Hindawi
Journal of Chemistry
Volume 2023, Article ID 1233867, 11 pages
https://doi.org/10.1155/2023/1233867

Research Article

Ultrasound-Assisted Enzymatic Extraction and Bioactivity Analysis of Polypeptides from *Cordyceps militaris*

Lina Xu, Shang Guo, Yanting Li, Weiwei Guo, Xiaofei Guo, and Shasha Hong

Shanxi Institute of Functional Food, Shanxi Agricultural University, Taiyuan 030031, Shanxi, China

Correspondence should be addressed to Shang Guo; 15364619285@163.com

Received 15 November 2022; Revised 17 December 2022; Accepted 26 December 2022; Published 10 January 2023

Academic Editor: Mahmood Ahmed

Cordyceps militaris is rich in protein, polysaccharide, cordycepin, and other active components, with anticancer and antioxidation functions. In order to improve the economic value of *C. militaris*, the protein was extracted from its fruiting body by alkali-soluble acid precipitation process, and the extraction technology was optimized by orthogonal test. The polypeptide was obtained by digesting those proteins with a complex enzyme. And the antimicrobial and anticancer activities of those polypeptides were evaluated by measuring inhibitory zone and cytotoxicity. The results showed that the optimal extraction conditions of protein were as follows: pH of 8.5, material-to-water ratio of 1 : 28, extraction time of 3.5 h, extraction three times, and the highest protein yield was 45.06%. The optimum enzymatic hydrolysis process of *C. militaris* polypeptide solution was as follows: the ratio of alkaline protease to papain was 4 : 3, the optimum temperature was 55°C, pH was 7.2, the enzyme dosage was 7000 U/mL, the enzymolysis time was 3.5 h, and the highest yield of peptide was 16.73%. Under those conditions, the polypeptides prepared from *C. militaris* (<3000 Da) showed good antibacterial activity against *Escherichia coli*, *Bacillus subtilis,* and *Staphylococcus aureus*, with inhibitory zones of (12.08 ± 0.22), (6.67 ± 0.12), and (10.32 ± 0.23) mm, respectively. The results showed that the SAO-S (IC_{50} = 0.49 mg/L) and T24 (IC_{50} = 0.23 mg/L) were significantly inhibited by *C. militaris* polypeptide. Results from this study suggest that polypeptides can be utilized as a new approach for bioactive compounds production from *C. militaris*.

1. Introduction

Cordyceps militaris (*C. militaris*), also called "Yong Chong Cao" in China, is a very valuable medicinal fungus [1]. *C. militaris,* also known as Cordyceps, is the complex composed of the fruiting body (the grass part) and the sclerotium (the dead body of insect), with the functions of improving immunity, antibacterial, anti-inflammatory, lowering blood glucose, and blood pressure [2]. China is the first country to cultivate the fruit body of *C. militaris* in the world. Due to its various bioactive components, it has received considerable attention as an important biomaterial for pharmaceutical and functional food applications [3, 4]. Among them, polypeptides play a key role in pharmacological functions of *C. militaris* such as immunomodulatory, antioxidant, anticancer, anti-inflammatory, hepatoprotective, antinociceptive, and antiaging activities [5–8].

C. militaris polypeptides are hydrolytic products extracted directly from the fruit bodies of *C. militaris* with strong biological activity and medicinal function [2]. Many sports showed that the polypeptide would mediate metabolism or control DNA transcription and translation. Also, the polypeptides could activate the reticuloendothelial system and macrophage and promote the transformation of lymphocytes. It could activate the immune competent cells (lymphocytes, lymphokines, mononuclear-macrophage system, and NK cells) to attack target cells and also exert its antiaging, anticoagulant, and lipid-lowering effects, enhance immunity, improve liver function, and delay aging [2, 9]. Immune competent cells are activated, for example, lymphocytes, lymphokines, monocyte-macrophage system, and NK cells, to attack target cells, and their antiaging and anticoagulant effects are exerted. Our team has been engaged in the research of *C. militaris* strain breeding and functional factor mining for many years and has obtained many

achievements [10, 11]. The self-cultivated *C. militaris* strain GS-8 is now stored in the laboratory of Functional Food Research Institute of Shanxi Agricultural University; the fruit body of *C. militaris* is orange in color, the stipe is uniform in thickness, the growth period is short, the head of the ascus is large, and the biological characteristics are stable and hereditary. Compared with the current five major production varieties in Liaoning Province (9906, Kangda, 988, Qingyi, and 582), the main nutrients are higher than the latter [12], in which the protein content is as high as 68%, and the amino acid structure is reasonable. According to the ideal protein requirements recommended by FAO/WHO, the *C. militaris* is a high-quality protein source, which is necessary for its further development and utilization.

Recently, many methods have been used for extracting peptide, for example, maceration, infusion, percolation, decoction, and Soxhlet extraction. However, there are many defects in those methods, such as poor performance, dangerous solvents, long extraction time, high cost, and large amount of raw materials [13]. Therefore, in order to solve these problems, nontraditional and new type methods were applied, such as supercritical fluid extraction, pressure liquid extraction, microwave assisted extraction, ultrasonic extraction, and hollow fiber membrane extraction [2, 14]. In recent years, ultrasound-assisted enzymatic extraction attracted more attention for its less solvent, short extraction time, low cost, and high performance of the extract [15]. In our study, single factor experiments and orthogonal experiments were used to investigate the influence of extraction conditions on the contents of protein and polypeptide from *C. militaris*. The extract under optimum conditions was tested in terms of anticancer and antioxidant activities in order to provide an important theory basis for the further research and development of *C. militaris* polypeptide. The antitumor and antioxidant activities of the polypeptide (<3000 Da) were tested, which provided an important theoretical basis for further research and development of *C. militaris* polypeptide.

2. Materials and Methods

2.1. Materials. *C. militaris* fruiting bodies were obtained from the Shanxi Institute of Functional Food, Shanxi Agricultural University, China. Enzymes (alkaline protease, neutral protease, papain, trypsin, and pepsin) were purchased from Qingdao Havesen Technology Co., LTD. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN), propidium iodide (PI) obtained from Sigma Chemical Co. (St. Louis, MO, USA). 96-well cell culture plates were purchased from Corning (USA). All other reagents used were of analytical grade.

3 kinds of pathogenic bacteria (*Staphylococcus aureus* ATCC 12600, *Bacillus subtilis* ATCC 29056, and *Escherichia coli* ATCC 23815) obtained from Shanxi University, China. Sao-S and T24 cells were obtained from Shanxi Provincial Cancer Hospital, China.

2.2. Methods

2.2.1. Determination of the Total Protein and Polypeptides. The protein and polypeptide contents were determined by the Kjeldahl method [16].

2.2.2. Analysis of the Amino Acid. Contents of amino acid were detected by S-433D automatic amino acid analyzer according to the methods mentioned by GB5009.3-2016.

2.2.3. Preparation of C. Militaris Protein Extract

(1) Comparison of Different Extraction Methods. The test samples (5 g) were extracted individually with heated reflux (50°C) and ultrasonic extraction (25°C, power 100 W) methods by stirring with 100 mL of distilled water according to the material-to-water ratio of 1 : 20 (g:mL) at a pH of 8.0 for 30 min and then centrifuged (5000 r/min) for 10 min, and the supernatant was collected and the protein content was determined by the method in Section 2.2.1.

(2) Effects of Single Factor in Ultrasonic Extraction Method on the Content of Protein. 4 factors (pH, material-to-water ratio, ultrasonic time, and numbers of extraction) were chosen to inquiry the effects on protein contents.

(3) Optimization of Extract Conditions by Orthogonal Experiments. Simultaneous effects of pH (8.0, 8.5, and 9.0), material-to-water ratio (1 : 25, 1 : 28, and 1 : 30), ultrasonic time (3.0, 3.2, and 3.5 h), and extract times (2, 3, and 4) on protein contents were investigated. Statistics were processed using Orthogonality Experiment Assistant 3.1 to build the graphs. The extraction conditions for the highest protein content were determined from the designs.

2.2.4. Preparation of Peptides in C. militaris

(1) Enzyme-Assisted Extraction. Effects of alkaline protease, neutral protease, papain, trypsin, and pepsin on peptide contents were appraised.

(2) The Effect of Single Factors of Enzymatic Hydrolysis on the Peptide Contents. 4 factors, including pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0), the compound of enzyme addition (2000, 4000, 6000, 8000, and 10000 U/mL), hydrolysis time (1, 2, 3, 4, 5, 6, and 7 h), and temperature (45, 50, 55, 60, 65, and 70°C) were appraised on peptide contents.

(3) Optimization of Enzymatic Hydrolysis Conditions by Orthogonal Experiments. An orthogonal experiment was designed to explore the suitable enzymatic hydrolysis condition of the peptides. In this experiment, the 4 factors including pH, the compound of enzyme addition, hydrolysis time, and hydrolysis temperature on peptide contents were investigated. The experimental factors and levels are shown in Table 1.

Table 1: Orthogonal factors table for polypeptide of *C. militaris*.

Levels	pH	Temperature (°C)	Enzyme addition (U/mL)	Time (h)
1	7.2	54	6000	3
2	7.5	55	7000	4
3	8.0	56	8000	5

2.2.5. Separation of C. militaris Polypeptide by the Ultrafiltration Membrane. The ultrafiltration membrane (relative molecular weight: 30 kDa, 10 kDa, and 3 kDa) was used to successively separate the hydrolysate, and the filtrate was collected and tested.

2.2.6. Bioactivities of the Peptides

(1) Antioxidant Activity. DPPH Radical Scavenging Assay. The antioxidant activity test was conducted using the methods described by Kang et al. [17], and its absorbance was monitored at 517 nm.

The scavenging activity (R_1) was calculated according to the following equation:

$$\frac{R_1}{\%} = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100, \quad (1)$$

where R_1 is the radical scavenging of peptides, A_0 is the optical density of the control sample, A_1 is the optical density of the extract, and A_2 is the blank control.

Hydroxyl Radical (OH) Assay. The radical assay was conducted according to Kang et al. [17]. Scavenging activity is obtained according to equation (1).

(2) Antibacterial Activity. The antibacterial activity of peptides was conducted with the disk diffusion method. Three kinds of bacteria (*Escherichia coli*, *Bacillus subtilis,* and *Staphylococcus aureus*) were investigated. Pure cultures of the microorganisms were subcultured on Mueller–Hinton agar. Sterile paper disks were placed on the agar plates, and then the filter paper (0.5 mm × 0.5 mm) soaked with 20 μL of 100 mg/L (w/v) peptides ($<3 \times 10^3$ Da) was applied to the disks. Cephalosporin g engine-grease (15 μg per disk) was used as the positive control and sterile distilled water as the negative control. The plates were incubated at 37°C for 24 hours. All tests were repeated three times.

(3) Anticancer Activity. The anticancer test was carried out by MTT method according to Shin et al. [18].

The percentage of cellular suppression (%/MTT) was calculated according to the following equation:

$$\frac{R_2}{\%} = \left(1 - \frac{A_3 - A_4}{A_0}\right) \times 100, \quad (2)$$

where A_0 is the optical density of the control sample, A_3 is the optical density of the extract, and A_4 is the blank control.

2.2.7. Data Analysis. Data are expressed as a mean-± standard deviation. Statistical significance between the groups was determined by a paired *t*-test and one-way ANOVA for repeated measures. Data were assessed using SPSS (version 16.0).

3. Results and Discussion

3.1. Analysis of the Amino Acid. The composition and amount of amino acids are presented in Table 2. 17 types of free amino acids were analyzed at the fruiting body of *C. militaris*. Mushroom is known as a good source of essential amino acids. Also, their proteins contain lots of nonessential amino acids [19, 20]. The sample detected in our study, contained 7 essential amino acids (tryptophan was not measured), and the content of essential amino acids is greater than 11.42%. The most abundant component of essential amino acid was lysine (2.37%), while the second essential amino acid was tyrosine (2.06%). The most abundant components of nonessential amino acid were glutamate (4.39%), then the second nonessential amino acid was glycine (4.31%). Many previous papers illustrated that the amino acid, such as Arg, Glu, Gly, and Pro, which plays a key role in regulating gene expression, cell signaling, blood flow, nutrient transport, antioxidative, and immune responses [21, 22]. In addition, Glu also helps to secrete gastric juice and maintain blood glucose levels [22]. Simultaneously, Gly, Try, Tyr, D-Ala, D-Asp, and D-Ser regulate neurological development and function and Leu stimulates protein synthesis and inhibits proteolysis [23]. The present studies suggest that the fruiting bodies of *C. militaris* are rich sources of amino acids. The result (Table 3) meets well the reference values of 0.6 recommended by FAO/WHO (1973).

3.2. Preparation of Protein in C. militaris

3.2.1. Selection of Appropriate Methods for Protein Extraction Technology. Figure 1 shows the effects of different methods for protein extraction. The ultrasonic extraction method gave the highest protein content than the heated reflux method, whose result is agreed with the previous research [24]. Because of the fruit bodies of *C. militaris* rich in proteins, so the best reaction temperature was 55°C in order to get higher contents of protein [7]. Above all, ultrasonic extraction would be a potential method to enhance the contents of the extract compounds than the others [25].

3.2.2. Effects of Single Factors on the Protein Contents. Effects of pH, material-to-water ratio, ultrasonic extraction time, and numbers of extraction on protein content are shown in Figures 2(a)–2(d). Figure 2(a) shows clearly that when the material-to-water ratio ranged from 1 : 15 to 1 : 25, the protein contents rose noticeably and then declined sharply as the ratio increased. As shown in Figure 2(b), the protein contents varied with the value of pH. Previous study showed that the optimal pH value for *C. militaris* protein extraction was greater than 7 [26]. In our study, when the pH value was excess of 8.0, the protein contents went down

Table 2: Composition and content of hydrolyzed amino acids in *C. militaris*.

Number	Name	Content (%)
1	Asp	3.63 ± 0.102
2	Thr	1.84 ± 0.113
3	Ser	1.70 ± 0.081
4	Glu	4.39 ± 0.001
5	Gly	4.31 ± 0.001
6	Ala	2.04 ± 0.001
7	Cys	0.007 ± 0.001
8	Val	1.96 ± 0.001
9	Met	0.58 ± 0.001
10	Ile	1.33 ± 0.001
11	Leu	2.02 ± 0.021
12	Tyr	2.06 ± 0.021
13	Phe	1.32 ± 0.011
14	His	1.51 ± 0.011
15	Lys	2.37 ± 0.021
16	Arg	0.14 ± 0.021
17	Pro	1.99 ± 0.011
EAA		11.42
NEAA		21.78
Total contents		33.20

Table 3: Analysis of essential amino acid composition in hydrolyzed amino acids in *C. militaris*.

Names	Fruiting body (%)	FAO/WHO model (%)	RAA	RC	AAS
Thr	5.56	4.00	1.39	1.22	139
Val	5.90	5.00	1.18	1.04	118
Met + Cys	1.77	3.50	0.51	0.45	51
Ile	4.01	4.00	1.00	1.01	100
Leu	6.08	7.00	0.87	0.88	87
Phe + Tyr	10.18	6.00	1.70	1.49	170
Lys	7.14	5.50	1.30	1.14	130
EAA	40.62	35.00	1.16	1.02	116

Amino acid ratio (RAA) = amino acid content in the sample/amino acid content in WHO/FAO model spectrum; amino acid ratio coefficient (RC) = amino acid ratio/amino acid ratio mean. Amino acid score (AAS) = amino acid content in the sample/amino acid content in WHO/FAO pattern spectrum × 100.

significantly. Why? These phenomena would be explained that the protein structure maybe damaged while the value of pH was too high [27]. Our results are consistent with published studies [28].

Figure 2(c) presents the effects of extraction time on the protein contents. Results showed that the contents of protein varied with the extraction time, and it got the highest value (41.60%) when extraction time was 3.0 h. This phenomenon was due to the fact that as the extraction time is less than 2.5 h, the cell may fragmentate incompletely, which resulted in a low extraction rate. So, an appropriate extension of extraction time is conducive to break the wall of cell completely to enhance the contents of the extract [29, 30].

Figure 2(d) presents that the contents of protein increased with twice times than only once. The value of protein contents got its highest value (42.50%) when extracting three times. It has also reported that repeated extraction has a positive effect on the extract content [30].

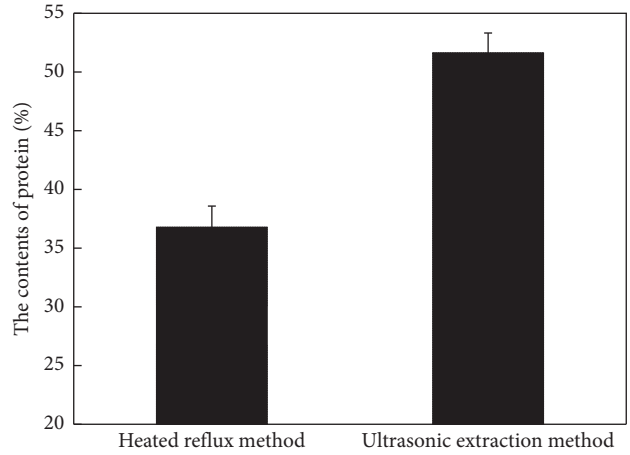

Figure 1: Effects of the extraction method on the contents of protein.

3.2.3. Orthogonal Experiments and Statistical Analysis of Protein Extraction. The single factor experiments indicated that the pH, material-to-water ratio, ultrasonic time, and numbers of extraction had significant influences on the protein contents. So, the 4 factors were chosen to design the orthogonal experiments. From Table 4, the theoretical optimum extraction conditions were as following: pH of 8.5, material-to-water ratio of 1 : 28, extraction time of 3.5 h, extraction three times, and the highest protein yield was 44.62%. In the verification experiment, the highest protein yield was 45.06%, which was 1.22 times higher than that of control. The results of variance analysis showed that the pH had a more significant impact on the protein content than other three factors.

3.3. The Preparation of Peptides in C. militaris

3.3.1. Enzyme-Assisted Extraction. Figure 3 presents the influence of different kinds of enzyme, namely, alkaline protease, neutral protease, papain, trypsin, and pepsin, on the contents of protein under the technology: pH of 8.5, material-to-water ratio of 1 : 28, extraction time of 3.5 h, and extraction three times. As can be seen from Figure 3, alkaline protease gave the highest value of protein. Our result is consistent with that reported by other researchers [31, 32]. Of course, it is also related to the fact that the fruiting bodies of *C. militaris* are rich in alkaline proteins. In addition, the composite enzyme could lead to a higher value of the extract compared to the single enzyme. Therefore, in our paper, composite enzyme (alkaline protease: papain = 4 : 3. v/v) was chosen to enhance the content of the protein.

3.3.2. Effects of Single Factors on the Polypeptide Content. The effects of pH, enzyme addition content, enzymatic hydrolysis time, and temperature on polypeptide contents are shown in Figures 4(a) and 4(b). Figure 4(a) shows that the contents of polypeptide enhanced sharply when pH value ranges from 5.5 to 7.5, but decreased when pH value reached over 7.5. Why? Maybe changes in

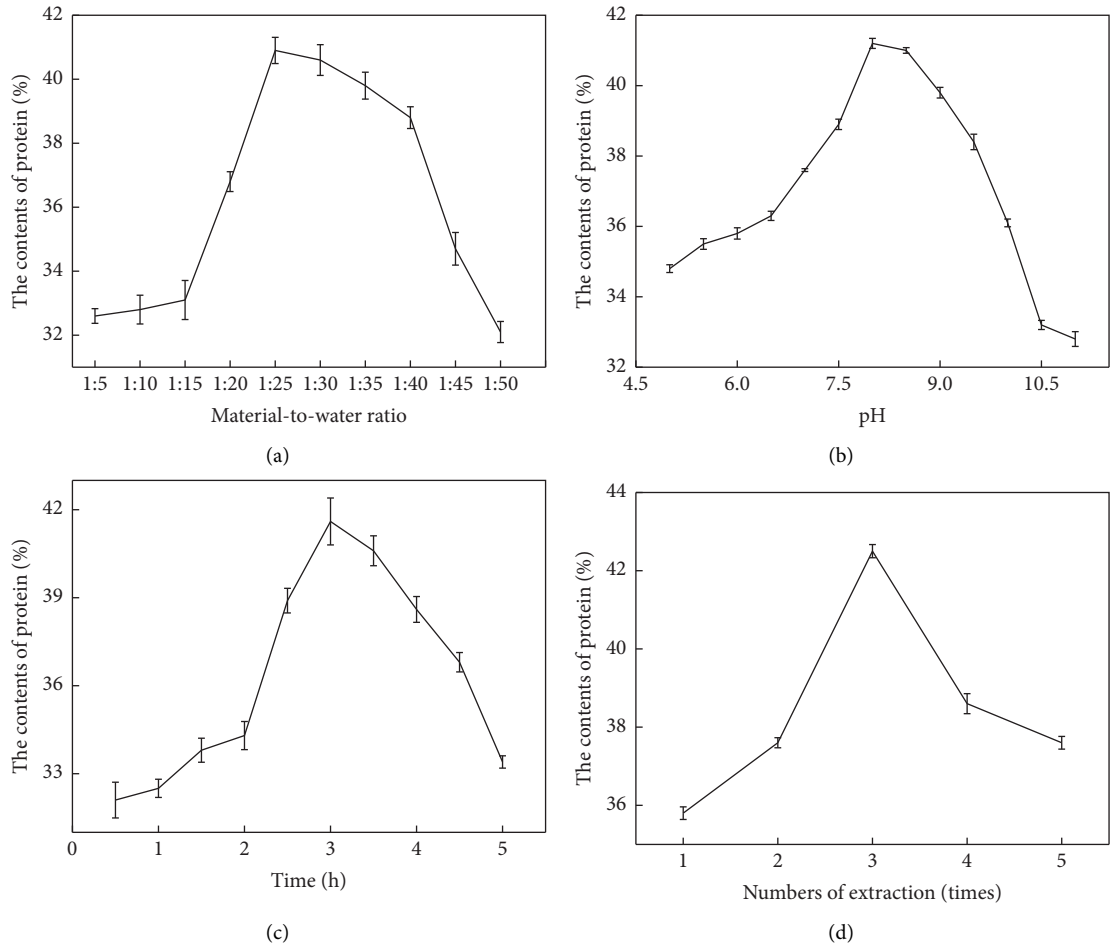

Figure 2: Effect of material-to-water (a), pH (b), time (c), and numbers of extraction (d) on the protein contents.

Table 4: Orthogonal optimization results of *C. militaris* protein extraction.

Number	pH	Material-to-water ratio (g/mL)	Ultrasonic extraction time (h)	Numbers of extraction (times)	Protein content (%)
1	1	1	1	1	$37.02 \pm 0.73^{*}$
2	1	2	2	2	$42.01 \pm 0.85^{*}$
3	1	3	3	3	$42.02 \pm 0.82^{*}$
4	2	1	2	3	$43.04 \pm 0.56^{*}$
5	2	2	3	1	$43.01 \pm 0.23^{*}$
6	2	3	1	2	$39.88 \pm 0.22^{*}$
7	2	2	3	2	$44.62 \pm 0.01^{*}$
8	3	2	1	3	$42.18 \pm 0.41^{*}$
9	3	3	2	1	$44.31 \pm 0.31^{*}$
K1	121.23	32.31	26.72	33.18	
K2	125.93	31.92	36.32	31.18	
K3	131.11	32.51	39.42	32.19	
k1	8.11	12.13	9.34	10.32	
k2	11.22	12.03	12.16	11.98	
k3	13.32	12.19	13.09	12.11	
R	5.01	0.13	4.19	0.67	
Order			*A* > *C* > *D* > *B*		
Optima levels	*A*2	*B*2	*C*3	*D*2	
Optimal combination			*A*2*B*2*C*3*D*2		

Data represent means ± standard deviation ($n = 5$). *Values in the same column with different superscripts are significantly different ($P < 0.05$).

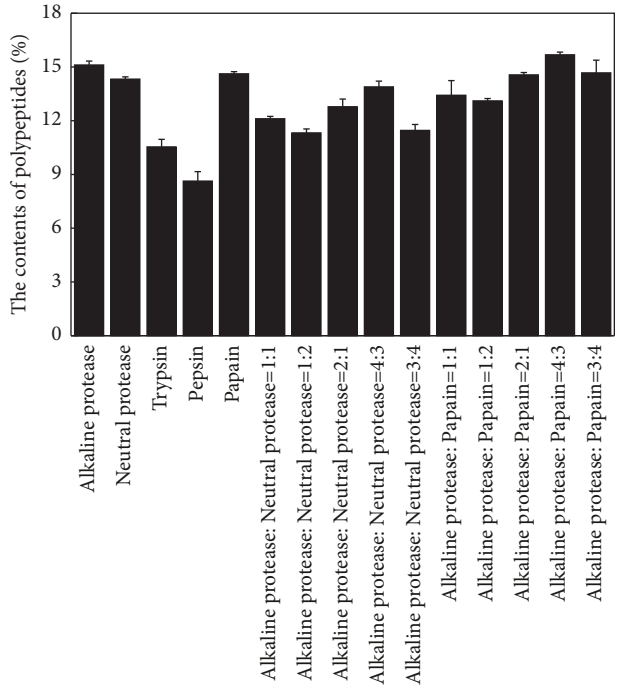

Figure 3: Peptide yield of enzymatic hydrolyzed in *C. militaris* protein.

pH would affect the structure and biological activity of enzyme [27]. Our results are same as Yin et al. [33] and Vuong Hoai et al. [34]. Many papers showed that pH acts as a key role in the hydrolysis extraction process. However, higher pH may inactivate the enzyme and adversely affect the solubility of the solvent and the target extracts.

Figure 4(b) illustrates the investigated range of enzyme addition of 2000–10000 U/mL. Results showed that when the enzyme addition enhanced from 2000 to 6000 U/mL, the polypeptide content increased significantly and then decreased noticeably according to the enzyme addition increased. Why? The results showed that the enzyme would do well only needed *n* a small amount [35]. As we all know, each enzyme has its specific biological activity and working concentration. The enzyme would no longer work while its concentration reaches a certain value [33]. Those results showed that the overuse of enzyme would limit its activity, also wastes resources of this enzyme. Why? I think there are several reasons. Firstly, hydrolysis time and temperature would affect the activity of enzyme. Secondly, the enzyme would affect the degradation of cell walls, it could degrade target extracts when overused or it could not break cell walls completely when used insufficiently.

Figure 4(c) shows that the contents of polypeptide enhanced obviously in the first 180 min. When the extraction time was 210 min, the contents of polypeptide reached its highest value. Results showed that prolonged enzymatic hydrolysis time could have a positive effect on the contents of polypeptide [36]. Those results were same as the previous reports [34, 35].

Figure 4(d) shows that the polypeptide contents varied from temperature and got its highest value (16.30%) at 55°C. Results showed that the high temperature would restrict the enzyme activity and damage protein structure [34, 37]. Our results are same as the reported research [38].

3.3.3. Orthogonal Experiments and Statistical Analysis of Polypeptide Extraction. The single factor experiments indicated that the pH, enzyme addition content, enzymatic hydrolysis time, and temperature had significant influences on the polypeptide contents. So, the 4 factors were chosen to design the orthogonal experiments. Table 5 indicates that the theoretical optimum extraction conditions were the following: pH (7.2), enzyme addition content (7000 U/mL), enzymatic hydrolysis time (210 min), temperature (55°C), and the highest polypeptide yield was 16.42%. In the verification experiment, the highest polypeptides yield was 16.73%, which was 1.02 times higher than that of the theoretical value. The results of variance analysis showed that the enzymatic hydrolysis time had a more significant impact on the protein content than other three factors [34].

Previous papers reported that the enzymatic category is a key factor in extracting process for target compounds [39]. Particularly, *C. militaris* is rich in protein, so the application of proteases could increase the contents of polypeptide.

3.4. Separation of C. militaris Polypeptide by the Ultrafiltration Membrane. *C. militaris* polypeptide obtained by enzymatic hydrolysis under the best extraction conditions was separated by ultrafiltration membranes (30 KDa, 10 KDa, and 3 KDa), respectively. Four components can be obtained I > 30 KDa, 10 KDa < II < 30 KDa, 3 KDa < III < 10 KDa, and IV < 3 KDa. Joen et al. [40] obtained 3 peptide fragments by enzymatic hydrolysis methods. Therefore, ultrafiltration can effectively separate peptides.

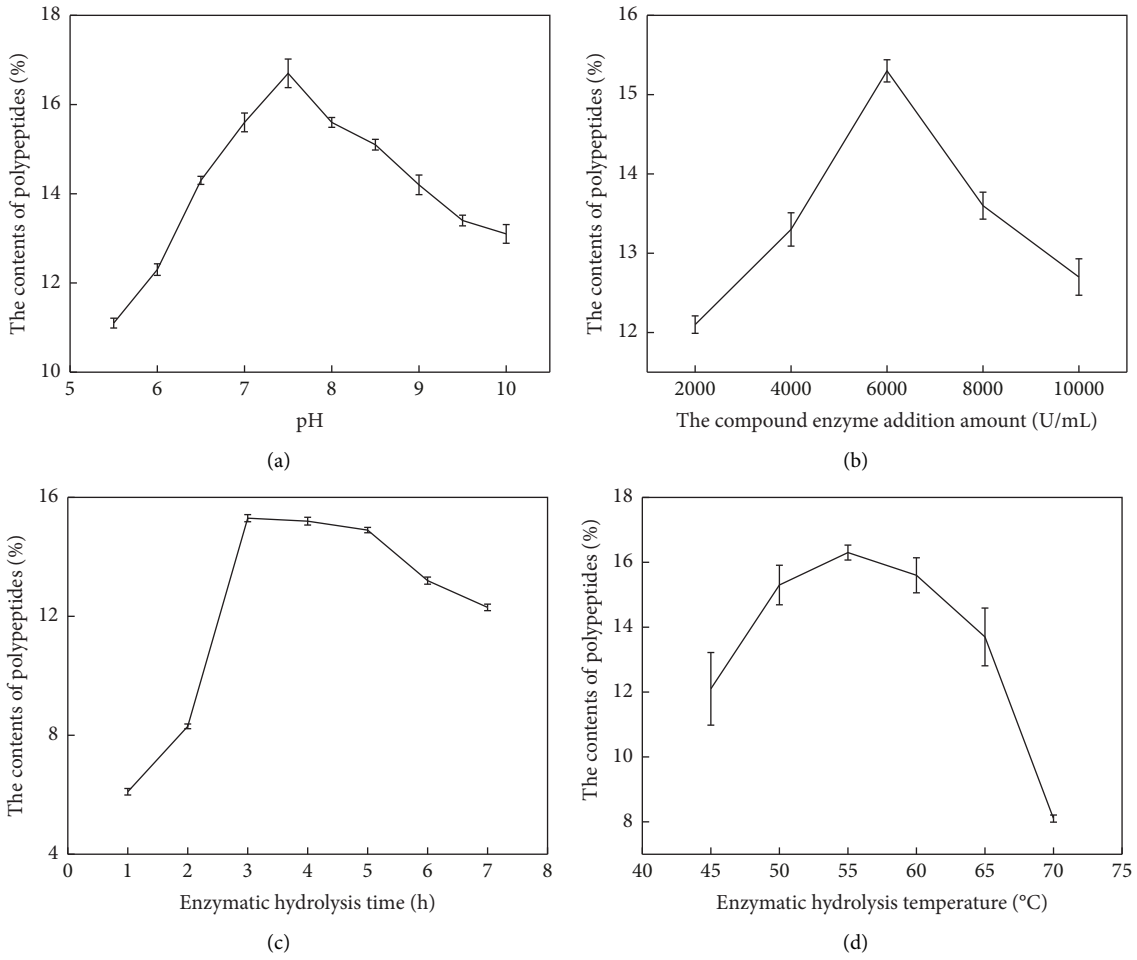

Figure 4: Effect of pH (a), the compound enzyme addition amount (b), enzymatic hydrolysis time (c), and temperature (d) on the polypeptide contents.

Table 5: Results and analysis of the orthogonal test of *C. militaris* polypeptides.

Number	pH	Addition enzyme content (U/mL)	Temperature (°C)	Time (h)	Polypeptide content (%)
1	1	1	1	1	$13.46 \pm 0.05^{*}$
2	1	2	2	2	$16.42 \pm 0.08^{*}$
3	1	3	3	3	$12.31 \pm 0.08^{*}$
4	2	1	2	3	$15.46 \pm 0.05^{*}$
5	2	2	3	1	$15.03 \pm 0.12^{*}$
6	2	3	1	2	$14.98 \pm 0.11^{*}$
7	3	1	3	2	$15.66 \pm 0.32^{*}$
8	3	2	1	3	$16.12 \pm 0.13^{*}$
9	3	3	2	1	$15.09 \pm 0.09^{*}$
K1	47.70	48.92	47.66	46.79	
K2	47.78	49.04	48.77	48.76	
K3	48.01	50.01	49.93	49.09	
k1	16.13	15.66	16.23	15.77	
k2	15.96	15.98	15.88	13.82	
K3	15.99	14.98	14.97	16.01	
R	0.26	2.01	2.13	1.09	
Order			$C > B > D > A$		
Optimal levels	*A*1	*B*2	*C*2	*D*2	
Optimal combination			*A*1*B*2*C*2*D*2		

Data represent means ± standard deviation ($n = 5$). *Values in the same column with different superscripts are significantly different ($P < 0.05$).

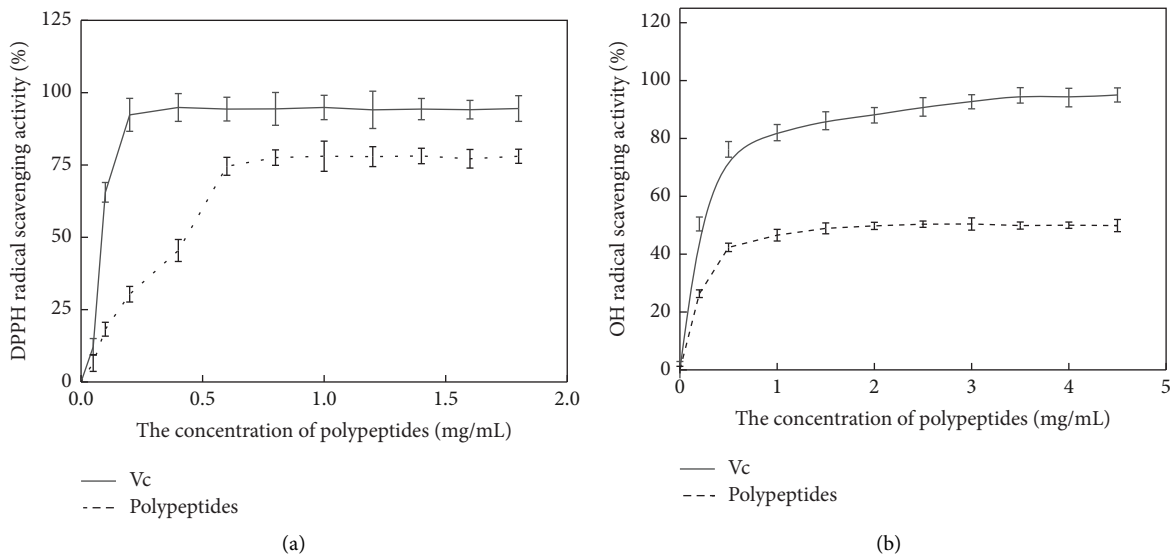

Figure 5: Scavenging rate of polypeptide in *C. militaris* to DPPH• (a) and •OH (b).

3.5. Antioxidant Activity

3.5.1. DPPH Radical Scavenging Activity. DPPH radical has a simple structure, easy to control the reaction, and has a characteristic absorption peak at 517 nm wavelength. It is widely used to detect the antioxidant ability of natural substances. DPPH radical has a single-free radical with relatively stable structure, and when it interacts with scavenger, the color of solution will change [41]. The scavenging activity of *C. militaris* polypeptide (<3 KDa) on hydroxyl radical and DPPH radical is shown in Figures 5(a) and 5(b). From Figure 5(a), the scavenging ability of DPPH free radicals is positively correlated with the mass concentration of samples in the range of 0–0.8 g/L. When the sample concentration was 1.0 mg/mL, the DPPH clearance rates of *C. militaris* polypeptide and ascorbic acid were 78.03% and 94.88%, respectively. Although there was a certain gap between *C. militaris* polypeptide and ascorbic acid (V_C), it also indicated that *C. militaris* polypeptide had a strong ability to provide electrons or hydrogen atoms.

3.5.2. Hydroxyl Radical. Hydroxyl-free radicals are extremely toxic and do great harm to the human body. In the body, they can cause the destruction of a variety of biological macromolecules, and finally lead to cell necrosis or mutation [42], and then cause diseases. If a substance with hydroxyl radical scavenging ability is added to the reaction system, it will compete with salicylic acid for hydroxyl freedom. Figure 5(b) implies that the scavenging effects of *C. militaris* polypeptide and ascorbic acid on hydroxyl-free radicals varied with the concentration. The hydroxyl-free radical rates of *C. militaris* polypeptide and ascorbic acid were 50.42% and 92.67%, respectively, while the peptide concentration was 3.0 g/L. Although there was a certain gap between them and ascorbic acid (V_C), it also indicated that *C. militaris* polypeptide had an ability to scavenge the hydroxyl-free radicals.

Table 6: Bacteriostatic effect of polypeptide.

Samples	Inhibitory zones (mm)
Staphylococcus aureus	$10.32 \pm 0.23^*$
Bacillus subtilis	$6.67 \pm 0.12^*$
Escherichia coli	$12.08 \pm 0.22^*$
Cephalosporin g engine-grease	$10.86 \pm 0.22^*$

Notes. *Values followed by different letters in each column are significant at $P < 0.05$ for each comparison among treatments.

3.6. Antibacterial Activity. It can be seen from the data in Table 6 that the polypeptide solution (<3 KDa) has different degrees of the inhibitory effect on three kinds of bacteria, and the inhibitory effect on *Bacillus subtilis* is poor, which may be related to its structure and strong resistance. The results are consistent with Zong and Li [41].The inhibitory effect of polypeptide (<3 KDa) on *Escherichia coli* is higher than that of the others, which may be related to the fact that *Escherichia coli* belongs to gram-negative bacteria. The cell wall of gram-negative bacteria is generally thin, so polypeptide is more likely to enter the cell through the cell wall, thus interfering with the physiological function of bacteria [43, 44]. Although the inhibitory effect of the peptide solution on the tested strains was not very different from that of the positive control group, the polypeptide obtained by the enzymatic method has low toxicity, high biocompatibility, and is safer than the direct use of antibiotics. Thus, results showed that polypeptide obtained in our study has the ability to against gram-negative bacteria.

3.7. Anticancer Activity. Results of the anticancer activity of polypeptide on two lines of osteosarcoma SAO-S cells and bladder cancer T24 cells are illustrated in Figures 6(a) and 6(b). Accordingly, polypeptide has ability to resist SAO-S and T24, with IC_{50} values of 0.49 mg/Land 0.23 mg/L, respectively. The anticancer activity of peptides on SAO-S (Figure 6(a)) and T24 (Figure 6(b)) was lower than that of

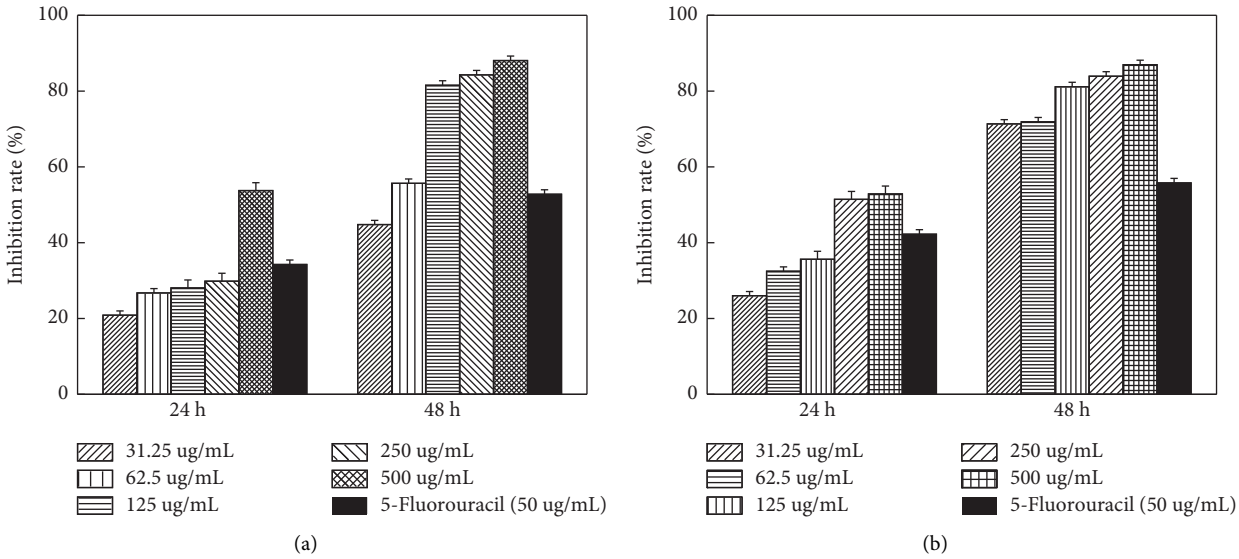

Figure 6: Inhibition rate of Sao-S cells in osteosarcoma (a) and bladder cancer T24 cells (b).

control (5-fluorouracil injection, 50 μg/mL). Cancer is a general term for many diseases in which abnormal cells grow and interfere the work of a normal cell. As mentioned above, peptides are one of the most common and key bioactive anticancer agents found in *Cordyceps* species [45].

4. Conclusion

In this study, the extraction conditions of *C. militaris* protein were optimized by single factor combined with the orthogonal test. When the extraction conditions were pH 8.5, material-to-water ratio 1 : 28, and extraction time 3.5 h, extraction was repeated three times and the highest content of protein was 45.06%. The preparation process of *C. militaris* polypeptide was optimized by a single factor combined with the orthogonal test. The optimal process was as follows: the ratio of alkaline protease to papain was 4 : 3, the optimum temperature of enzymatic hydrolysis was 55°C, pH7.2, the enzyme addition was 7000 U/mL, the enzymatic hydrolysis time was 3.5 h, and the maximum peptide content was 16.73%. Under the conditions of complex enzymatic hydrolysis established in this paper, the polypeptide showed a potential antibacterial activity against gram-negative bacteria and cancer. Thus, the application of these peptide based on our findings may lead to valuable discoveries in various fields, including medical devices and in the pharmaceutical and biomedical industries. Toxicity studies of these peptides using human pathogens may open the door to a new range of antibacterial agents and anticancer drugs.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors are grateful to the support and facilities given by Shanxi Youth Science Research Project (202103021223160); Doctoral Research Project of Shanxi Agricultural University (2021BQ45); Shanxi Province Excellent Doctoral Work Award-Scientific Research Project (SXBYKY2021086); and Research Innovation Team Special Project of Shanxi Academy of Agricultural Sciences (YGC2019TD03).

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