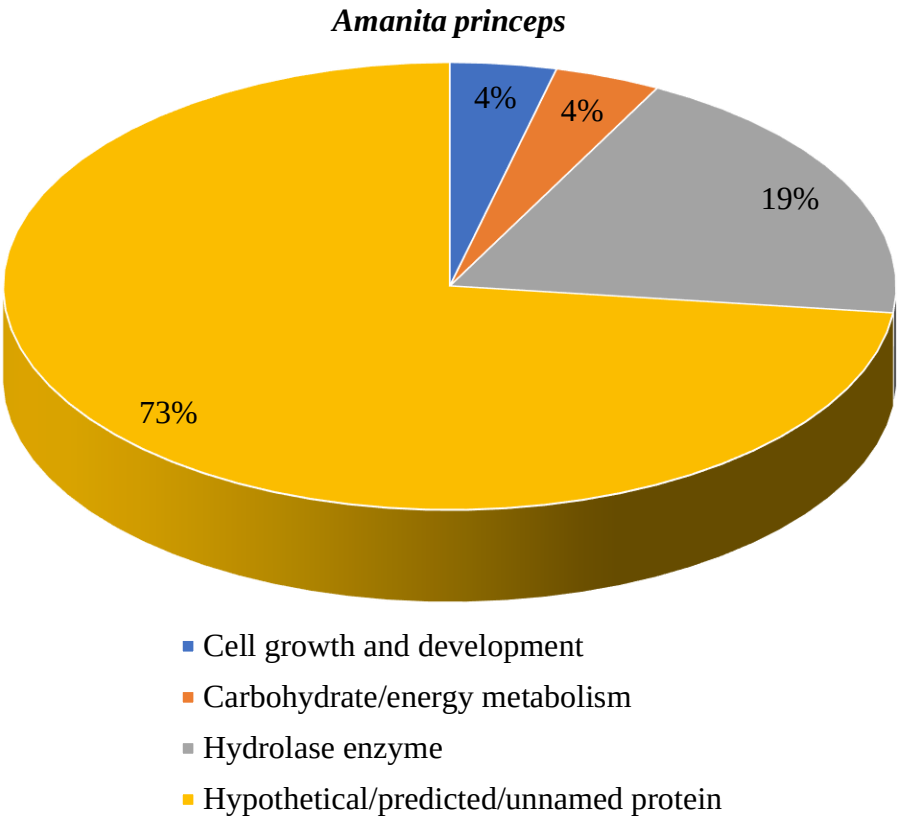

Figure 3. Classified proteins of *Amanita princeps* according to their biological function.

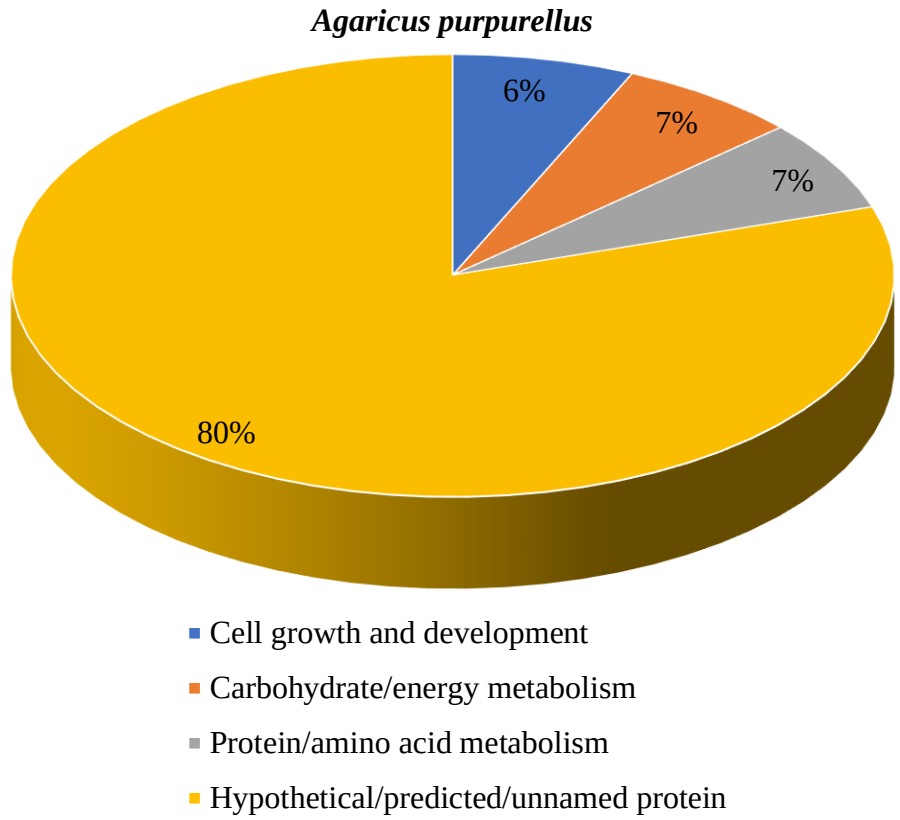

Figure 4. Classified proteins of *Agaricus purpurellus* according to their biological function.

Conclusion

The results show that proteins from both wild mushrooms (*A. princeps* and *A. purpurellus*) could be classified into four groups including protein/amino acid metabolism, cell growth and development, carbohydrate/energy metabolism, and hydrolase enzyme. However, further experiments addressing the function of the differentially expressed proteins in a wide range of mushroom species are required to identify unique proteins for edible and inedible mushrooms.

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