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Bioactivity of Peptides from Two Mushrooms

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***Abstract*—**Mushrooms or macro-fungi, as an important superfood, contain many bioactive compounds, particularly bio-peptides. In this research, mushroom proteins were extracted by buffer or buffer plus salt (0.15 M), along with ultrasound bath to extract the intercellular protein. As a result, the highest amount of proteins in mushrooms were categorized into albumin. Proteins were also hydrolyzed and changed into peptides through endogenous and exogenous proteases, including gastrointestinal enzymes. The potency of endogenous proteases was also higher in *Agaricus bisporus* than *Terfezia claveryi*, as their activity ended at 75 for 15 min. The blanching process, endogenous enzymes, the mixture of gastrointestinal enzymes (pepsin-trypsin-α-chymotrypsin or trypsin- α-chymotrypsin) produced the different antioxidant and antibacterial hydrolysates. The peptide fractions produced with different cut-off ultrafilters also had various levels of radical scavenging, lipid peroxidation inhibition, and antibacterial activities. The bio-peptides with the superior bio-activities (less than 3 kD of *T. claveryi*) were resistance to various environmental conditions (pH and temperatures). Therefore, they are good options to be added in nutraceutical and pharmaceutical preparations or functional foods, even during processing.

***Keywords*—**Bio-peptides, mushrooms, gastrointestinal enzymes, bioactivities.

I. Introduction

TO date, extensive research has been conducted on bioactive peptides derived from animals and plants. However, there has been a limited number of studies focusing on bioactive peptides found in edible mushrooms. It is worth noting that there are currently over 12,000 species of mushrooms, with approximately 2,000 of them being edible. Out of these, around 200 species are collected for consumption or as ingredients in pharmaceutical products [1]. This indicates that edible mushrooms possess a vast and largely unexplored reservoir of resources. The diverse range of bioactive compounds present in different mushroom species can be utilized in the development of various functional foods. Edible mushrooms are considered unique functional foods and nutritional supplements due to their abundance of bioactive metabolites, such as proteins, polysaccharides, enzymes (e.g., superoxide dismutase), dietary fiber, and numerous other biomolecules [2]. In comparison to peptides derived from animals, mushroom bio-peptides serve as excellent raw materials for the production of healthy and functional products. They possess numerous advantages such as abundant resources, safety, affordability, and the ability to be produced on a large scale, resulting in valuable nutrients for maintaining health and preventing diseases. The complete absorption of mushroom bio-peptides by the intestine allows for direct partial benefits in the digestive tract without adding any functional burden to the gastrointestinal tract [3]. Furthermore, mushroom bio-peptides can enter the circulatory system in their intact form, exerting beneficial physiological effects. They exhibit high organizational affinity [4], low toxicity, and remarkable stability [5]. Additionally, mushroom bio-peptides offer a wide range of nutritional, functional, and biological properties. Endogenous mushroom bioactive peptides have the capacity to demonstrate antioxidant and anti-inflammatory effects through the regulation of antioxidant pathways and activation of immune cells [6]. Moreover, these peptides may possess analgesic or opioid properties, thereby augmenting memory and cognitive function in nerve cells [7]. Conversely, exogenous mushroom bio-peptides can be harnessed as functional foods and medicines to alleviate inflammation, hyperglycemia, hypertension, high cholesterol, and other degenerative diseases [8]. As a result of variations in growing environments and mushroom species, the extraction methods employed for mushroom bio-peptides are diverse, leading to disparities in their structures and bioactivities [9]. To enhance extraction efficiency, the utilization of innovative extraction techniques could prove advantageous. Ultrasound waves are categorized into three types based on their frequency: low (20–100 kHz), high (100–1000 kHz), and diagnostic (1–500 MHz) frequency ultrasonic waves [10]. Low-intensity waves are commonly used in food processing and bioactive extraction [11]. The utilization of ultrasound in hydrolysis processes brings about changes in protein conformation by influencing hydrogen bonds and hydrophobic interactions. These waves cause acoustic cavitation, which leads to changes in temperatures and pressures, enabling plant cells to release their intercellular compounds. Several factors, including treatment time, pH, temperature, and ultrasound intensity, play a crucial role in determining the extraction yield [12]. However, the solubility can be enhanced through enzyme-assisted or ultrasound-assisted protein extraction. Furthermore, ultrasound technology reduces the size of protein aggregates, increases protein surface hydrophobicity and flexibility, and promotes the formation of short-chain peptides with low molecular weight. Consequently, it has the potential to enhance the functional properties as well as the bioactive properties (including antioxidant and antihypertensive effects) of protein hydrolysates and peptides [11]. The application of ultrasound as a pretreatment method improves the conversion rate of protein and reduces the hydrolysis time [13].

There are essentially two main methods for preparing

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mushroom bio-peptides. The first method involves directly extracting endogenous bio-peptides from mushrooms. These endogenous bio-peptides can be obtained from fresh fruiting bodies, dried powders, or fermented powders [6]. On the other hand, the second approach utilizes proteolytic reactions of exogenous enzymes to release peptide fragments from mushroom proteins. These proteins are isolated from edible mushrooms or their mycelia and are indirectly subjected to hydrolysis, for instance, through the use of gastrointestinal enzymes, plant proteases, etc. This process liberates potent peptides from intact proteins [9], [14], [15]. In fact, enzymatic hydrolysis has proven effective in obtaining bio-peptides with diverse biological activities, including anti-oxidative, antibacterial, anti-inflammatory, anti-aging, anti-tumor, and anti-diabetic properties [6], [14].

Ultrafiltration, hydrophilic interaction chromatography, fast protein liquid chromatography, ion exchange chromatography, size exclusion chromatography, gel filtration chromatography, and high-performance liquid chromatography are commonly employed methods for purifying substances after extraction and conducting preliminary bioactivity screening [6], [15].

As a result of distinct raw materials and preparation processes, mushroom bio-peptides may possess diverse targeting functions in terms of antioxidant activities. The antioxidant mechanisms of these bio-peptides can be classified into those involving the regulation of reactive oxygen species (ROS) production, antioxidant enzyme activities, and antioxidant pathways. Mushroom bio-peptides have the ability to scavenge free radicals by providing protons, electrons, and chelating metal ions, thereby regulating ROS production [16].

Infections can be effectively treated with antibiotics. However, the misuse and overuse of antibiotics have led to the emergence of antibiotic resistance [17]. As an alternative to antibiotics and conventional drugs, natural bioactive peptides have gained attention due to their high efficacy, stability, and low toxicity [18]. Antimicrobial activities of mushroom bio-peptides have been extensively studied, and numerous antimicrobial peptides have been extracted and purified from edible mushrooms [14], [15], [19]. In many cases, the mushroom bio-peptides remain inactive within their parent proteins and only become potent peptides upon hydrolysis. Enzymatic hydrolysis of low molecular weight hydrolysates and peptides often results in enhanced biological activities compared to the original proteins. It should be emphasized that peptides with higher levels of hydrophobic amino acid residues and low molecular weight may exhibit improved performance [6].

The aim of the article is to investigate the stability, anti-oxidative and antibacterial properties of exogenous and endogenous bioactive peptides extracted from two edible mushrooms (*Agaricus bisporus* and *Terfezia claveryi*) through applying ultrasound technique and gastrointestinal enzymes.

II. Methods and Materials

A. Materials

A. bisporus fruiting bodies were acquired from a nearby market, while *T. claveryi*, a wild mushroom, was gathered from Shiraz in the southern region of Iran. All chemical substances for extraction and assessment of the anti-oxidative and antibacterial properties were supplied from Merck (Germany) and Sigma-Aldrich (USA) companies as well.

B. Protein Extraction

The fruiting bodies of every individual species underwent a thorough washing process, followed by chopping and homogenization in either the Tris-HCl buffer solution (pH of 7.3) or the Tris-HCl buffer (pH 7.3) plus NaCl (0.15 M). This was done at a ratio of 1:2 (w/v) using a blender. Afterwards, they left in a bath Sonicator filled with water and ice, operating at a power of 320 W, a frequency of 35 kHz for 10 minutes. Subsequently, it was put into a shaker incubator (150 rpm and 4 °C for 1-2 hours). To eliminate any sediments and waste substances, it underwent centrifugation using a refrigerated centrifuge at 10000 rpm for 30 minutes [14].

C. Blanching

In order to eliminate any potential proteolytic enzymes and anti-trypsin compounds in two Macro-fungi, two different temperatures were applied: 60 °C for 30 minutes and 75 °C for 15 minutes. The 60 ml aqueous extracts from both mushrooms were then blanched at mentioned temperatures [20].

D. Precipitation of Protein with Sulphate Ammonium

Proteins were precipitated from the non-blanched and blanched mushroom extracts using ammonium Sulphate at 90% saturation. The protein pellet was obtained through re-centrifugation at 10,000 rpm for 30 minutes. Subsequently, the proteins were dialyzed against water at 4 °C for 48 hours [14].

E. Protein Assay

The protein content was determined using the Bradford [21] technique with some modification through using a Nano-Drop Spectrophotometer [14], [15].

F. In vitro Gastrointestinal Proteolysis

The buffers used for proteolytic hydrolysis were Tris-HCl buffer (pH 7.8) for trypsin and α-chymotrypsin, and HCl-KCl buffer (pH of 2) for pepsin. Protein, pepsin, the mixture of trypsin and α-chymotrypsin were adjusted to 4 mg/ml each. Aliquots of pepsin, and subsequently a mixture of trypsin and α-chymotrypsin were added to each protein sample with an enzyme/substrate ratio of 1:10. The samples were then incubated at 37 °C for 5 hours in a Thermomixer (Eppendorf, Germany), as the hydrolysis process was carried out with pepsin for 2.5 hours, followed by the trypsin-α-chymotrypsin mixture for another 2.5 hours [14].

G. Determination of Hydrolysis Degree for Endogenous and Exogenous Enzymes

The O-phthaldialdehyde (OPA) based on spectroscopic assay was employed to assess the degree of hydrolysis (DH) by intrinsic and gastrointestinal enzymes [14].

H. Purification of Bioactive Peptide Using the Ultra-filtration Method

The hydrolysates underwent fractionation via ultrafiltration membranes, which had different molecular weight cut-offs of 30, 10, 5, and 3-kDa. This process was carried out using 20 ml ultrafiltration centrifugal concentrators manufactured by Sartorius, Germany.

I. Assay of Antioxidant Activity

1. DPPH Radical Scavenging Capacity

This evaluation was carried out according to Farzaneh et al. [14]. Briefly, 25 µl of protein or hydrolysates (0.25 mg/ml) were added to 975 µl of DPPH solution (2 mg/100 ml methanol) and mixed thoroughly. After incubation in darkness for 30 minutes, the absorbance at 517 nm was measured using a NanoDrop® Spectrophotometer to determine the reduction of DPPH. The DPPH radical scavenging activity was calculated using the formula: scavenging activity (%) = (1-(A1-A2)/A0) × 100, where A0 represents the absorbance of the control, A1 represents the absorbance of the sample, and A2 represents the absorbance of the sample with methanol instead of DPPH. EC_{50} of the superior peptide fractions of *T. claveryi* and *A. bisporus* hydrolysates was also determined.

2. Inhibition of Lipid Peroxidation

Protein, hydrolysates, and peptide fractions were dissolved in 500 µl of Tris-HCl buffer (pH 7) and subsequently introduced into a mixture comprising 6.5 µl of 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 using distilled water. The resulting mixture was then incubated at 40 ± 1 °C in the absence of light. The oxidation of linoleic acid was assessed at 24-hour intervals for 5 days by measuring the ferric thiocyanate values. The percentage of inhibition of linoleic acid peroxidation was calculated using the formula: Linoleic acid peroxidation percentage = (1 - (As/Ac)) × 100, where AC represents the absorbance of the control (buffer instead of sample) and AS represents the absorbance of the sample [14], [15].

J. Antibacterial Activity

The antimicrobial activity of proteins and their hydrolysates was assessed using the 96-well microplate-based broth dilution method, following the protocols established by Farzaneh et al. [14], [15] at a concentration of 0.25 mg/ml. The initial bacterial population was adjusted to 0.5 McFarland using optical density. For each plate setup, 200 µl of Moller-Hinton Broth, 6 µl of different samples, and 10 µl of bacterial suspensions were added to each well. The microplate was then incubated at 37 °C for 24 hours. The extent of bacterial growth was measured at 630 nm using the ELX800 Microplate Reader (Bio-Tek Instrument, Inc., USA). The inhibitory concentration of the samples was determined as a percentage of OD= (ODw-(ODs-ODc)/ODw) × 100, where ODw represents the absorbance of the bacterial growth control, ODs represents the absorbance of bacterial suspensions containing varying amounts of mushroom proteins and their hydrolysates, and ODc represents the absorbance of the well containing the intended sample without any bacteria added.

MIC and MBC of the best peptide fractions were measured to identify bacteriostatic and bactericide concentrations, respectively. As 190 µl Moller-Hinton Broth, 50 µl peptide fractions of less than 3 kD (0.125, 0.185, 0.5, 1. 1.16 mg/ml), and 10 µl of each bacterial culture (1×10^6CFU/ml) were transferred to a 96-well microplate and incubated overnight at 37 °C [15].

K. The Effect of Environmental Conditions on the Best Antioxidant and Antibacterial Peptides Obtained by Trypsin-Chymotrypsin Enzymes

To determine the stability of antioxidant activity in the selected peptides, peptides were subjected to various pH (4, 6, and 7) and temperatures (50, 70, and 90 ºC) for 30 minutes. The control was peptide fraction stored at 4 ºC [22], [23].

L. Statistical Assay

The experiments were conducted in triplicate or more. In order to identify any significant variations ($P < .05$) among the averages, ANOVA and Duncan's multiple range tests were carried out. The statistical analyses were conducted using SPSS-24.

III. Results and Discussion

A. Protein Quantity

In order to determine the protein content in 100 mg of dry matter, the macro-fungi's extract and protein pellet were subjected to freezing drying method (-55 °C and 0.0012 bar pressure) for 24 hours. The quantity of protein is shown in Table I.

TABLE I
THE PROTEIN AMOUNT IN TWO MUSHROOMS

Samples	Protein concentration (mg/100 mg dw)	
	Agaricus bisporus	*Terfezia claveryi*
Extract with buffer	3.84 ± 1.12^{Ab}	$12.22 \pm 0.^{94Aa}$
Extract with buffer + salt	2.38 ± 1.01^{B}	10.02 ± 1.28^{B}
Protein pellet (buffer)	6.24 ± 0.02	14.10 ± 0.73
Protein pellet (buffer + salt)	5.46 ± 0.02	14.68 ± 0.08
Blanched protein (60 °C, 30 min) (buffer)	1.98 ± 0.014	6.08 ± 0.34
Blanched Protein (60 °C, 30 min) (buffer + salt)	1.98 ± 0.10	5.08 ± 0.33
Blanched Protein (75 °C, 15 min) (buffer)	0.46 ± 0.10	0.50 ± 0.02
Blanched Protein (75 °C, 15 min) (buffer + salt)	0.44 ± 0.05	0.45 ± 0.04

Each value is the mean ± standard deviation of three replicates. In each column, different capital superscript letters mean significant differences ($P < 0.05$). Also, in each raw, the little superscript letters mean significant differences ($P < 0.05$).

The amount of soluble protein in *T. claveryi* was higher that of *A. bisporus*. Generally, the buffer extraction method yielded a higher amount of soluble protein in both macro-fungi compared to the salt-buffer extraction method, and this difference was statistically significant ($P < 0.05$). This finding aligns with the study of Petrovska [24], which indicated that albumins and then globulins are the predominant protein components in macro-fungi, with albumin being soluble in

water and globulin being soluble in water plus salt. The average abundance of protein components of the studied macro-fungi as a percentage of the total protein includes albumins (24.78%), globulins (12.07%), glutelins (11.46%), and glutelin-like substances (7.43%). %), prolamins (5.67%) and prolamin-like substances (5.31%) [24]. According to different treatments, it was found that the best extraction method was with salt-free buffer. The amount of soluble protein in the blanched samples decreased significantly, due to the protein denaturation resulting from heating [20].

B. Blanching Effect on Endogenous Proteases and Anti-Trypsin Compounds and Hydrolysis Degree

In order to determine the activity of endogenous enzymes in each macro-fungus, a control sample without the addition of digestive enzymes was utilized at pH 2 and 7.8. However, it was observed that the endogenous enzymes in both macro-fungi were not active at pH 2. To assess the heat effect on the proteolytic enzymes and anti-trypsin compounds in macro-fungi and the biological activity of two macro-fungi proteins, two different temperatures were employed: 60 °C for 30 minutes and 75 °C for 15 minutes. Based on the relative activity of protease enzymes and anti-trypsin effect at 60 °C, a temperature of 75 °C was chosen for blanching the mushroom extracts. The results indicated that the protease enzymes in *A. bisporus* exhibited higher activity compared to *T. claveryi*. Furthermore, the study revealed that the activity of endogenous proteases in *A. bisporus* ceased after 3 hours (DH = 20.52 ± 1.34%), whereas *T. claveryi* displayed lower protease activity and ceased within the first half hour (DH = 7.98 ± 1.06%). It was found that *A. bisporus* proteases underwent autolysis during purification, so its activity decreased after one hour at 35 °C in pH 6.5-10 where the enzyme was highly active [25]. The hydrolysis degree for the all samples has been shown in Table II.

C. Anti-Oxidative Activity Assay

As shown in Table III, the best result in this analysis for *A. bisporus* was pepsin-trypsin-chymotrypsin enzyme hydrolysate and then trypsin-chymotrypsin enzyme hydrolysate. For *T. claveryi*, the non-blanched hydrolysate of trypsin-chymotrypsin enzyme mixture had the best free radical inhibition. Also, at the concentration of 0.5 mg/ml, the amount of DPPH radical scavenging by hydrolysates of *A. bisporus* was more than 90%. The increase in free radical scavenging activity after protein hydrolysis can result from the solubility of smaller peptides, the formation of active peptides from different sources and the release of free amino acids [6].

The amount of lipid peroxidation compared to synthetic (butyl hydroxyl-toluene) and natural (ascorbic acid) antioxidants was measured during 5 days. The hydrolysates of both macro-fungi, especially the non-blanched type, showed more inhibitory activity than the positive control. In the case of *A. bisporus*, the non-blanched trypsin-chymotrypsin hydrolysate was the best example in preventing the peroxidation of linoleic acid, which were comparable to ascorbic acid and BHT. Also, the best sample for *T. claveryi* macro-fungi was trypsin-chymotrypsin hydrolysate as well. Enzymatic hydrolysis creates smaller peptide chains with hydrophobic-hydrophilic balance and greater solubility, which can rapidly organize at the interface of lipid droplets. The formed interfacial layer protects lipid molecules from aqueous phase radicals such as metal ions [26].

TABLE II
HYDROLYSIS DEGREE (DH) OF PROTEINS OF TWO MUSHROOMS THROUGH ENDOGENOUS AND EXOGENOUS ENZYMES

Samples		Hydrolysis	DH (%)
A	B	Endogenous enzyme at pH 7.8	0k
		Endogenous enzyme at pH 2	0k
		Pepsin-trypsin-chymotrypsin enzymes	26.53 ± 0.81d
		Trypsin-chymotrypsin enzymes	19.67 ± 0.81fg
	NB	Endogenous enzyme at pH 7.8	20.52 ± 1.34f
		Endogenous enzyme at pH 2	0k
		Pepsin-trypsin-chymotrypsin enzymes	23.45 ± 1.34e
		Trypsin-chymotrypsin enzymes	43.87 ± 1.34c
T	B	Endogenous enzyme at pH 7.8	0k
		Endogenous enzyme at pH 2	0k
		Pepsin-trypsin-chymotrypsin enzymes	18.70 ± 0.29g
		Trypsin-chymotrypsin enzymes	11.89 ± 0.29i
	NB	Endogenous enzyme at pH 7.8	7.98 ± 1.06j
		Endogenous enzyme at pH 2	0k
		Pepsin-trypsin-chymotrypsin enzymes	45.45 ± 1.06b
		Trypsin-chymotrypsin enzymes	52.87 ± 10.6a

Each value is the mean ± standard deviation of three replicates. In each column, different capital superscript letters mean significant differences ($P < 0.05$). NB: Non-blanched; B: Blanched; *A: A. bisporus; T: T. claveryi*.

TABLE III
ANTIOXIDANT ACTIVITY OF PROTEIN AND HYDROLYSATES OF TWO MUSHROOMS

Samples		Hydrolysis (0.25 mg/ml)	DPPH radical scavenging (%)	Inhibition of linoleic oxidation after 5 days (%)
A	B	With no enzyme	28.66 ± 1.79	67.58 ± 0.81
		Pepsin-trypsin-chymotrypsin enzymes	77.77 ± 1.79	-
		Trypsin-chymotrypsin enzymes	53.34 ± 1.79	70.09 ± 0.81
	NB	Endogenous enzyme at pH 7.8	23.39 ± 1.79	52.10 ± 0.81
		Pepsin-trypsin-chymotrypsin enzymes	73.68 ± 1.79	-
		Trypsin-chymotrypsin enzymes	52.63 ± 1.79	75.21 ± 0.81
T	B	With no enzyme	30.41 ± 2.01	74.85 ± 0.82
		Pepsin-trypsin-chymotrypsin enzymes	-	-
		Trypsin-chymotrypsin enzymes	28.65 ± 2.01	82.28 ± 0.82
	NB	Endogenous enzyme at pH 7.8	9.18 ± 2.01	67.84 ± 0.82
		Pepsin-trypsin-chymotrypsin enzymes	14.62 ± 2.01	63.44 ± 0.82
		Trypsin-chymotrypsin enzymes	51.50 ± 2.01	85.85 ± 0.82

Each value is the mean ± standard deviation of three replicates. In each column, different capital superscript letters mean significant differences ($P < 0.05$).

(-) No measure because of insolubility of pepsin-trypsin-chymotrypsin hydrolysates of *T.claveryi.* B: Blanched; NB: Non-blanched; *A: A. bisporus; T: T. claveryi.*

D. Antibacterial Activity Assay

The study revealed that certain hydrolysates exhibited greater antimicrobial activity compared to their constituent proteins. Among the macro-fungi tested, the trypsin-

chymotrypsin hydrolysates were found to be the most effective against the majority of microorganisms (refer to Table IV). Previous research has indicated that excessive heating of food can cause protein degradation to an extent where not only are peptides not released through enzymatic digestion, but they also lose their biological activity [27]. In this particular case, the blanched hydrolysate of *A. bisporus* and *T. claveryi* demonstrated no efficacy against *S. aureus* and *L. monocitogenous*, respectively. Conversely, a report suggests that heating can generate resistant peptide bands that exhibit different properties compared to their natural counterparts [28]. For instance, blanched protein and hydrolysates of *A. bisporus* and *T. claveryi* had more impact on *Pseudomonas aeruginosa* and *B. cereus* than those of the non-blanched. On the other hand, some hydrolysates of *A. bisporus* and *T. claveryi* roughly exhibited more antibacterial activity on the tested microorganisms than the samples with no enzyme.

TABLE IV
THE EFFECT OF PROTEIN AND HYDROLYSATES OF TWO MUSHROOMS ON THE TESTED BACTERIA

Samples 0.25 mg/ml		Hydrolysis	*Escherichia coli*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	*Bacillus cereus*
A	B	1	9.40 ± 1.30c	0e	10.85 ± 1.06c	20.5 ± 0.79a	0f
		2	8.87 ± 1.30c	0e	0f	15.22 ± 0.79c	0f
		3	6.79 ± 1.30d	0e	19.54 ± 1.06a	17.89 ± 0.79b	5.92 ± 0.70e
	NB	4	12.07 ± 1.30b	0e	8.09 ± 1.06d	6.47 ± 0.79ef	0f
		2	0f	0e	20.61 ± 1.06a	13.83 ± 0.79d	0f
		3	14.01 ± 1.30a	10.58 ± 0.58d	0f	13.76 ± 0.79d	14.75 ± 0.70b
T	B	1	6.08 ± 0.88d	17.63 ± 0.95b	0f	6.77 ± 0.64ef	19.22 ± 0.62a
		2	11.91 ± 0.88b	0e	0f	7.14 ± 0.64e	12.76 ± 0.62d
		3	11.34 ± 0.88b	20.43 ± 0.95a	0f	0h	18.72 ± 0.62a
	NB	4	6.20 ± 0.88d	16.63 ± 0.95b	3.41 ± 0.93e	4.03 ± 0.64g	0f
		2	0f	11.77 ± 0.95c	17.66 ± 0.93b	5.66 ± 0.64f	13.37 ± 0.62cd
		3	3.00 ± 0.88e	21.14 ± 0.95a	8.92 ± 0.93d	17.06 ± 0.64b	13.88 ± 0.62c

Each value is the mean ± standard deviation of three replicates. In each column, different capital superscript letters mean significant differences ($P < 0.05$). B: Blanched; NB: Non-Blanched; A: *A. bisporus*; T: *T. claveryi; 1:* With no enzyme; 2: Pepsin-trypsin-chymotrypsin enzymes; 3: Trypsin-chymotrypsin enzymes; 4: Endogenous enzyme at pH 7.8

TABLE V
ANTIOXIDANT ACTIVITY OF PEPTIDE FRACTIONS IN TWO MUSHROOMS

Samples 0.125 mg/ml	Size of ultrafilter (kDa)	DPPH radical scavenging percentage	Lipid peroxidation inhibition (%) after 5 days
Agaricus bisporus	More than 30	21.84 ± 1.50f	86.60 ± 1.38a
	10-30	37.48 ± 1.50d	93.88 ± 1.38a
	5-10	56.29 ± 1.50c	80.71 ± 1.38a
	3-5	14.44 ± 1.50g	87.76 ± 1.38a
	Less than 3	27.15 ± 1.50e	86.59 ± 1.38a
Terfezia claveryi	More than 30	5.93 ± 1.50h	5.93 ± 1.38b
	10-30	25.56 ± 1.50e	86.37 ± 1.38a
	5-10	62.81 ± 1.50b	90.35 ± 1.38a
	3-5	94.73 ± 1.50a	95.43 ± 1.38a
	Less than 3	93.33 ± 1.50a	94.42 ± 1.38a

Each value is the mean ± standard deviation of three replicates. In each column, different capital superscript letters mean significant differences ($P < 0.05$).

E. Frication of Trypsin-Chymotrypsin Hydrolysates of Two Mushrooms Using Ultrafilters

The hydrolysates obtained through the action of trypsin-chymotrypsin enzymes exhibited superior antioxidant and antibacterial properties compared to other samples. The study revealed that the fractions of 3-5 and less than 3 kDa of trypsin-chymotrypsin hydrolysate from *T. claveryi* demonstrated the highest DPPH radical scavenging percentage, exceeding 90% at a concentration of 0.125 mg/ml (Table V). Peptide molecular weight plays a crucial role in determining biological activity. Typically, antioxidant peptides containing 2-20 amino acids exhibit greater potential than their parent protein molecules, which usually consist of 50-200 amino acids or more [6]. The EC_{50} value for the superior fraction (5-10 kDa) of *A. bisporus* was 0.125 mg/ml for antioxidant activity, whereas the values for the fractions of 3-5 and less than 3 kDa from *T. claveryi* were 0.038 and 0.024 mg/ml, respectively.

The inhibition of Linoleic acid peroxidation in *T. claveryi* fractions was more than those of *A. bisporus*, and fraction of 3-5 and less than 3 kDa of *T.claveryi* had the most activity than larger fractions. Lower molecular weight peptides are believed to inhibit more efficiently lipid peroxidation than their parental proteins and peptides with larger sizes [29]. The antioxidant activity of two mushroom peptide fractions is shown in Table V.

The environmental factors (pH and temperatures) had no significant effect on the values of DPPH radical scavenging activity and lipid peroxidation inhibition of *T. claveryi* peptide fractions (3-5 and less than 3 kDa) ($p < 0.05$). Proteins of macro-fungi have been proven to be unique and different from animal, plant and microbial proteins, and the majority of them are resistant to heat and pH [1]. As a result, the peptides produced from the hydrolysis of these proteins can remain active against environmental factors such as temperature and pH changes.

The effect of *T. claveryi* peptide fractions was more than that of *A. bisporus* on some tested bacteria (no shown data about *A. bisporus*). In different researches, antimicrobial peptides with a variable number of amino acids have been introduced, such that oligopeptides with a length of 5 to more than 100 amino acids

[30] or between 10 and 50 amino acids with a molecular weight of 2-9 kDa [31]. In this study, lower size of peptides separated from *T. claveryi* had more antibacterial effect on *P. aeruginosa* and *B. cereus,* while *S. aureus* and *E. coli* were more influenced by peptides with larger molecular weight (Table VI).

In MBC/MIC experiments, the 3-kDa peptide fraction of *T. claveryi* was used. Nisin (positive control) was prepared and tested in the amount of 1000 and 500 Au/ml. *P. aeruginosa* and *S. aureus* grew at 500 Au/ml. At 1000 Au/ml, only *P. aeruginosa* grew. In the highest concentration, 3-kDa peptide fraction of *T. claveryi* was considered a bactericide compound against *B. cereus* and a bacteriostatic compound for *S. aureus* and *L. monocytogenes* (Table VI).

TABLE VI
ANTIBACTERIAL ACTIVITY OF DIFFERENT PEPTIDE FRACTIONS OF *T. CLAVERYI* AND MBC/MIC OF THE BEST FRACTION

Fractions (kD)	B	S	L	P	E
1	-	-	-	18.34 ± 0.79c	31.25 ± 0.81a
2	6.94 ± 1.18c	19.89 ± 1.55a	-	27.62 ± 0.79b	5.06 ± 0.81b
3	7.94 ± 1.18c	5.36 ± 1.55b	-	26.24 ± 0.79b	-
4	47.52 ± 1.18b	-	-	27.84 ± 0.79b	-
5	90.84 ± 1.18a	-	-	40.30 ± 0.79a	-
MIC/MBC of less than 3 (mg/ml)	B	S	L	P	E
MIC	0.5	1.16	1.16	More than 1.16	More than 1.16
MBC	1	-	-	-	-

Each value is the mean ± standard deviation of three replicates. In each column, different capital superscript letters mean significant differences ($P < 0.05$).
1: More than 30; 2: 10-30; 3: 5-10; 4: 3-5; 5: Less than 3; B: *Bacillus cereus; S: Staphylococcus aureus; L: Listeria monocytogenes; P: Pseudomonas aeruginosa; E: E. Coli.*

The environmental factors (pH and temperatures) had no significant effect on antibacterial properties of peptide fraction of less than 3 kDa. The interaction of pH and temperatures had no significant effect on this property as well. The reason of this insignificant effect could be associated with the stability of protein and the resultant peptides from *T. claveryi* against pH and temperature [1].

IV. CONCLUSION

The bioactive peptide derived from mushrooms, through the action of both endogenous and exogenous enzymes (specifically gastrointestinal enzymes), can be extracted using innovative techniques such as ultrasound. These bioactive peptides exhibit unique properties and can be effectively utilized throughout the entire production process of functional foods. This is primarily due to the remarkable stability of mushroom bio-peptides across a wide range of pH levels and temperatures. It is evident that mushroom protein and hydrolysates can be regarded as a valuable bioactive source, offering diverse health advantages.

REFERENCES

[1] J. Erjavec, J. Kos, M. Ravnikar, T. Dreo, J. Sabotic, "Proteins of higher fungi--from forest to application," Trends Biotechnol, vol. 30, pp. 259–273, 2012.
[2] K. Balakrishnan, D. Dhanasekaran, V. Krishnaraj, A. Anbukumaran, T. Ramasamy, M. Manickam, "Edible Mushrooms: A Promising Bioresource for Prebiotics," In Advances in Probiotics; Elsevier, Amsterdam, The Netherlands, pp. 81–97, 2021.
[3] Bhandari, D.; Rafiq, S.; Gat, Y.; Gat, P.; Waghmare, R.; Kumar, V. "A Review on Bioactive Peptides: Physiological Functions, Bioavailability and Safety," Int. J. Pept. Res. Ther. Vol. 26, pp. 139–150, 2019.
[4] E. B. Daliri, D. H. Oh, B. H. Lee, "Bioactive Peptides," Foods, vol. 6, no. 32. 2017.
[5] J. Mishra, R. Rajput, K. Singh, S. Puri, M. Goyal, A. Bansal, K. Misra, "Antibacterial Natural Peptide Fractions from Indian Ganoderma lucidum," Int. J. Pept. Res. Ther. Vol. 24, pp. 543–554, 2017.
[6] H. Li, J. Gao, F. Zhao, X. Liu, B. Ma, "Bioactive Peptides from Edible Mushrooms—The Preparation, Mechanisms, Structure—Activity Relationships and Prospects," Foods, vol, 12, pp. 2935, 2023.
[7] K. F. Chai, A. Y. H. Voo, W. N. Chen, "Bioactive peptides from food fermentation: A comprehensive review of their sources, bioactivities, applications, and future development," Compr. Rev. Food Sci. Food Saf., vol. 19, pp. 3825–3885, 2020.
[8] J. M. Lorenzo, P. E. S. Munekata, B. Gómez, F. J. Barba, L. Mora, C. Pérez-Santaescolástica, F. Toldrá, "Bioactive peptides as natural antioxidants in food products—A review," Trends Food Sci. Technol. Vol. 79, pp. 136–147, 2018.
[9] J. Zhou, M. Chen, S. Wu, X. Liao, J. Wang, Q. Wu, M. Zhuang, Y. Ding, "A review on mushroom-derived bioactive peptides: Preparation and biological activities," Food Res. Int., vol. 134, no. 109230, 2020.
[10] M. Dabbour, R. He, H. Ma, A. Musa, "Optimization of ultrasound assisted extraction of protein from sunflower meal and its physicochemical and functional properties," J. Food Process. Eng., vol. 41, pp. 1–11, 2018.
[11] D. Tawalbeh, W. A. N. Wan Ahmad, N. M. Sarbon, "Effect of ultrasound pretreatment on the functional and bioactive properties of legumes protein hydrolysates and peptides: A comprehensive review," Food Rev. Int., vol. 39, no. 8, pp. 5423-5445, 2022.
[12] M. Kumar, M. Tomar, J. Potkule, R. Verma, S. Punia, A. Mahapatra, T. Belwal, A., Dahuja, S. Joshi, M. Berwal, V. Satankar, A. G. Bhoite, R. Amarowicz, C. Kaur, J. F. Kennedy, "Advances in the plant protein extraction: mechanism and recommendations, Food Hydrocoll. Vol. 115, no.106595, 2021.
[13] L. Mora, F. Toldra, "Advanced enzymatic hydrolysis of food proteins for the production of bioactive peptides," Curr. Opin. Food Sci., vol. 49, no. 100973, 2023.
[14] P. Farzaneh, M. Khanahmadi, M. R. Ehsani, A. Sharifan, "Bioactive properties of Agaricus bisporus and Terfezia claveryi proteins hydrolyzed by gastrointestinal proteases," LWT-Food Sci. Technol., vol. 91, pp. 322-329, 2018.
[15] P. Farzaneh, M. Khanahmadi, M. R. Ehsani, A. Sharifan, A. "Characterization of bio-peptides purified from Terfezia claveryi hydrolysate and their antibacterial effect on raw milk. LWT-Food Sci. Technol., vol. 116, no. 108522, 2019.
[16] F. Tonolo, A. Folda, L. Cesaro, V. Scalcon, O. Marin, S. Ferro, A. Bindoli, M. P. Rigobello, "Milk-derived bioactive peptides exhibit antioxidant activity through the Keap1-Nrf2 signaling pathway," J. Funct. Foods, vol. 64, no. 103696, 2020.
[17] G. Theron, J. Limberis, R. Venter, L. Smith, E. Pietersen, A. Esmail, G. Calligaro, J. Te Riele, M. de Kock, P. van Helden, et al. "Bacterial and host determinants of cough aerosol culture positivity in patients with drug-resistant versus drug-susceptible tuberculosis," Nat. Med. Vol. 26, pp. 1435–1443, 2020.
[18] M. Krishnan, J. Choi, A. Jang, Y. A. Kim, "Novel Peptide Antibiotic, Pro10-1D, Designed from Insect Defensin Shows Antibacterial and Anti-Inflammatory Activities in Sepsis Models," Int. J. Mol. Sci. vol. 21, pp.

6216, 2020.
[19] P. H. Ngai, Z. Zhao, T. B. Ng, "Agrocybin, an antifungal peptide from the edible mushroom Agrocybe cylindracea," Peptides, vol. 26, pp. 191–196, 2005.
[20] X. F. Cheng, M. Zhang, B. Adhikari, "The inactivation kinetics of polyphenol oxidase in mushroom (*Agaricus bisporus)* during thermal and thermosonic treatments," Ultrason. Sonochem. Vol. 20, pp. 674-679, 2013.
[21] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," Anal. Biochem., vol. 72, pp. 248-254, 1976.
[22] S. Zheng, Q. Liu, G. Zhang, H. Wang, T. B. Ng, "Purification and characterization of an antibacterial protein from dried fruiting bodies of the wild mushroom *Clitocybe sinopica*," ACTA Biochimica Polonica, vol. 57, no. 1, pp. 43-48, 2010.
[23] A. Alvarez-Ordonez, M. Begley, T. Clifford, T. H. Deasy, K. Considine, C. Hill, "Structure-activity relationship of synthetic variants of the milk derived antimicrobial peptide α_{S2}-casein f (183-207)," AEM, vol. 79, no. 17, pp. 5179-5185, 2013.
[24] B. B. Petrovska, "Protein fraction in edible Macedonian mushrooms," Eur. Food Res. Technol. vol. 212, pp. 469-472, 2001.
[25] K. S. Burton, D. A. Wood, C. F. Thurston, P. J. Barker, "Purification and characterization of a serine proteinase from senescent sporophores of the commercial mushroom (*Agaricus bisporus*)," J. Gen. Microbiol., vol 139, pp. 1379–1386, 1993.
[26] Y. L. Xiong, "Antioxidant peptides," in Bioactive proteins and peptides as functional foods and nutraceuticals (Eds Y. mine, E. Li-Chan, B. Jiang), Blackwell Publishing Ltd and Institute of Food Technologists, pp. 29-42., 2010
[27] H. Korhonen, A. Pihlanto, "Food-derived bioactive peptides–opportunities for designing future foods," Curr. Pharm. Des. vol 9, pp. 1297–1308, 2003.
[28] K. J. Rutherfurd-Markwick, P. J. Moughan, Bioactive peptides derived from food. J. AOAC Int. vol. 88, pp. 955–966, 2005.
[29] S. Ranathunga, N. Rajapakse, S. K. Kim, "Purification and characterization of antioxidative peptide derived from muscle of conger eel (Conger myriaster). Eur. Food Res. Technol. Vol. 222, no. 3-4, pp. 310-315, 2006.
[30] A. A. Bahar, R. Dacheng, "Antimicrobial peptides," J. Pharm, vol. 6, pp.1543-1575, 2013.
[31] R. Nawrot, J. Barylski, G. Nowicki, J. Broniarczyk, W. Buchwald, A. Gozdzicka-Jozafiak, "Plant antimicrobial peptides," Folia Microbiol., vol. 59, no. 3, pp. 181-196, 2013.