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Bioactive properties of *Agaricus bisporus* and *Terfezia claveryi* proteins hydrolyzed by gastrointestinal proteases

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ABSTRACT

The proteins of *Terfezia claveryi* and *Agaricus bisporus* were hydrolyzed by gastrointestinal enzymes. The degree of hydrolysis, type of enzymes and mushrooms, and blanching as pretreatment influenced the bioactivity properties of produced peptides. Some produced peptides from both mushrooms had more antioxidative and antimicrobial activity than their respective initial proteins ($P < .05$). The most effective samples were non-blanched intact protein of *A. bisporus* with absorbance of 0.305 ± 0.005 for reducing power and non-blanched and blanched pepsin-trypsin-α-chymotrypsin hydrolysates with 73.68 ± 1.79% and 77.77 ± 1.79% for DPPH scavenging activity, respectively. However, non-blanched Trypsin-α-chymotrypsin and non-blanched trypsin hydrolysates of *T. claveryi* were more effective for inhibition of linoleic acid oxidation with 85.85 ± 0.82% and chelating activity with 21.36 ± 0.84%, respectively. Besides, inhibition of growth of *Pseudomonas aeruginosa* and *Bacillus cereus*, as the most sensitive microorganisms, were 26.64 ± 0.79% by blanched trypsin hydrolysate of *A. bisporus* and 27.44 ± 0.62% and 27.00 ± 0.62% by the blanched trypsin and α-chymotrypsin hydrolysates of *T. claveryi*, respectively.

1. Introduction

Endogenous metabolic processes may cause the production of reactive oxygen species (ROS) that are able to damage cellular substances, eventually leading to the expansion of many diseases such as cardiovascular disease, cancer, diabetes (Mirzaei, Mirdamadi, Ehsani, Aminlari, & Hosseini, 2015; Ozturk et al., 2011). In addition, oxidation in food processing and storage is critical in the food industry as contributing factor to lipid and protein oxidation as well as to the formation of disagreeable color, texture and flavor (Nawar, 1996). Hence, the use of synthetic antioxidants in foods, including butylated hydroxyl toluene, butylated hydroxyl anisole, have been common. In spite of being more effective than their natural antioxidant counterpart, however, these antioxidants are suspected to be carcinogenic and liver damaging (Ozturk et al., 2011). As such, the discovery of new and natural antioxidants derived from foodstuff has recently received much attention (Mirzaei et al., 2015).

On the other hand, the bacterial resistance to common and synthetic antibiotic compounds and their serious side effects on human health are one of the most important problems, that have led to the recognition of the necessity in exploring alternative, safe and new antibiotics (Finch, 1998). Thus, the transition to using natural antimicrobial agents to replace current artificial antimicrobial ones in the food industry seems to be essential (Shahidi & Zhong, 2008).

Recently, proteins and biopeptides have been considered not only as nutraceuticals but also as functional and bioactive compounds (Shahidi & Zhong, 2008). Bioactivity of produced peptides on human health, which depends on the source of proteins, degree of hydrolysis (DH), type of proteases etc. (Salami et al., 2011), includes antimicrobial (Esmaeilpour, Ehsani, Aminlari, Shekarforoush, & Hosseini, 2016), antihypertensive (Mirzaei et al., 2015; Salami et al., 2011), antioxidant (Mirzaei et al., 2015; Salami et al., 2011), etc.

As regards protein sources of bioactive peptides, one of the promising foodstuffs in the reduction of oxidation and bacterial resistance, are mushrooms. Mushrooms are a functional food and a source of bioactive compounds (Lau, Abdullah, Suriza Shuib, & Aminudin, 2012; Ozturk et al., 2011). One of the main components of mushrooms is protein, thus making them a good source of bioactive peptides (Lau et al., 2012). To this end, one of the most consumed and economically important mushrooms is *Agaricus bisporus*, which has been utilized in traditional medicine (Liu, Jia, Kan, & Jin, 2013). Another mushroom that is considered as one of the oldest foodstuffs with nutritional value,

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comparable to fish and meat is truffle (Janakat, Al-Fakirs & Sallal, 2004). Desert truffles, like *Terfezia claveryi,* are socio-economically and medicinally important fungi grown in the Mediterranean, Middle Eastern and North African regions (Al-Laith, 2010; Janakat, Al-Fakhiri, & Sallal, 2005).

In the majority of previous research, the main compounds studied with regard to antioxidant and antimicrobial properties of mushrooms have been second metabolites and polysaccharides (Al-Laith, 2010; Ozturk et al., 2011; Vaz et al., 2011). Research on the proteins of mushrooms and their hydrolysates has, however, been limited. The main objective of this article was to review the antioxidant and antimicrobial properties of hydrolysates generated from the hydrolysis of the proteins of *A. bisporus* and *T. claveryi* via gastrointestinal enzymes, and to compare the DH with these bioactive properties of protein extracts and their hydrolysates.

2. Materials and methods

2.1. Materials

2,2-diphenyl-1-picryhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-2,4-triazine-p-p'-disulfonic acid mono Sodium salt hydrate (FerroZine), disodium ethylene-diamine-tetra-acetate (Na_2EDTA), 2,6- Di-tert-butyl-4-methylphenol,6-Di-tert-butyl-p-cresol (BHT), linoleic acid, nisin, O-phthadialdedehyde, α-Chymotrypsin (EC 3.4.21.1, containing ≥40 units/mg protein, Bovine Pancreas), Trypsin (EC 3.4.21.4; containing 1000–2000 BAEE units/mg protein, porcine pancreas) and pepsin (EC 3.4.23.1, 800–2500 units/mg protein, porcine gastric mucosa) were purchased from Sigma-Aldrich Chemie GmbH, Germany. Other chemicals were purchased from Merck, Germany. All chemicals were of analytical grade.

Cultures of *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19114, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027 were from Persian-type culture collection of the Iranian Research Organization for Science and Technology (IROST).

2.2. Protein extraction

Fruiting bodies of *A. bisporus* were purchased from a local market. However, *T. claveryi* as wild mushroom was collected from Shiraz in the southern part of Iran. The fruiting bodies of each species were washed, chopped, homogenized in Tris-HCl buffer solution, pH 7.3, at a ratio of 1:2 (w/v) with a blender (Houshdar Tehrani, Fakhrehoseini, Kamali Nejad, Mehregan & Hakemi-Vala, 2012). Then the mixtures were centrifuged to remove unwanted debris. Using ammonium sulphate 90% saturation, Proteins were precipitated out from the mushroom extracts. The pellet protein was collected by re-centrifugation (10 000 rpm, 30 min). Then they were dialyzed against water at 4 °C for 48 h (Lau, 2013).

2.3. Protein assay

Protein was measured by the Bradford (1976) method using bovine serum albumin (BSA) as a standard to prepare the calibration curve.

2.4. In-vitro proteolysis of mushroom proteins

Lyophilized samples were dissolved in Tris-HCl buffer, pH 7.8 for trypsin and α-chymotrypsin and HCl-KCl buffer, pH 2, for pepsin, to obtain a final concentration of 4 mg/ml. Aliquots of trypsin, α-chymotrypsin, pepsin, mix of trypsin and α-chymotrypsin at concentration of 4 mg/ml were added to each protein sample with enzyme/substrate ratio of 1:10 and then incubated at 37 °C for 5 h (Mirzaei et al., 2015) in Thermomixer (Eppendorf, Germany). To determine the effect of gastrointestinal enzymes on substrates without interfering with endogenous proteases, mushroom extracts were blanched, 75 °C for 15 min, before precipitating protein. Hydrolysis by the pepsin-trypsin-α-chymotrypsin mixture was done at first for 2.5 h by pepsin and subsequently by trypsin-α-chymotrypsin mixture for another 2.5 h (Lau, 2013).

2.5. Determination of hydrolysis degree

To measure the degree of hydrolysis (DH) of treated proteins following the action of hydrolytic enzymes, a sensitive and easy method, O-phthaldialdehyde (OPA) based spectroscopic assay, was applied (Mirzaei et al., 2015). The solution absorbance at 340 nm was determined using a NanoDrop® ND-1000 Spectrophotometer (USA). L-serine was used to produce standard curve (Mirzaei et al., 2015; Nielsen, Petersen, & Dambmann, 2001).

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The SDS-PAGE analysis was done for protein and its hydrolysates, according to the modified method of Laemmli (Laemmli, 1970). Electrophoresis was conducted at a constant current of 80 V to move the proteins through the stacking gel (5 g polyacrylamide/100 ml solution), followed by 140 V to resolve the proteins in the separating gel (12.5 g polyacrylamide/100 ml solution). Then, the gel was fixed with a solution including of 45 ml/100 ml H_2O methanol and 1 ml/100 ml H_2O acetic acid for 30 min consequently, the protein bands were stained by coomassie brilliant blue staining method.

2.7. Antioxidant activity assay

2.7.1. DPPH radical scavenging capacity

The ability of proteins and hydrolysates of each mushroom to scavenge the free radical DPPH was evaluated according to the procedures described by Kalogeropoulos, Yanni, Koutrotsios, and Aloupi (2013); Liu et al. (2013); Mirzaei et al. (2015), with some modifications. Briefly, 0.25 mg/ml of protein or hydrolysates (25 µl) was added in 975 µl DPPH (2 mg/100 ml methanol) and vortexed. The reduction of DPPH was determined by measuring the absorbance at 517 nm by NanoDrop® Spectrophotometer, after the mixture was incubated for 30 min in darkness. The DPPH radical scavenging activity was calculated by scavenging activity (%) = $(1-(A_1-A_2)/A_0) \times 100$, Where A_0 is the absorbance of the control, A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample with methanol instead of DPPH.

2.7.1.1. Inhibition of lipid peroxidation. Lipid peroxidation inhibition was measured in a linoleic acid model system according to the method of Osawa and Namiki (1985), with some modifications. 0.25 mg/ml of proteins or hydrolysates was dissolved in 500 µl of Tris-HCl buffer (pH 7) and then added into a mixture containing 6.5 µl linoleic acid and 500 µl of ethanol (99.5 ml/100 ml H_2O) in Eppendorf tubes. The final volume was adjusted to 1.25 ml with distilled water. The resultant mixture was incubated at 40 ± 1 °C in the dark. Linoleic acid oxidation was determined at 24 h intervals by measuring the ferric thiocyanate values according to the method of Chandrasekara and Shahidi (2012). The percentage of inhibition of linoleic acid peroxidation was calculated by Linoleic acid peroxidation percentage = $(1-(A_s/A_c)) \times 100$, Where A_C is the absorbance of the control (buffer instead of sample), A_S is the absorbance of the sample.

2.7.1.2. Metal ion chelating agent assay. The chelating activity on Fe^{+2} was determined, using the method of Memarpoor-Yazdi, Mahaki, and Zare-Zardini (2013).

2.7.1.3. Reducing power assay. The reducing power was measured according to Memarpoor-Yazdi et al. (2013).