



Comparative study of protein pattern changes and mineral composition in cultivated and newly domesticated wild *Flammulina velutipes*

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Abstract

A wild strain of *Flammulina velutipes* indigenous to Iran has been recently adapted for artificial cultivation, requiring nutritional information in order to improve its utilization as a medicinal-culinary mushroom. Thus, the current study aimed to evaluate protein profile of this domesticated wild *F. velutipes* strain over six different developmental stages, in comparison with a commercially cultivated strain of *F. velutipes*. Various macro- and microelements were also investigated in the mature sporomes cultivated in a lignocellulosic substrate composed of 40% wheat straw +40% sawdust +18% wheat bran +1% lime +1% gypsum. The lowest and highest amounts of protein were observed in primordia (33% of dry matter) and sporomes picked at the third or fifth day of harvesting (57% of dry matter), respectively ($p \leq 0.05$), followed by an obvious decrease in protein content at the late stages of harvesting ($p \leq 0.05$). Further SDS-PAGE analyses revealed that the protein molecular mass increased from less than 30 kDa in primordia to 235 kDa in mature sporomes. However, no significant difference in protein profiling was noticed between the wild and the reference strains of any given stage ($p \geq 0.05$). While nutritional elements such as K, P and Mg were found at 886.9, 745.2, and 142.7 mg/100 g of dry weight, respectively, no heavy metals was found at levels higher than the provisional maximum tolerable intake in the sporomes. This study may be considered an initial step towards recognition of the wild *F. velutipes* as a non-toxic functional food, warranting further proteomics studies.

Key words – Developmental stages – Nutritional elements – PAGE – Protein content – SDS – Wild mushrooms

Introduction

Continuous in-depth evaluation of nutritional values of cultivated medicinal mushrooms has demonstrated that they are one of the most prominent sources of nutraceuticals. Some of the most recently published researches on well-known cultivated medicinal mushrooms include *Lentinula edodes* (Berk.) Pegler (Li et al. 2018), *Flammulina velutipes* (Curtis) Singer (Tsai et al. 2017), and *Pleurotus ostreatus* (Jacq.) P. Kumm. (Tolera & Abera 2017). However, these types of studies do

not necessarily reflect the nutritional potentiality of all the wild-growing mushroom species. Of approximately 14,000 taxonomically recognized mushroom species, about 200 are experimentally grown, 100 economically cultivated, around 60 commercially cultivated, and more than 10 have reached industrial scale in commercial cultivation in many countries (Wasser 2014, Miles & Chang 2004). Moreover, the commercially cultivated species may have numerous wild counterparts growing in different habitats, remaining a huge genetic diversity among wild mushrooms to be explored.

In addition to mushroom cultivars, a growing number of researches have been also done on nutritional properties and composition of wild mushrooms, focusing on medicinal mushrooms directly collected from the wild (Quarcoo et al. 2014, Sharma & Atri 2014, Mridu & Atri 2017, Sharif et al. 2017). By contrast, very limited studies have evaluated nutritional composition and medicinal properties of sporomes of wild mushrooms domesticated in artificial substrates, for example *Ganoderma neo-japonicum* Imazeki (Tan et al. 2015), the HS isolate of wild *Pleurotus* spp. (Sulistiany et al. 2016), and *Ganoderma australe* (Fr.) Pat. (Luangharn et al. 2017). Domestication of wild mushrooms in locally available lignocellulosic substrates may provide benefits for conservation of rare wild species as well as regional economy for mushroom growers. In addition, it provides an alternative way to overcome the limited supply of wild mushrooms (Tan et al. 2015). The continuous mushroom production in high amounts and in a standardized quality is also important to facilitate reproducibility of biomedical or nutraceutical research on wild mushrooms (Lindequist et al. 2005).

Culinary-medicinal mushroom Enoki, *F. velutipes*, ranks fifth among cultivated mushrooms in the world (Royse et al. 2017), comprising of commercially cultivated strains and wild types. Recently, we reported the successful domestication and evaluated agronomic characterization of an Iranian wild Enoki strain (in comparison with a commercial strain) on various locally available agro-wastes (Rezaeian & Pourianfar 2017). Following domestication of wild mushrooms, assessment of nutritional/toxic minerals and protein content in sporomes of edible newly cultivated wild mushrooms should be investigated, as it directly associates with human food safety and protein nutrition or malnutrition particularly in developing countries (Jaworska et al. 2014, Kalač 2009, Sharma & Gautam 2015). Besides, proteins present in sporomes of wild mushrooms have a variety of important biological activities (Lau et al. 2012). Thus, the current study sought to address protein content, protein profiling over different developmental stages, and the presence of macro and micro dietary elements in Iranian native newly domesticated *F. velutipes* in comparison with a reference commercial strain.

Materials & Methods

Chemicals & reagents

The following reagents (analytical grade) including acetic acid (AC), methanol (MeOH), Tris (hydroxymethyl) aminomethane, glycine, N, N-methyl-enebisacrylamide (Bis), acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), sodium carbonate, mercaptoethanol, formaldehyde, tetramethylethylenediamine (TEMED), bovine serum albumin (BSA), and sodium thiosulfate were all purchased from Merck (Darmstadt, Germany). Standard protein ladders and phenylmethane sulfonyl fluoride (PMSF) were prepared from Cinnagen Co. (Iran) and Sigma, respectively. Malt extract agar from Merck (Darmstadt, Germany) was also used as solid media for growing and maintenance of mycelial cultures of the tested Enoki mushrooms. In all the experimental steps, deionized water was utilized to prepare solutions and buffers.

Production of fresh Enoki sporomes

The wild-growing strain of *F. velutipes* mushroom utilized in this study has been previously collected and identified by our group (Rezaeian & Pourianfar 2017). In addition, a commercially cultivated strain of *F. velutipes* (M4622-mcup-2) purchased from Mycelia (Belgium) was used as the reference. Based on the findings of the previous study (Rezaeian & Pourianfar 2017), the

following substrate was selected for production of fresh sporomes: 40% wheat straw +40% sawdust +18% wheat bran +1% lime +1% gypsum. Mycelial culture, spawn preparation, and mushroom fructification were carried out by procedures previously described (Rezaeian & Pourianfar 2017) with modifications. The fresh sporomes were sampled related to six developmental stages including, primordia and mature sporomes harvested at three, five, seven, nine and eleven days after appearance of primordia. Harvested mushrooms in each developmental stage were immediately stored at -80°C in order to prevent protein disintegration before use. The harvested sporomes were further dried using lyophilization (Buchi B-191, Switzerland).

Total protein extraction

Total protein was extracted from the lyophilized powder of each sporome harvested at various developmental stages, based on a modified method reported elsewhere (Liu et al. 2012). In brief, 1X phosphate buffered saline (PBS) was manually made at a pH of 7.4. In order to neutralize protease enzymes, 1% PMSF (v/v) was added to the PBS solution. Then, 40 mg of the mushroom powder was re-suspended in one mL of PBS containing PMSF followed by grinding technique using liquid nitrogen for five minutes. After centrifugation (2880×g, 2 min, 4°C), the supernatant was collected to serve as soluble crude proteins.

Protein measurements

The total protein content was determined using the Bradford method (Bradford 1976) based on the reaction of extracted proteins with Coomassie Brilliant Blue. Five mL of Bradford Reagent (1x, 0.1 mg/mL in MeOH) was added into 100 µL of BSA (at various concentrations) followed by incubation at 4°C for 20 min. The absorbance was then measured at 595 nm with a UV/VIS spectrophotometer (Epoch 2 Microplate Spectrophotometer from BioTek). Absorbance values for BSA generated a standard curve in the range of 200-1000 µg/mL with the following equation: $Y = 0.2728X + 0.423$ ($R^2 = 0.9822$). The same procedure was carried out with the soluble crude proteins extracted from Enoki mushrooms. Total protein content was then extrapolated using the curve equation and expressed as percentage of mushroom dry weight.

Protein analysis

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to obtain the primary profile of total protein extracts obtained from each developmental stage using a vertical slab gel apparatus. Sample buffer and crude proteins were mixed at a ratio of 1:1 (v/v) and boiled for 5 min at 96°C. Seven mL of a mixture of broad range protein ladder was loaded into the wells. Electrophoresis was conducted at a constant current of 110 V to move the proteins through both the stacking and resolving gel (4% and 12% polyacrylamide, respectively). Following electrophoresis, the gel was fixed with a solution consisting of 40% (v/v) MeOH and 10% (v/v) AC for 20 minutes. The protein bands were then stained by Coomassie brilliant blue for three hours.

Macro- and microelement content in sporomes

Nutritional and toxic elements present in the tested sporomes of *F. velutipes* were determined using an ICP-UV/VIS instrument (Spectra ARCOS, Germany, Model number 76004555). The limit of detection was determined and the quantitative analyses were carried out under the instrumental condition for all studied elements (Table 1).

Statistical analysis

Each experiment was independently repeated at least three times. Graph prism version 5 was utilized to conduct statistical analyses and ANOVA tests. Means were compared using Tukey method with a significance level (p value) of 0.05. For protein analysis, the background was normalized with advanced imageJ software using rolling ball algorithm (optimum radius, 50) and protein signals were detected by GelAnalyzer software followed by PlotDigitizer (Zarei et al. 2015).

Table 1 Instrumental conditions of ICP-UV/VIS for all studied elements.

Instrument name	Type
Nebulizer	Cross flow
Rf power	1400 w
Nebulizer gas (argon) flow rate	1 L/min
Introduction of the sample	1.5 mL/min
Pump speed	30 rotations per minute
Number of replicates	1

Results

Determination of total protein content

To overcome the inaccuracies borne in mushroom moisture contents, total protein measurements were determined as a percentage of dry matter. As illustrated in Fig. 1, total protein content of *F. velutipes* harvested in different maturation stages displayed an age-dependent bell-shaped curve. The lowest amount of protein (33%) was observed in primordia as the initial step of fructification. Following stipe elongation and cap expansion, the highest protein content (57%) was measured in sporomes harvested at the third and fifth days of harvesting ($p \leq 0.05$), as compared to other stages of fructification. Then, an obvious decrease in protein content was seen in mature mushrooms (at the ninth day of harvesting) ($p \leq 0.05$). However, no significant difference in protein content was noticed between the wild and the reference strains of *F. velutipes* at any given stage ($p \geq 0.05$).

Protein profiling

Total crude proteins of the wild *F. velutipes* were analyzed by SDS-PAGE in order to compare changes in protein patterns obtained from six developmental stages, using 12% acrylamide gel. Both clear distinct bands and unclear bands were observed in the protein banding patterns, with molecular masses ranged from 9 kDa to 235 kDa. The number of protein bands increased over mushroom maturation from 12 clear distinct protein bands at the primordial stage to 21 bands in the 11th day of harvesting. The SDS-PAGE analysis also showed variations in the molecular size of protein bands observed over the different stages of harvesting ($p \leq 0.05$). According to Fig. 2, the primordial phase showed mostly proteins of low molecular mass (< 30 kDa), whilst mushrooms harvested at the late phases showed higher molecular mass proteins.

Elemental profile

Various elements were observed in both wild and the reference strains, including essential elements and some heavy metals. However, there was no significant difference in the presence or the quantity of elements between the wild and reference strain ($p \geq 0.05$). As shown in Fig. 3, K was estimated to be 876.7 and 886.9 mg/100 g of dry weight for the reference and wild strain, respectively. In the wild strain, P was also recorded at a high content (745.2 mg/100g of dry weight) followed by Mg (142.7 mg/100g of dry weight) none of which showed significant difference from those of the reference strain. Fe, Ca, Na and Zn were also present in concentrations of 8.102, 29.23, 37.24 and 6.961 mg/100g, respectively. In addition, trace amounts (2-6 mg/100g of dry weight) of Cr, Pb, Cu, and Mn were also found, while concentrations of Ni, Cd, and Ar were less than 0.08 mg/100g of dry weight. Co, Li and Ag were below detection levels.

Discussion

The current study for the first time showed that the protein content of a newly domesticated wild Enoki mushroom is similar to that of the commercially cultivated Enoki mushroom in all the stages of sporome development. The findings of this study also showed that the highest protein content (57% per dry matter) is present in sporomes harvested at the third and fifth days after

appearance of primordia. At the present, data on changes in protein contents of edible wild-growing mushrooms over different stages of sporomes (i.e. primordium, immature or mature sporome) is limited to some edible wild mushrooms, despite the global trend in growing and domestication of various wild mushroom species (Herawati et al. 2016, Salmones et al. 2018, Shahtahmasebi et al. 2018, Arana-Gabriel et al. 2018, Debnath et al. 2019). A few proteomic studies have addressed changes in protein expression at molecular level (related to mushroom development or specific proteins such as lectin) during different developmental stages of wild or cultivated edible mushrooms (Chen et al. 2012, Nikitina et al. 2017, Rahmad et al. 2014). It should be noted that protein contents of mushrooms not only are dependent on developmental processes but also are affected by mushroom species/strain, environmental conditions, and even formulation and structure of the substrate (Hoa et al. 2015, Li et al. 2017). Thus, in this study, it was ensured to employ the same conditions for cultivation of both the wild and the reference *F. velutipes* in terms of variables such as humidity, temperature, substrate, and other controlled environmental parameters. In addition, since post-harvesting conditions may affect the protein content (Lau et al. 2012), all the harvested sporomes were stored at -80°C until use.

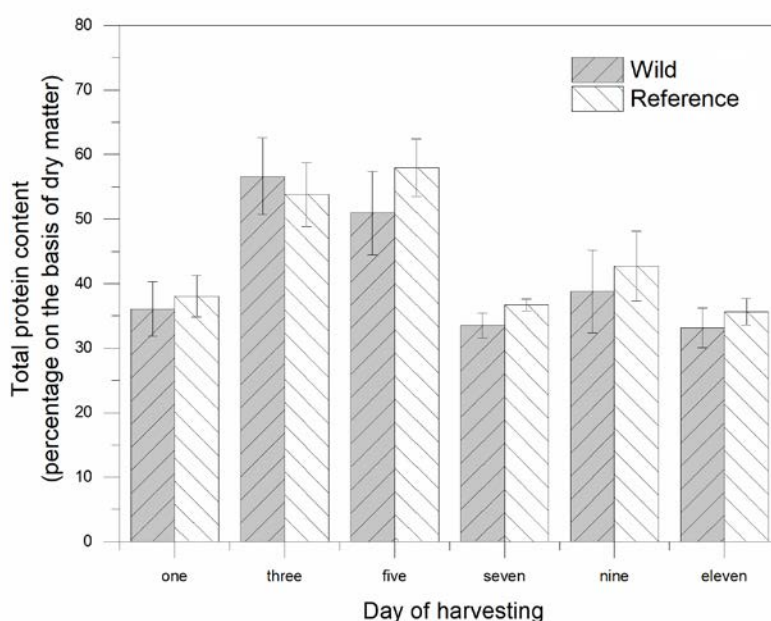


Fig. 1 – Total protein through PBS buffer extraction was obtained from sporomes of both wild-growing and the reference *F. velutipes*. The sporomes were harvested in six maturation stages (i.e. primordia and three, five, seven, nine, and eleven days after appearance of primordia). Total protein was determined using extrapolation of the calibration curve of BSA and expressed as a percentage of dry matter. For each replication of each maturing stage, 30 sporomes were harvested and weighed followed by lyophilization. Then, 100 mg of freeze-dried sporomes were used to extract and determine the total protein content. Data represent the means of three independent replications from which standard deviations were calculated.

The quantified data showed that total protein of the tested mushroom increased until the fifth day of harvesting after which a decline was noticed in the last days. These findings may immediately suggest optimum days for harvesting of this mushroom in terms of protein content. Further SDS-PAGE protein analysis confirmed changes in total protein content over the stages of harvesting. The confirmation was based on changes in intensities of the bands which might have direct relationships with concentration of proteins. On the other hand, the yield performance was also determined at each stage of harvesting. While the number of harvested sporomes was constantly 30 for each stage, fresh sporomes harvested at different stages showed different yield performance, including 2, 6, 15, 20, 27, and 33 g for primordial and mushrooms harvested at the

third day, fifth day, seventh day, ninth day, and eleventh day, respectively. Thus, the optimum day for harvesting Enoki mushrooms in terms of protein contents (i.e. the fifth day) might not be necessarily the best time for harvesting high yielding mushrooms. Some other studies also suggested an early harvesting day as the optimum time for harvesting the wild mushrooms of highest protein content, which may confirm our findings in this study (Castronuovo et al. 2019). These findings also provide insights into the relationship between changes in protein content and changes in the use of N by the fungus.

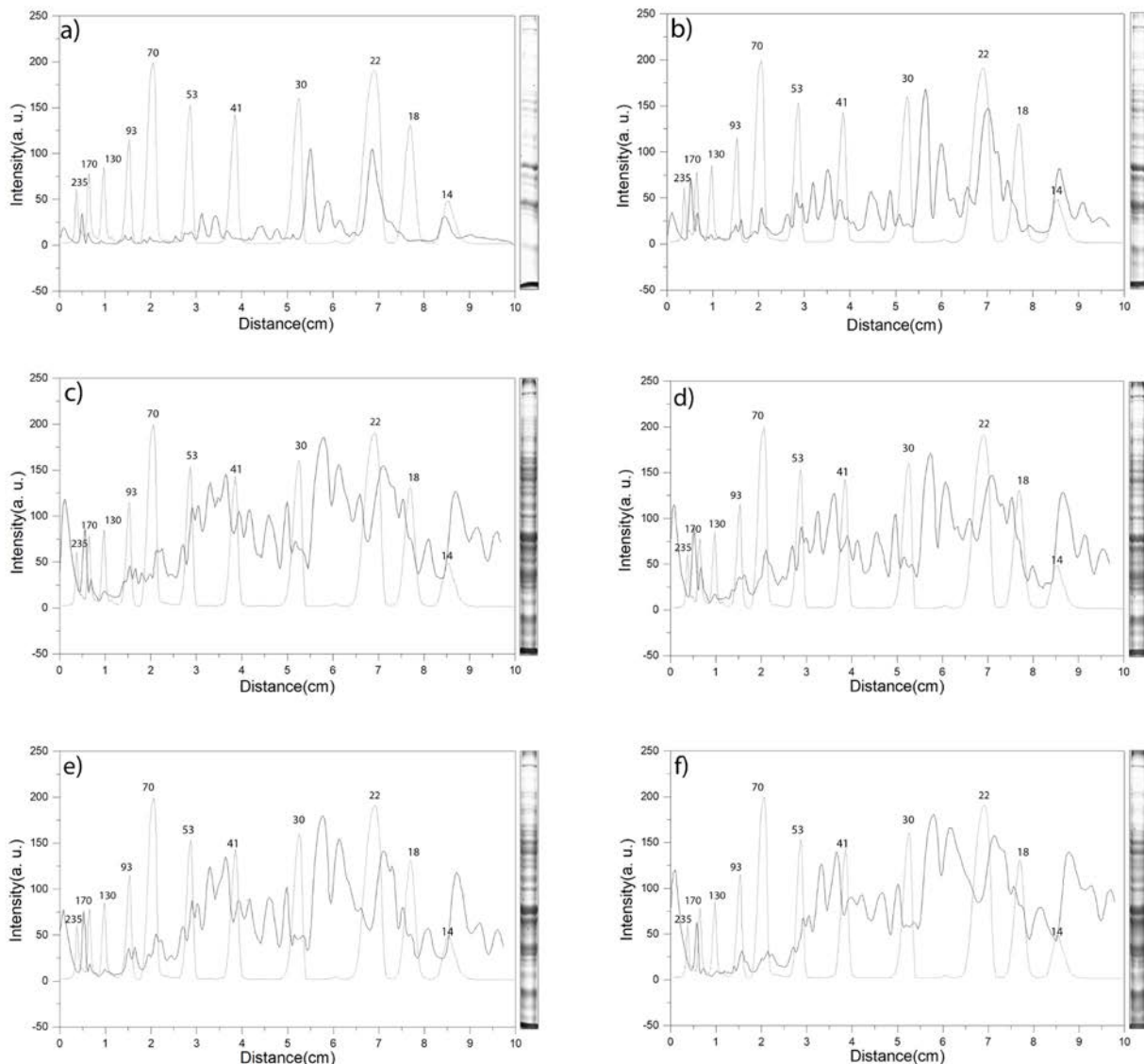


Fig. 2 – The SDS-PAGE electropherograms of total proteins were constructed for each stage of the wild *F. velutipes*, including primordia (a) and sporomes harvested in three days (b), five days (c), seven days (d), nine days (e), and eleven days (f) after appearance of primordia. For each replication of each maturing stage, 30 sporomes were harvested and weighed followed by lyophilization. Then, 100 mg of freeze-dried sporomes were used to extract and determine the total protein content. In electrophoresis, 10 μ L (at a concentration of 10 ppm) of each protein sample was loaded into the gel. The image of protein bands observed in SDS-PAGE were converted to signals using GelAnalyzer software followed by PlotDigitizer. The background was normalized with advanced imageJ software using rolling ball algorithm (optimum radius, 50). Dash lines represent the protein ladder and molecular weight values (kDa), while lines represent the Enoki mushroom samples. The intensities of the signals in each stage of harvesting (a to f) confirmed data obtained from crude protein of that stage (depicted in Fig. 1)

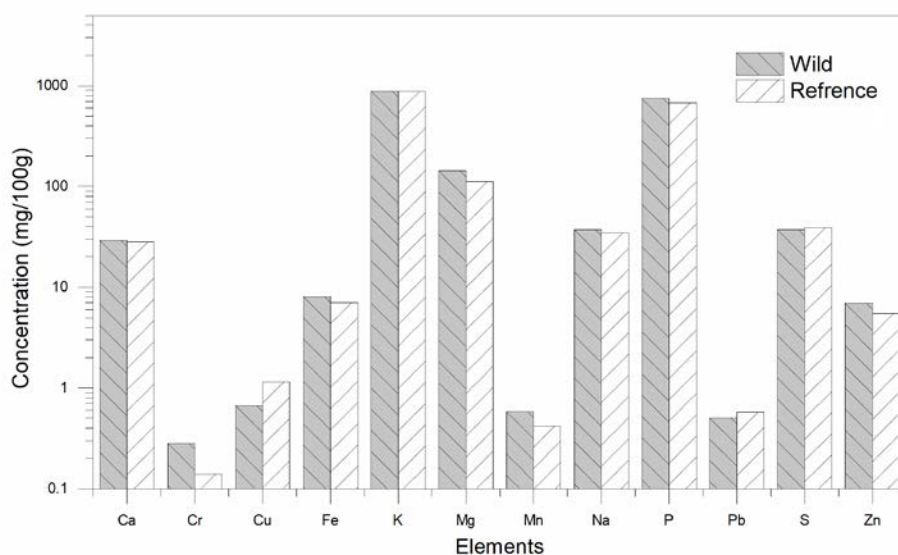


Fig. 3 – Nutritional elements obtained by ICP-UV/VIS in both wild and the reference strains of Enoki mushrooms (of dry weight). The Y-axis is presented in the logarithmic scale. Co, Ag, Li, Ni, and Se were below detection limit and are not presented in the graph.

The N content of the lignocellulosic substrate is the immediate source to be consumed during vegetative growth of mycelia. As the sporomes develop and grow up, the fungus continues to store N to form proteins until the middle stages of harvesting (Al-Obaidi 2016, Hsu et al. 2002). Then, as the N in substrate becomes limited, the fungus utilizes its stored nitrogen source for cell wall formation and weight increase (Rahmad et al. 2014), leading to a decline in total protein content of sporomes.

Changes in protein molecular mass during sporome formation might also have biological implications. In primordia and early stages of sporome maturation, proteins were found to be mostly of 25–32 kDa. These proteins normally represent the ribosome inactivating proteins, known to possess antifungal, antiviral, immunomodulatory and antitumor/anti-proliferative activities (Erjavec et al. 2012, Ng 2004). In the mature sporomes proteins with higher molecular weight were determined which are assumed to be up-regulated during the sporome formation and maturation (Rahmad et al. 2014). Previous researches have showed that high molecular weight proteins belong to oxidoreductases enzymes (such as tyrosinase (42-55 kDa) and pyranose 2-oxidase (61-70 kDa) which play important roles in both aerobic and anaerobic metabolisms of mushrooms (Ng 2004). Consistent with this assumption, high molecular weight proteins of the tested *F. velutipes* in this study could have been present in the mature sporomes. However, proteins of Enoki mushroom (like any other mushroom) may be depolymerized during gastrointestinal digestion to very small peptides, making it essential to differentiate between nutritional and biomedical applications of proteins. Accordingly, proteins produced in any stage of fructification of wild Enoki may be directly used for biological activity, while the effectiveness of proteins for human nutrition depends on their composition of amino acids.

All the elements observed in the reference *F. velutipes* were also present in the wild strain without any significant difference. High concentrations of K, Ca and Mg along with low amounts of Na present in the tested mushrooms would make them reliable sources of nutrition for preventing hypertension, neuromuscular irritability, osteoporosis, type 2 diabetes, and Alzheimer (Larsson & Wolk 2007, Mallikarjuna et al. 2013, Moyer et al. 2013, Ozawa et al. 2012). In addition, functional foods such as mushrooms containing high amounts of Ca and protein likely possess high concentrations of P (Takeda et al. 2012). This fact may be confirmed in mushrooms by their ability to accumulate P in their sporomes (Kalač 2009) which make them natural remedies for patients with symptoms of low P (such as joint or bone pain, loss of appetite, irritability or

anxiety and fatigue). It is noteworthy that the concentrations of the elements found in *F. velutipes* in this study are comparable to some other fungi species such as *Pleurotus giganteus* (Phan et al. 2019). These findings may suggest the potential value of the newly cultivated wild Iranian Enoki to be considered as a nutritious food. Similarly, it has been shown that Iranian wild genotypes have significant genetic differences with commercial cultivars which cause in resistance of the sporomes against diseases, which resulted to obtain healthier products (Rokni et al. 2019)

The occurrence and distribution of different toxic components in wild mushrooms are mycological problems with environmental and toxicological impacts (Mallikarjuna et al. 2013). In the present work, heavy metals such as Cu, Fe, Zn and Mn were at levels lower than the provisional maximum tolerable intake which is 2.2, 28-30, 15.5 and 5.5 mg/day, respectively (Mallikarjuna et al. 2013). According to the established provisional tolerable weekly intake of heavy metals by FAO/WHO (Kalač 2010), it can be estimated that a weekly intake of 1000 g of fresh wild Enoki mushrooms (having dry matter content of 100 g) for an adult with 70 kg bodyweight would be permissible. Thus, our findings would confirm that wild Enoki mushrooms cultivated in the substrate formulated in this study may be considered as a non-toxic food source in terms of heavy metals. It should be noted that substrate composition of a mushroom also plays a key role in quantity and quality of elements found in the sporomes, in addition to the individual difference in metals uptake among mushroom species. Thus, the use of unpolluted substrates for adaptation of wild mushrooms would lead to low accumulation of the detrimental elements.

Conclusion

This study might be considered as the first step towards recognition of an Iranian wild Enoki mushroom as a safe, non-toxic functional food, adapted to cultivation in lignocellulosic substrate composed of 40% wheat straw +40% sawdust +18% wheat bran +1% lime +1% gypsum. This study may also suggest that the best time for consumption of the wild Enoki as a culinary mushroom appears to be around 3-5 days after appearance of primordia, where sporomes produce high amounts of protein with a various range of high and low molecular weights. Therefore, the findings of this study may strongly encourage further attempts in order to gain deep insight into proteome of wild *F. velutipes* and its amino acid composition of proteins during different developmental stages.

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